Upregtilation of neuronal α7 nicotinic acetylcholine receptors and preconditioning

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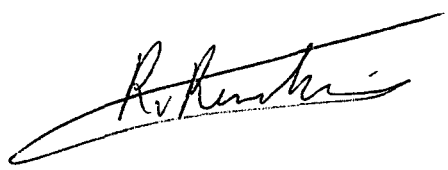
Candidates Declaration

I confirm that no part of the materials presented has previously been submitted for a degree in this or any other University. If materials have been generated through joint work, my independent contribution has been clearly indicated. In all other cases, materials form this work of others has been clearly indicated, acknowledged and quotations and paraphrases indicated.



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ABSTRACT

The upregulation of alpha 7 nicotinic acetylcholine receptors (α7 nAChRs) are putatively reported to play a role in *in vivo* cortical spreading depression-elicited neuroprotection. In this study, a reliable *in vitro* spreading depression model was created for studying this phenomenon. In contradiction to previous studies, it was, however, shown that functional α7 nAChRs are down-regulated upon chronic depolarisation with KCl, although the activity of this receptor subtype remained essential for the preconditioning mechanism. Evidence was provided for a differential mechanism underlying protection against NMDA-mediated and hypotonic-shock induced cell loss. Non-pharmacological upregulation of the α7 nAChRs in pure neuronal cortical cultures by means of a recombinant adenovirus led to the increased cell death subsequent to an excitotoxic glutamate insult. In addition, in order to study the relationship between α7 nAChRs and its function-dependent regulator Ric3, a novel anti-Ric3 antibody and a recombinant adenovirus expressing the *ric3* gene were created. Ric3 was found to be expressed in many important brain structures, including hippocampus, perhinal cortex, thalamic and hypothalamic paraventricular nucleus, structures implicated in both cognitive and emotional behaviours. Interestingly, Ric3 was also expressed in the choroid plexus, a non-neuronal cell type not known to α7 nAChRs, indicating additional roles for this protein. The recombinant constructs expressing *ric3*, α7 nAChRs and dual *ric3*/ α7 nAChRs were all validated *in vitro*.

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LIST OF ABBREVIATIONS

αbtx	-	Alpha-Bungarotoxin
aa	-	Amino acid
ACh	-	Acetylcholine
AChBP	-	Acetylcholine binding protein
AdV	-	Adenovirus
Amp	-	Ampicillin
AMPA	-	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	-	Adenosine-5'triphosphate
bp	-	Base pair
BDNF	-	Brain-derived neurotrophic factor
BSA	-	Bovine serum albumin
°C	-	Degree Celsius
CaM-kinase II	-	Ca^{2+}-calmodulin dependent kinase
C. elegans	-	*Caenorhabditis elegans*
CMV	-	Cytomegalovirus
cpe	-	Cytopathic effect
CRE	-	Calcium response element
CSD	-	Cortical spreading depression
C-terminal	-	Carboxyl terminal
DAB	-	3, 3'-Diaminobenzidine
ddH_2O	-	Deionized distilled water
DHβE	-	Dihydro-β-erythroidine
DIV	-	Day *in vitro*

DMPP	-	1,1-Dimethyl-4-phenylpiperazinium
DMSO	-	Dimethyl sulfoxide
DNA	-	Deoxyribonucleic acid
dNTP	-	2'-deoxynucleoside-5'triphosphate
DTT	-	Dithiothreitol
E1	-	Early gene 1
EBS	-	Exposure buffer saline
EC_{50}	-	H*alf maximal effective concentration*
E. coli	-	*Escherichia coli*
EDTA	-	Ethylenediaminetetra-acetic acid
EGFP	-	Enhanced green fluorescent protein
Egr-1	-	Early growth response-1
ER	-	Endoplasmic Reticulum
FBS	-	Fetal bovine serum
FCS	-	Fetal calf serum
FITC	-	Fluorescein isothiocyanate
GABA	-	γ-aminobutyric acid
GFP	-	Green fluorescent protein
HBSS	-	Hanks balanced salt solution
HEK	-	Human embryonic kidney
HPLC	-	High performance liquid chromatography
hric	-	Human resistant to inhibitors of cholinesterase
HRP	-	Horseradish peroxidase
5-HT	-	5-Hydroxytryptamine
ICD	-	Ion channel domain

IEG	-	Immediate early gene
IC50	-	Half maximal inhibitory concentration
Ig	-	Immunoglobulin
IRES	-	Internal ribosome entry site
KATP	-	ATP-sensitive K^+
kb	-	Kilobase
kDa	-	Kilodalton
K_i	-	Kinetic Inhibitor
KRE	-	Krox response element
LDH	-	Lactate dehydrogenase
L-NAME	-	N^G-nitro-L-arginine methyl ester
µg	-	Microgram
µl	-	Microlitre
µM	-	Micromolar
M	-	Molar
mA	-	Milliampere
MBS	-	3-Maleimimdobenzoic acid N-hydroxysuccinimide ester
MCAO	-	Middle cerebral artery occlusion
MCS	-	Multiple cloning site
MEM	-	Minimum essential medium
mGluR	-	Metabotropic glutamate receptor
MK801	-	(1)-5-methyl-10, 11-dihydro-5H-dibenzo [a, d] cyclohepten-5, 10-imine maleate
ml	-	Millilitre
MLA	-	Methyllycaconitine

mM	-	Millimolar
MOI	-	Multiplicity of infection
MPTP	-	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
Mr	-	Relative molecular weight
mric	-	Mouse resistant to inhibitors of cholinesterase
mRNA	-	Messenger ribonucleic acid
MSR	-	Membrane spanning region
MTT	-	1-(4, 5-Dimethylthiazol-2-yl)-3,5-diphenylformazan
mV	-	Millivolt
nAChR	-	Nicotinic acetylcholine receptor
NCBI	-	National Centre for Biotechnology Information
NGF	-	Nerve growth factor
NMDA	-	N-methyl-D-aspartic acid
NOS	-	Nitric oxide synthase
NPS@	-	Network Protein Sequence Analysis program
NTD	-	N-terminal domain
N-terminal	-	Amino terminal
NaAc	-	Sodium acetate
ng	-	Nanogram
nm	-	Nanometre
O.D.	-	Optical density
OGD	-	Oxygen glucose deprivation
PAGE	-	Polyacrylamide gel electrophoresis
PBS	-	Phosphate buffered saline
PBS^{2+}	-	Phosphate buffered saline with calcium and magnesium

PC12	-	Pheochromocytoma 12
PCR	-	Polymerase chain reaction
pfu	-	Plaque forming units
PKA	-	Protein kinase A
PKC	-	Protein kinase C
pmol	-	Picomolar
PNMT	-	*Phenylethanolamine* N-*methyltransferase*
PSB	-	Protein solvent buffer
PVN	-	Paraventricular nucleus
Ric	-	Resistant to inhibitors of cholinesterase
RNA	-	Ribonucleic acid
rpm	-	Revolutions per minute
SD	-	Spreading depression
SDS	-	Sodium dodecyl sulphate
S.E.M	-	Standard error of the mean
SRE	-	Serum response element
SynI	-	Synapsin-1
TARP	-	Transmembrane AMPA receptor regulatory protein
TBS	-	Tris buffer saline
TEMED	-	N,N,N',N'-tetramethylethelenediamide
Tet	-	Tetracycline hydrochloride
Tm	-	Temperature
Tris	-	Tris(hydroxymethyl)-aminomethane
U	-	Units
UCL	-	University College London

USF1	-	Upstream stimulatory factor-1
UV	-	Ultraviolet
V	-	Volt
v/v	-	Volume per volume
WPRE	-	Woodchuck hepatitis virus posttranscriptional regulatory element
w/v	-	Weight per volume

CHAPTER 1: Literature Review and Aims

1.1.1.1. Cortical Spreading Depression

Spreading depression (SD) is a transient suppression of neuronal activity, resulting from a temporary disruption of local brain ionic homeostasis by a propagating wave of neuronal hyperexcitability over the cerebral cortex and was first identified by Leão (1944) in the cerebral cortex of rabbits (Lauritzen, 1994). Cortical spreading depression (CSD) has been hypothesized to form the basis of the aura and spreading neurovascular events associated with migraines (Young and Van Vliet, 1992; Lauritzen, 1994; Lauritzen, 2001), as well as being associated with the cerebral pathology following ischaemia (Nedergaard, 1988; Nedergaard and Hansen, 1993; Obrenovitch and Zilkha, 1995) or trauma (Oka *et al.*, 1977; Sunami *et al.*, 1989; Strong *et al.*, 2002; Fabricius *et al.*, 2006).

In an *in vivo* environment, it is generally thought that the propagating wave of neuronal hyperexcitability is merely triggered by the application of high concentrations of potassium ions (K^+) but is sustained by the efflux of intracellular K^+, which diffuses to the surrounding tissue to promote further cell firing and depolarisation (Gardner-Medwin, 1981; Obrenovitch and Zilkha, 1995; Koroleva and Bures, 1996). Experimentally, SD can be reliably achieved *in vivo* by increasing the extracellular K^+ concentration, which has long been known to reach levels of 50 mM or more during normal SD (Szerb, 1991; Herreras and Somjen1993; Martins-Ferreira *et al.* 2000). Numerous attempts to produce CSD using different stimuli have been attempted: chemical stimuli (Van Harreveld, 1959; Aquino-Cias *et al.*, 1967; Obrenovitch and Zilkha, 1995, Menna *et al.*, 2000), mechanical stimuli e.g. puncture of the cortex (Nellgard and Wieloch, 1992; Lambert *et al.*, 1999; Ebersberger *et al.*, 2001) and electrical stimuli e.g. cathodal stimulation (Koroleva and

Bure, 1980; Reid *et al.*, 1987; Golanov and Reis, 1999). Despite this, only endothelin (ET)-1 has been found to reflect the potency and reliability achieved with KCl-induced CSD *in vivo* (Dreier *et al.*, 2002; Petzold *et al.*, 2003; Somjen 2001). It should, however, be noted that endothelin-1 induced CSD may take place through a mechanism distinct from that of KCl (Kleeberg *et al.*, 2004).

The CSD phenomenon is restricted to the brain and appears to involve both neuronal and glial cell populations (Smith *et al.*, 2006). CSD is not associated with neuronal injury in the normal brain (Nedergaard and Hansen, 1988; Nedergaard and Hansen, 1993). This differs from *ex vivo* experiments, such as in isolated tissue slices, where continuous K^+–depolarisation was associated with neuronal injury, indicating that the mechanism or the characteristics associated with the processes and environments might differ (Jing *et al.*, 1991). This also emphasizes the effect of vascular clearance on the concentration gradients of KCl in intact tissue (Gardner-Medwin *et al.*, 1981; Gardner-Medwin, 1983).

1.1.1.2. Cortical Spreading Depression and Preconditioning

Application of high concentrations of K^+-triggered spreading depression or a minor non-injurious ischemic event accompanied by the associated CSD can also induce tolerance to a subsequent sustained ischemic insult *in vivo*, a phenomenon known as preconditioning (Kawahara *et al.*, 1995; Kobayashi *et al.*, 1995; Matsushima *et al.*, 1996; Kawahara *et al.*, 1997; Taga *et al.*, 1997). The first documented case of *in vivo* preconditioning was performed by Dahl and Balfour in 1964 where it was discovered that brief anoxia leads to an acute increase in the capacity of the rat brain for anaerobic glycolysis with an increased survival time of the animal subsequent to prolonged anoxic exposure (Dahl and

Balfour, 1964). Preconditioning, or ischemic tolerance, is a latent cerebroprotective phenotype that has been evolutionarily conserved as a form of cerebral plasticity that occurs in invertebrates and vertebrates (Gidday, 2006).

Preconditioning triggers one of two adaptive cytoprotective responses: acute and chronic phase protection. Acute phase protection is the short-lasting tolerance gained to an insult only a few minutes subsequent to preconditioning, whereas with chronic phase protection the insult can be applied days after the preconditioning event and has a longer window of protection than exists with the acute phase preconditioning. The acute phase, or rapid preconditioning as it is more commonly referred to, is mostly due to changes in the ion channel permeability, protein phosphorylation and other post-translational modifications (Perez-Pinzon *et al.*, 1997; Pérez-Pinzón and Born, 1999; Stagliano *et al.*, 1999; Lu *et al.*, 2005). Chronic phase preconditioning, the classical and more attractive model, requires time for pro-survival gene activation with *de novo* protein synthesis, and the suppression of strenuous metabolic pathways and pro-apoptotic/ necrotic genes to generate resistance to the delayed ischemic insult (Shimazaki *et al.*, 1994; Shimizu *et al.* 2001; Trendelenburg *et al.*, 2002; Stenzel-Poore *et al.*, 2003; Wu *et al.*, 2003). Kitagawa *et al.* (1990) was the first to illustrate that the disturbance of cellular metabolism caused by the preconditioning event, requires time for transcriptional regulation and cellular adaptation to occur. This reprogramming of the cell's genomic response to an ischemic insult can be produced by various stimuli activating a wide array of cellular mechanisms with different degrees of successful protection spanning different windows of opportunity e.g. hypoxia (Pérez-Pinzón *et al.*, 1996; Emerson *et al.*, 1999; Gidday *et al.*, 1999; Centeno *et al.*, 1999), seizures (Plamondon *et al.*, 1999; Borges *et al.*, 2007), hyperthermia (Chopp *et al.*, 1989; Kitagawa *et al.*, 1991), adenosine (Heurteaux *et al.*, 1995; Pérez-Pinzón and Born,

1999; Blondeau *et al.*, 2000), and oxidative phosphorylation inhibitors (Riepe *et al.*, 1997; Riepe and Ludolph, 1997; Christophe and Nicolas, 2006). Although the triggering and propagation of CSD might have been attributed to K^+ ions, it is highly probable that there are other biological processes that are necessary or, at the very least, contribute to the elicitation and propagation of CSD and its subsequent neuroprotective effect.

The number of consecutive recurrent CSD induced i*n vivo* is the main determinant as to whether or not tolerance to an ischemic event will be induced and is subject to the environmental conditions that affect the metabolic rate of the subject during the preconditioning period. Horiguchi *et al.* (2005) illustrated that the application of 0.5 M KCl for a period of 120 minutes on the intact brain dura mater overlaying the occipital cortex of a rat treated with 1% halothane elicited approximately 12 CSD, effectively inducing tolerance to an ischemic insult four days later. Tolerance was not induced under the same conditions, however, when the rats were anaesthetised with either 0.5% or 2% halothane. The animals experienced enhanced metabolic stress under 0.5% halothane with an average of 27 CSD being elicited in the two hour period. Contrary to this, 2% halothane reduced the metabolic rate with an average of only 6 CSD being induced; supporting previous findings that CSD-induced tolerance can be abrogated by the reduction in metabolic rate by means of e.g. halothane or hypothermia (Saito *et al.*, 1995; Wada *et al.*, 1997; Horiguchi *et al.*, 2005). It is possible to induce tolerance in animals with a lowered metabolic rate by utilizing a higher concentration of KCl for a shorter time period, e.g. 1 M KCl for 90 minutes and 3 M KCl for 30 minutes both produce 10 consecutive CSD in the presence of 2% anaesthetic (Taga *et al.*, 1997; Chazot *et al.*, 2002). The chronic window of protection generated by this treatment spans from between 24 hours up to seven days (Obrenovitch and Chazot, personal communication).

1.1.1.3. The Molecular Mechanism of Cortical Spreading Depression

The fundamental molecular mechanisms of CSD-induced ischemic tolerance have yet to be fully unravelled. During an ischemic event, spontaneous, recurrent CSD can be found in the penumbra region – the area surrounding the ischaemic core with blood flow below what is needed to sustain electrical activity, but above that which is required to preserve cellular ionic gradients (Obrenovitch and Zilkha, 1995). The presence of CSD in this region may promote lesion progression and worsen the outcome in stroke models (Back *et al.*, 1996; Busch *et al.*, 1996; Hossmann, 1996; Back *et al.*, 2004) despite high potassium-induced CSD not being associated with irreversible damage under normal circumstances (Nedergaard and Hansen, 1988, Nedergaard and Hansen, 1993). The number of naturally occurring CSD during focal ischaemia correlates directly with or, in the case of superimposing K^+-induced CSD on middle cerebral artery occlusion (MCAO), worsens the extent of damage observed (Alexis *et al.*, 1996; Back *et al.*, 1996; Tatlisumak *et al.*, 1998; Tatlisumak *et al.*, 2000). It is also possible to reduce the infarct size of the ischemic lesion by the application of CSD blockers, such as the N-methyl-D-aspartate (NMDA) channel antagonist MK801 (Gill *et al.*, 1992; Iijima *et al.*, 1992) or magnesium ions (van den Bergh *et al.*, 2002).

Due to this association between ischaemia and CSD, it was thought that the preconditioning models had similar mechanisms of activation and tolerance. In a seminal paper by Heurteaux *et al.* (1995) it was shown that the first period of ischemic preconditioning releases adenosine and the resultant activation of adenosine A1 receptors then activates the ATP-sensitive K^+ (K_{ATP}) channels, leading to the generation of ischemic tolerance. Ischemia-generated tolerance can be fully blocked by the application of adenosine A1

antagonists and partial protection is gained by the application of adenosine A1 receptor agonists (Heurteaux *et al.*, 1995). The partial protection indicates that although activation of the K_{ATP} channels is necessary, it is not the only prerequisite for the full complement of the cell's survival-promoting mechanisms. This, however, is not the case for CSD preconditioning where the inhibition of either K_{ATP} channels or adenosine A1 receptors had no effect on the development of CSD-induced tolerance to ischaemia (Horiguchi *et al.*, 2005). In the same preconditioning model, N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase (NOS), effectively blocked CSD-induced tolerance to an ischemic insult (Horiguchi *et al.*, 2005b).

The neurotransmitter glutamate, with enhanced NMDA-receptor activation and a subsequent Ca^{2+} influx (Rosen *et al.*, 1994; Iida *et al.*, 2001), has been proposed to play an active role in CSD preconditioning. During CSD, a marked transient increase in extracellular glutamate has been detected (Fabricius *et al.*, 1993; Zilkha *et al.*, 1995; Basarsky *et al.*, 1999) and it has been found that CSD (McLachlan, 1992; Nellgard and Wieloch, 1992) and its tolerance to an ischemic insult (Kitagawa *et al.*, 1990; Kitagawa *et al.*, 1991; Kato *et al.*, 1992; Taga *et al.*, 1997) can be blocked by various NMDA-receptor antagonists.

Of note for the reduction of the cells' Ca^{2+} permeability and subsequent metabotropic demands, are the changes to the cell surface receptors associated with the acquisition of an ischaemia-tolerant phenotype. There seems to exist a relative shift between excitatory glutamate receptors and inhibitory γ-aminobutyric acid $(GABA)_A$ receptors post-ischaemia which may contribute to endogenous neuroprotection with the glutamate receptors being largely, but not exclusively, downregulated and the $GABA_A$ receptors

being upregulated (Sommer *et al.*, 2002). For example, with regard to glutamate receptor expression: a decrease, or no change, of the NMDA receptor subunits NR2A, NR2B and NR1 (Shamloo and Wieloch, 1999; Chazot *et al.*, 2002), a decrease in Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit expression GluR 1 and GluR 2 (Chazot *et al.*, 2002 Sommer and Kiessling, 2002) or upregulation of GluR 4 (Ying *et al.*, 1997); a decrease in metabotropic glutamate receptors (mGluRs) subtypes 1b and 5 of group I but not subtypes 2 and 3 of group II mGluRs (Sommer *et al.*, 2000), a down-regulation of the glial glutamate transporter isoforms EAAT2 and EAAT1 on glial cells (Douen *et al.*, 2000). The balance between excitatory and inhibitory receptors is, therefore, not clearly defined.

Of more importance to this study is the observation by Chazot *et al.* (2002) of a 12–15-fold increase in α7 nicotinic acetylcholine receptor (nAChR) following CSD in the mouse cerebral cortex. This is a selective upregulation as can be seen from the unaltered expression of α4 nAChR subunit (Chazot *et al.*, 2002). This change might contribute to the increased tolerance to ischaemia. The concept that α7 nAChR is a key contributor to adaptive neuroprotection is also supported by data obtained from the literature and is the focal point of this study.

1.1.2.1. Alpha 7 Nicotinic Acetylcholine Receptors

Neuronal nicotinic acetylcholine receptors (nAChRs) mediate synaptic transmission in many parts of the vertebrate central nervous system, as well as in autonomic ganglia, retinal and adrenal medulla, and may play a role in perception, cognition and emotion (Zhao *et al.*, 2003). These membrane-bound receptors belong to the ligand-gated ion channel superfamily (Patrick *et al.*, 1987; Vijayaraghavan *et al.*, 1992; Alkondon and Albuquerque, 2004). There are at least 17 different nAChR subunits that can be divided into two categories: muscle type (α1, β1, δ, γ and ε) and neuronal type (α2 - α10 and β2 - β4) subunits (Lukas *et al.*, 1999). The neuronal nAChR channel is composed of five subunits, which can be subdivided into two groups: subunits carrying the principal acetylcholine binding site component (α2 – α10) and subunits carrying the complementary component of the acetylcholine binding site (non-α or β2 - β4) (Cooper *et al.*, 1991; Galzi *et al.*, 1991; Lukas and Bencherif, 1992). The channels formed by these subunits can be either homomeric, consisting of five subunits comprising the same type (α7 - α9), or heteromeric, where the pentameric channel consists of two or more different subunits. It was first discovered in 1973 by Salvaterra and Moore that the curaremimetic neurotoxin α-bungarotoxin (αbtx), a component of the venom of the Formosan krait, *Bungurus multicinctus*, bound selectively to a subset of nAChRs in the brain (Salvaterra and Moore, 1973; Eterović and Bennett, 1974; McQuarrie *et al.*, 1976; Oswald and Freeman, 1981; Elgoyhen *et al.*, 2001). It was only much later that it was shown that this subgroup is actually the homo-oligomeric receptors, pentameric channels consisting of either α7, α8 or α9 subunits. The αbtx-binding subgroup of nAChRs has a lower affinity for binding nicotine, in contrast to the heteromeric nAChRs, consisting of both α and β subunits, which bind nicotine with a high affinity and αbtx with low affinity

(Marks *et al.*, 1986; Wonnacott, 1986; Goldman *et al.*, 1987; Schoepfer *et al.*, 1990; Amar *et al.*, 1993; Zhang *et al.*, 1994). Although it is possible for α7 subunits to combine *in vitro* with β-subunits to form heteromeric α7-containing channels with significant pharmacological differences, such heteromeric receptors have not been unequivocally proven to exist *in vivo* (Palma *et al.*, 1999; Khiroug *et al.*, 2002).

Alpha 7 nicotinic acetylcholine receptors are the only homomeric nicotinic receptors expressed in the human brain and is one of the two most abundantly expressed nicotinic receptor subtypes in the brain, the other being the heterodimer consisting of α4 and β2 subunits (Clarke *et al.*, 1985; Gault *et al.*, 1998; Belluardo *et al.* 2000; Alkondon and Albuquerque, 2004). The α8 nAChR is exclusively found in the brain of chickens (Schoepfer *et al.*, 1990), whereas α9 homomeric channel formations can be found in the cochlear hair cells and the pituitary, and also in non-neuronal tissues such as tonsil, immortalized B-cells, cultured T-cells and peripheral blood lymphocytes (Elgoyhen *et al.*, 1994; Lustig *et al.*, 2001). The distribution pattern and expression levels of α7 nAChRs in the brain differ greatly depending on the detection technique employed and the species investigated. Low levels of α7 nAChRs expression can be detected in most nuclei throughout the human brain. High density α7 nAChR can be found in the nucleus reticularis, the lateral and medial geniculate bodies, pontine nucleus, the horizontal limb of the diagonal band of Broca, the nucleus basalis of Meynert, and the inferior olivary nucleus. Moderate density α7 nAChRs can be located in the hippocampus, the hypothalamus, pons, and the medulla, with low density expression in the cortex and the cerebellum (Rubboli *et al.*, 1994; Breese *et al.*, 1997; Spurden *et al.*, 1997; Summarized in Paterson and Nordberg, 2000).

1.1.2.2. Function of Alpha 7 Nicotinic Acetylcholine Receptors

The release of acetylcholine (ACh) and several other neurotransmitters throughout the CNS is modulated by presynaptic and preterminal nAChRs. Stimulation of a nAChR induces the influx of Ca^{2+} through the nAChR channels or through the opening of voltage-gated calcium channel, which results in the release of neurotransmitters. The synaptic release of each neurotransmitter is regulated by a specific nAChR depending on its location in the CNS. The α7 nAChR regulates the release of GABA in the hippocampus and the spinal cord, as well as the regulation of glutamatergic neurotransmission throughout the brain (Wonnacott, 1997; Sher *et al.*, 2004).

Homopentameric nAChR complexes have greater relative Ca^{2+} permeabilities and faster desensitization rates than their heteromeric counterparts (Jensen *et al.*, 2005). α7 nAChRs are selectively permeable to high levels of Ca^{2+} and have an even greater relative Ca^{2+} permeability than the other nAChRs (Vijayaraghavan *et al.*, 1992). This permeability is even greater than that of the NMDA subtype of glutamate receptors, which is known to be potentially excitotoxic (Choi *et al.* 1987; Séguéla *et al.* 1993; Rothman and Olney, 1995). However, the α7 nAChR is much more rapidly desensitized in the presence of an agonist than NMDA receptors and may in this way be able to self-limit intracellular Ca^{2+}. The mutated, non-desensitizing form of α7 nAChRs was found to reduce cell viability, apparently due to intracellular calcium-overload (Treinin and Chalfie, 1995). The mode of entry and levels of intracellular Ca^{2+} achieved appears to be decisive to the outcome of intracellular signalling. An influx of Ca^{2+} through α7 nAChRs may play a role in activating beneficial downstream calcium-dependent mechanisms (Séguéla *et al.* 1993, Donnely-Roberts *et al.* 1996).

The receptor has been implicated in diverse functions, such as stabilizing synapse formation during development, learning and memory, and neuronal survival (Gotti and Clementi, 2004). It is purported that α7 nAChRs might play a role in a range of diseases including epilepsy, schizophrenia, Alzheimer's disease and anxiety disorders (Kem, 2000; Picciotto *et al.*, 2001; Martin and Freedman, 2007; Morissette *et al.*, 2007). To further elucidate the role of α7 nAChR in the brain, knock-out mice with no detectable αbtx-binding sites were created (Orr-Urtreger *et al.*, 1997). Taking into account the α7 nAChR subunit's prevalence in the brain, it came as a surprise that a plethora of behavioural tests yielded no significant deviations when compared with wildtype animals. Subsequent to this, a knock-in mouse model was created expressing hypersensitive mutated Leu250Thr α7 nAChRs (Orr-Urtreger *et al.*, 2000). Transgenic mice homozygous for this trait died within hours of birth – the increased Ca^{2+} influx through the non-desensitizing α7 mutant receptors lead to substantial neuronal apoptosis in the somatosensory cortex. Heteromeric α7 nAChRs containing Leu250Thr mutated and wildtype α7 subunits desensitize more slowly when exposed to an agonist, although the heterozygous knock-in mice expressing this mixed population of α7 nAChR subunits once again showed no apparent behavioural dysfunction (Orr-Urtreger *et al.*, 2000).

1.1.2.3. Structure of the Alpha 7 Nicotinic Acetylcholine Receptor Gene

The human α7 neuronal nicotinic acetylcholine receptor cDNA consists of 1509 bp, including the ATG start and the TAA stop codons, and is translated into a 502 amino acid protein (Peng *et al.*, 1994). There exists a high level of amino acid conservation for the α7 subunit between species with 94% amino acid identity between human and rat sequences, and 92% homology between humans and chickens (Schoepfer *et al.*, 1990;

Séguéla *et al.* 1993; Peng *et al.*, 1994.) This serves to indicate the importance of the amino acid sequence for correct formation of functional α7 receptors. The human gene itself spans a minimum of 75 kb in the chromosome 15q13 region and contains of 10 exons (Gault *et al.*, 1998). The splice junctions, as presented in Table 1, have locations identical to those in the chick α7 gene (Matter- Sadzinski *et al.*, 1992, Gault *et al.*, 1998).

Table 1 Exon–Intron Splice Junctions of the Human α7 nAChR

Exon No.	**Exon size (bp)**	**cDNA position**	**Peptide size in amino acids**	**Intron No.**	**Intron size (kb)**
1	55	1–55	18.3	1	0.3
2	140	56–195	46.7	2	>25
3	45	196–240	15	3	9.0
4	110	241–350	36.7	4	>25
5	80	351–430	26.7	5	4.0
6	168	431–598	56	6	0.6
7	195	599–793	65	7	1.0
8	87	794–880	29	8	3.5
9	110	881–990	36.7	9	5.0
10	519	991–1509	173		

*Created from data provided in Gault *et al.*, 1998.

Of note is the possibility for the human α7 nAChR gene to undergo alternative splicing, yielding an array of mostly non-functional α7-like proteins (Gault *et al.*, 1998; Garcia-Guzman *et al.*, 1995). Also, a duplication of exons 5 – 10 of the α7 nAChR gene can be found within 1 Mb and centromeric to the full-length α7 gene on chromosome 15 of the human genome. This duplicated region differs from the α7 gene by either an adenosine substitution at base position 690 or a 2-bp (TG) deletion at position 497–498 of exon 6. Four novel non-α7 exons, 5'-α7D, α7C, α7B, and α7A-3', have also been discovered that bind to the duplicated α7 exons. Variation is also generated by the alternative splicing of

the non-α7 sequence, α7B, in some of the transcripts. These transcripts, however, miss the α7 signal peptide that normally occur in exons 1 and 2 of the α7 nAChR gene, which is necessary for assembly and surface presentation of functional receptors (Gault *et al.*, 1998). The function of these alternatively spliced and duplicate transcripts has yet to be elucidated although the duplicated region with its 4 novel N-terminal exons and the possibility for alternative splicing might account, in part, for the non-specificity of primary antibodies directed at α7 nAChRs (Jones and Wonnacott, 2005; Moser *et al.*, 2007). This problem becomes apparent in transgenic animals in which the α7 nAChR gene has been deleted. Despite the absence of this gene, antibodies specifically targeted at amino acid sequences from within the α7 nAChR polypeptide still produce immunoreactivity indicating the non-specificity of the antibodies (Herber *et al.*, 2004; Moser *et al.*, 2007; Jones and Wonnacott, 2005).

1.1.2.4. Transcription of the Alpha 7 Nicotinic Acetylcholine Receptor Gene

High levels of the α7 nAChR gene expression is driven by a cell-specific 178-base pair promoter in rat neurons. This sequence has a high GC-content with no CAAT- or TATA-boxes and is located upstream between –275 and –97 relative to the translation start codon. A negative regulatory element is present at position –172. Several transcription factors interact in close proximity to control the expression of the α7 nAChR gene. Upstream stimulatory factor-1 (USF1) positively regulates α7 expression as a homodimer by binding to an E-box at position –116. SP1 and/ or SP3 binding occurs downstream to or overlapping an early growth response-1 (Egr-1) binding site, which is located directly next to the E-box (Nagavarapu *et al.* 2001).

Sp1 is a ubiquitous mammalian transcription factor that frequently initiates transcriptional activation in housekeeping genes that lack the TATA and the CAAT boxes (Boisclair *et al.* 1993). The interaction between Sp1 and Egr-1 transcription factors has been studied for the rat phenylethanolamine *N*-methyltransferase (PNMT) gene promoter where both these factors bind specifically to overlapping elements. It was determined that the binding is mutually exclusive in a concentration-dependent fashion and also depends on the binding affinity of each factor for the site (Ebert and Wong, 1995).

The presence of the USF1 binding may stabilise Egr-1 binding to the α7-promoter in the rat model since the removal of the USF1 binding site results in no Egr-1 binding but binding of Sp1 and Sp3 (Nagavarapu *et al.* 2001; Carrasco-Serrano *et al.* 1998). It has been shown repeatedly that Egr-1 stimulation and binding increase the α7-promoter activity by up to 14-fold (Carrasco-Serrano *et al.* 2000; Carrasco-Serrano *et al.* 1998; Carrasco-Serrano and Criado 2004). However, Nagavarapu *et al.* (2001) found that when treating pheochromocytoma (PC12) cells with nerve growth factor (NGF), egr-1 RNA increased many fold while α7 RNA levels decreased. However, the evidence is stacked against the Nagavarapu *et al.* (2001) paper. Egr-1 (also known as Krox-24, NGFI-A, Zif268, Tis8 and Zenk) is an immediate early gene (IEG) whose activity does not require new protein synthesis and is activated within minutes. Diverse biological processes modulate the expression of the Egr-1 gene. These include cell growth, differentiation and following cellular depolarization. The promoter region of Egr-1 is complex and can be activated through many possible mechanisms depending on the stimulant and the cell type. The 5' upstream promoter region contains four (human) or five (mouse) serum response elements (SREs), an SP1-like motif, one Krox response element (KRE), one

calcium response element (CRE)-like, one AP-1-like motif and AP-2-like binding sequences and two CCAATT sequences (Tsai-Morris *et al.* 1988). Egr-1 can autoregulate its own transcription by binding with high affinity to the KRE or GSG box in its own promoter (Cao *et al.* 1993). However, the focus here will remain on activation through a potassium-induced depolarization mechanism. The activation of Egr-1 corresponds with the increased expression of α7 nAChR. Egr-1 mRNA levels increase dramatically following membrane depolarization both *in vitro* and *in vivo* (Sukhatme *et al.* 1988; Enslen and Soderling 1994) and transient focal ischaemia induced by middle cerebral artery occlusion led to increased egr-1 mRNA expression in the cerebral cortex (Abe *et al.,* 1991). Application of 1 M KCl to rat cortical surfaces induced cortical spreading depression where Egr-1 was expressed in all cortical layers but with different intensities of immunoreactivity (Herdegen *et al.* 1993). Egr-1 showed a prominent expression in layers II, IV, and VI. It was also found that Egr-1 proteins were expressed in neurons but not in glial cell populations (Herdegen *et al.* 1993). Most importantly, however, Utsugisawa *et al.* (1999) found that the relative amount of α7 subunit mRNA compared with that before transient hypoxia of PC12 cells decreased to 84% immediately after hypoxia, but then began to increase at 6 h after hypoxia, reaching 171% at 12 h.

The human α7 nAChR gene promoter region also contains an Egr-1 transcription factor binding site (Gault *et al.* 1998). This might be indicative of a conserved mechanism for the activation of α7 expression. It does not appear that any of the other known α-subunit nicotinic receptor promoter regions contain an Egr-1 binding site (α2, Bessis *et al.* 1993; α2, Milton *et al.* 1995; α3, Yang *et al.* 1995; α3, Liu *et al.* 1999; α3 and α5, Du *et al.* 1998; α5, Campos-Caro *et al.* 2001; α6, Ebihara *et al.* 2002; α9, Valor *et al.* 2003; α9,

Lustig and Peng 2002; α10, Lustig *et al.* 2001). No information concerning the promoter region of the α4 subunit gene is currently available.

Consequent to an increase in cytosolic Ca^{+2}, multiple pathways may be recruited to promote cell survival, which can differ in the pattern of intracellular signalling between different types of neurons. The signal transduction machinery of cortical neurons differs from other neuron populations in a number of significant ways. For example: it has been shown that the survival of retinal ganglion cells promoted by brain-derived neurotrophic factor (BDNF) is dependent on cAMP. Yet in cortical neurons it was shown that cAMP attenuates the ability of BDNF to rescue the cells from serum deprivation (Poser *et al.* 2003). Further investigation is still required.

1.1.2.5. Structure of the Alpha 7 Nicotinic Acetylcholine Receptor

Upon completing translation of the α7 nAChR polypeptide, the first 22 amino acids of the mature protein, as coded for by exon 1 and the start of exon 2 and which forms the signal peptide, is removed by cleavage between Q22 and G23 (Séguéla *et al.* 1993; Avramopoulou *et al.*, 2004). The remaining 480 amino acids become a large membrane bound protein, which form part of a homopentameric α7 nAChR channel. The N-terminal 208 amino acids form an extracellular region that contains all the loops necessary for the binding of agonists and competitive antagonists (Arias, 1997). The rest of the peptide meanders between the extracellular and the cells' cytoplasmic environments by crossing the hydrophobic membrane four times; peptide areas referred to as membrane spanning regions (MSR) I – IV (Séguéla *et al.* 1993; Gault *et al.*, 1998). The assembly of these two

regions of the five separate α7 subunits into a functional α7 nAChR creates the N-terminal domain (NTD) and the ion channel domain (ICD) of the pentameric complex.

Attempts at obtaining x-ray crystallographic images of the structure of any AChR have met with little success (Avramopoulou *et al.*, 2004). The recent uncovering of the crystal structure of a mollusk *Lymnaea stagnalis* acetylcholine binding protein (AChBP) has shed more light on the structure of the N-terminal domain of α7 nAChR (Brejc *et al.*, 2001; Celie *et al.*, 2004; Celie *et al.*, 2005). The mature free-floating AChBP acts as a buffer to cholinergic neurotransmission by binding to acetylcholine (Smit *et al.*, 2001). The 210 amino acid long AChBP shows a significant 24% homology with the extracellular N-terminal 208 amino acids of α7 nAChR, as well as having all the structural hallmarks that can be found in the α7 extracellular domain (Smit *et al.*, 2003). In concordance with these structural similarities AChBP is able to form a homopentamer that actively binds αbtx and other α7 nAChR cholinergic ligands with comparable affinities, making it a suitable structural model for the α7 nAChR (Smit *et al* 2001; Brejc *et al.*, 2001; Smit *et al.*, 2003).

The acetylcholine binding protein monomer has a short α-helix at the N-terminus, followed by two short 3_{10} helices and ten β-strands, termed β1 – β10 (Brejc *et al.*, 2001). The protein conforms to a modified immunoglobulin (Ig) topology in which an inner β-sheet (composed of β1, β2, β3, β5, β6 and β8) is linked to an outer β-sheet (composed of β4, β7, β9 and β10) by means of a "Cys-loop" disulfide bridge between Cys123 and Cys136 and organized in a curled β-sandwich (Brejc *et al.*, 2001; Smit *et al.*, 2003). The Cys-loop is a feature of the N-terminal of the acetylcholine ion channels superfamily (other members being $GABA_A$ and glycine receptors), and can be found between Cys128

and Cys142 of α7 nAChR (Galzi *et al.*, 1991; Séguéla *et al.* 1993; Avramopoulou *et al.*, 2004). The only contacts between subunits of a pentamer are the two dimer interfaces of each protomer. There are five orthosteric nAChR ligand-binding sites, which can be located at these five protomer interfaces, close to the outside surface of the pentameric AChBP ring. The residues forming the orthosteric binding pockets are highly conserved between LGIC superfamily members, contradicting the low homology of the residues lining the interface between two protomers (Brejc *et al.*, 2001; Smit *et al.*, 2003). In addition, the α7 nAChR subunit contains three potential sites of N-linked glycosylation at N22, N68 and N111 within the extracellular domain, although, due to their distance from the ligand-binding sites, the absence of glycosylation does not appear to affect ligand binding (Marinou *et al.*, 2003). The binding of either an agonist or antagonist to the orthosteric binding pockets in the NTD cause changes in the molecular structure of the receptor, which gets translated down to the ICD resulting in the opening or closing of the ion channel.

The ion channel domain of α7 nAChR is composed of four putative α-helix membrane spanning regions consisting of 19 – 24 amino acids (MSR I – 24 aa, II – 19 aa, III – 20 aa, and IV – 19 aa) (Séguéla *et al.* 1993; Gault *et al.*, 1998). There exists a short cytoplasmic region between MSR I and II (7 aa), and a short extracellular region between MSR II and III (15 aa). A large intracellular domain exists, however, between MSR III and IV. This hydrophilic 154 amino acid domain contains multiple putative sites for the phosphorylation at S343 by cAMP-dependent protein kinase, at T393 and S405 by casein kinase II, and at Y420 by tyrosine kinase (Kemp and Pearson, 1990). No predicted site for protein kinase C was detected in the MSR III and IV cytoplasmic domain. After MSR

IV, the remaining 14 amino acid hydrophilic C-terminal of the peptide exits on the extracellular side of the membrane (Séguéla *et al.* 1993; Gault *et al.*, 1998).

Seminal work by Unwin and co-workers on the AChR at the neuromuscular junction of the electric ray *Torpedo californica* [$(\alpha 1)_2\beta 1\gamma\delta$] serves as model for the ion channel domain of α7 nAChR (Unwin *et al.*, 1988; Unwin, 1993; Unwin, 1995; Unwin, 1996). When the five α7 nAChR subunits come together to form a homopentameric ion channel, the central ion channel is mostly composed of the five α-helix-folded MSR II regions arranged symmetrically around an axis perpendicular to the membrane. The MSR II central core is supported by the other three MSR's in what appears to be a configuration resembling a 'starfish' (Bertrand *et al.*, 1993; Karlin, 1993; Unwin, 1993; Ortells and Lunt, 1996). In keeping with the 'starfish' analogy, the MSR IV α-helix forms the outside tip of a single pointed arm; the adjoining α-helix-turn-β-sheet MSR III and the β-sheet MSR I from the same peptide form the base of each arm surrounding the MSR II core channel (Unwin, 1993; Görne-Tschelnokow *et al.*, 1994; Ortells and Lunt, 1996). There exists minimal contact between the MSR II ion pore and the surrounding MSR I and III helical regions, with these two regions forming a scaffolding around the MSR II channel and sequestering it from the surrounding membrane. This water-filled cavity provides the space needed for the agonist-induced conformational changes in the ion channel which allows the influx of Ca^{2+} and cellular signalling (Unwin, 1995).

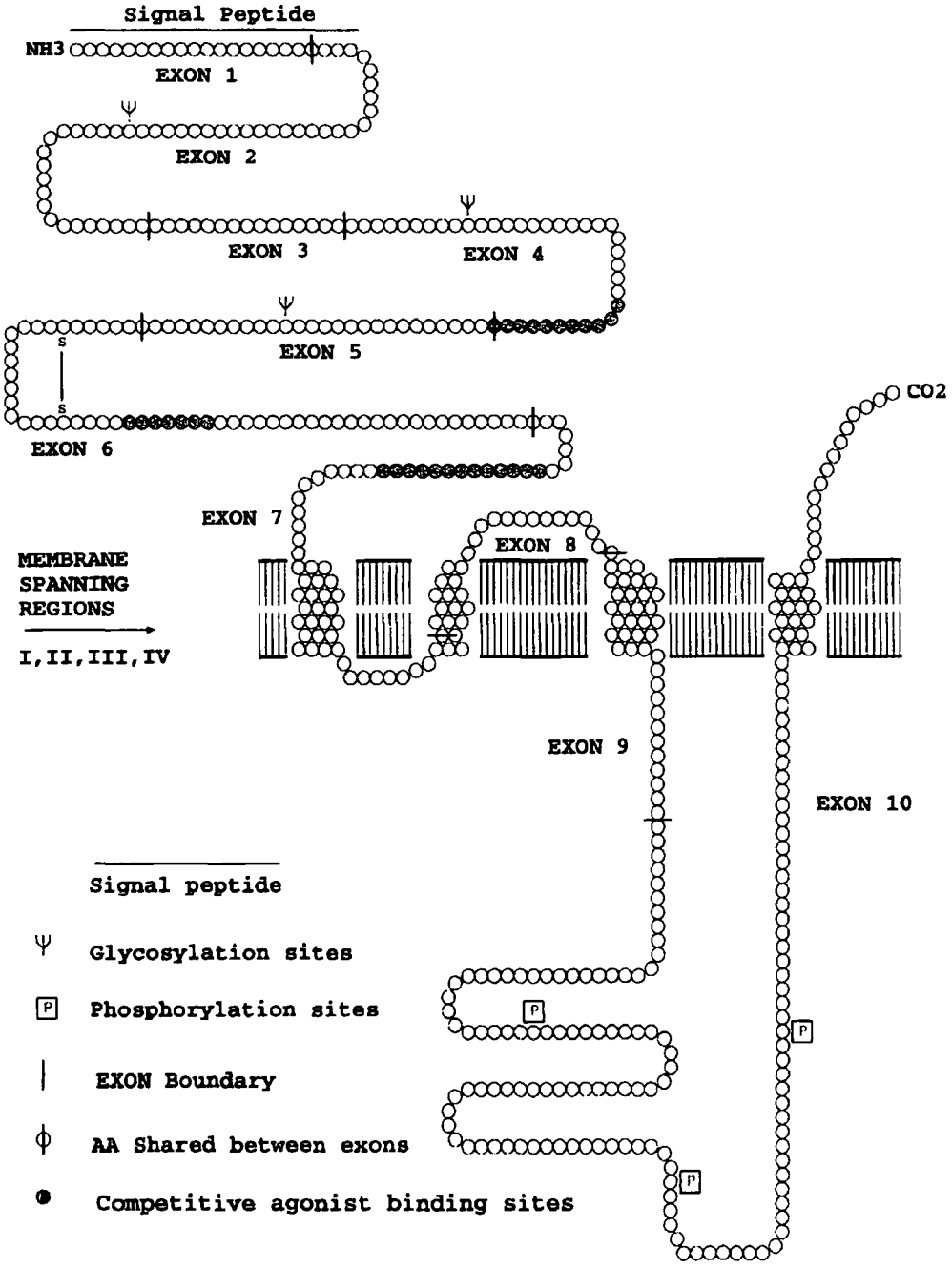


Diagram of the human α7 neuronal nicotinic receptor subunit (Gault *et al.*, 1998).

1.1.3. Ric3

Halevi *et al.* (2002) created a dominant mutation in the pore-forming transmembrane domain of DEG-3 acetylcholine receptor subunit of *Caenorhabditis elegans* (*C. elegans*) in a search for genes required for receptor activity. When assembled, this specific mutation in the DEG-3 subunit form a non-desensitizing channel in the receptor, ultimately leading to degeneration and necrosis of the neuron in which it is being expressed (Treinin and Chalfie, 1995; Treinin *et al.*, 1998) This experiment led to the identification of *ric3* (resistant to inhibitors of cholinesterase), miss-folding of which suppressed the DEG-3 mutational effect, providing the first indication that it was required for receptor functionality (Halevi *et al.*, 2002). Further investigation revealed that *ric-3* activity is required for the functional expression of multiple acetylcholine-specific ligand-gated ion channels, including the alpha 7 acetylcholine receptor, in both neuronal and muscle cells but is not necessary for the function of $GABA_A$, glycine and glutamate receptors in the same cells. A possible link between α7 nAChRs and Ric3 was first observed by Dominguez del Toro *et al.* (1994) who described how regions with high levels of *ric3* transcript expression correlate with α7 expression in the mouse brain, thus indicating an interaction between the two proteins.

Receptor subunits are folded and assembled into pentamers in the Endoplasmic Reticulum (ER) before being exported through the Golgi apparatus to the plasma membrane (Keller *et al.*, 2001). Coimmunoprecipitation experiments have provided evidence that ER-localised Ric3 associates with the unassembled acetylcholine receptor subunits – whether for correct folding or assembly is not known (Lansdell *et al.*, 2005; Williams *et al.*, 2005). The absence of Ric3 expression prevents the transportation of receptors from their assembly point in the

ER to the cell surface. Cell surface expression of the α-7 acetylcholine receptor has always been thought to be cell type specific (Cooper and Millar, 1997; Sweileh *et al.*, 2000). The heterologous expression of Ric3 greatly enhanced the rat α7 acetylcholine receptor activity in *Xenopus laevis* oocytes (Halevi *et al.*, 2003).

Ric3 is the only protein to date that has been described as specific to the maturation pathway of acetylcholine receptor processing pathway. Other non-specific proteins that have been implicated in acetylcholine receptor biogenesis are BiP, calnexin and 14-3-3η (Blount and Merlie, 1991; Chang *et al.*, 1997; Jeanclos *et al.*, 2001). Ric3's ability to specifically recognize and individually process different receptors is thought to provide an additional regulatory mechanism to distinguish between different groups of receptors for cell surface expression.

Sequence homology alignments indicate that *ric3* is a member of a conserved gene family with representatives in both vertebrates and invertebrates. Like its *C. elegans* homolog, the human *ric3* homolog (*hric3*) can also be heterologously expressed in *Xenopus laevis* oocytes leading to enhanced whole-cell current amplitudes when co-expressing α7 nAChR. The same enhanced amplitudes can be attained when co-expressing *hric3* with either rat α7 nAChR or *C. elegans* DEG-3/DES-2 receptor in *Xenopus* oocytes. This implies an evolutionary conservation of the interactive regions of *ric3* and respective receptor partners. It should be noted, however, that this conservation does not extend to all the members of the nAChR family. Contrary to the results attained with other members of the nAChR family, the co-expression of *hric3* with α4β2 and α3β4 receptors in the oocytes lead to a decrease in the whole-cell current amplitude (Halevi *et al.*, 2003). This, however, is not the case where expression of *hric3* with α4β2 and α3β4 receptors occurs in homologous

mammalian cells. Ric3 significantly enhances the functional expression levels of these receptors, as well as those of heteromeric receptors α3β2 and α4β4 in mammalian cells. This phenomenon seems to indicate that the effect of Ric3 upon the functional expression of nAChRs can be influenced by other host cell factors (Lansdell *et al.*, 2005). Of the homomeric nAChRs, only α7 and closely the related α8 had enhanced levels of receptor formation in the presence of Ric3 in otherwise nonpermissive mammalian cells (Lansdell *et al.*, 2005; Williams *et al.*, 2005).

Multiple transcripts for the *hric-3* resulting from editing, alternative splicing, and alternative promoters are differentially expressed in a variety of tissues, including non-excitable ones. The purpose for this complexity is unknown at this stage although three possible options remain: 1) functional proteins that differentially affect the maturation machinery are produced; 2) functional proteins are produced whose function is unrelated to nAChR maturation; or 3) the shorter isoforms are non-functional (Halevi *et al.*, 2003).

Initial analyses of the Ric3 amino acid interspecies sequences indicated that all of the proteins consist of two transmembrane domains, separated by a proline-rich spacer and followed by extensive coiled-coil domains (Halevi *et al.*, 2002 and 2003). Using more recent databases, Cheng *et al.*, (2005) concluded that only a single transmembrane region can be detected in the Ric3 amino acid sequence and that the first transmembrane region described by Halevi *et al.* (2002) is in reality a signal sequence. Both the N – and C-terminus coiled-coil are predicted to be cytosolic, implying that Ric3 acts as an adapter protein – bringing together receptors and proteins in a multiprotein maturation complex. It is possible that the proline-rich spacer between the two transmembrane domains serve as a

structural scaffold on which interacting partners can be anchored. The transmembrane domain may also serve a similar function.

1.1.4. Alpha 7 Nicotinic Acetylcholine Receptor Upregulation and Neuroprotection

The generally accepted paradigm for receptor regulation is for receptors to be down regulated when over exposed to an agonist and upregulated after overexposure to an antagonist. It appears, however, that α7 nicotinic acetylcholine receptors (nAChRs) and nicotinic receptors in general, contradict this convention. This phenomenon becomes apparent in postmortem binding studies using [^{3}H] nicotine and [^{3}H] acetylcholine when comparing the brains of smokers and non-smokers, with a positive correlation existing between the amount of cigarettes smoked and an increase in the number of binding sites (Benwell *et al.*, 1988; Breese *et al.*, 1997; Nybäck *et al.*, 1989). The number of binding sites was, however, lower in ex-smokers than in non-smokers. In rodents, chronic treatment *in vivo* with nicotine also results in a comparable increase in the number of high affinity binding to [^{3}H] nicotine and [^{125}I] α bungarotoxin (αbtx) – an effect that reflects an increase in the number of high affinity nAChRs with no increase in the receptor's affinity (Romanelli *et al.*, 1988; Wonnacott, 1990; Flores *et al.*, 1992; Barrantes *et al.*, 1995; Rowell and Li, 1997; Gopalakrishnan *et al.*, 1997). (–)-Nicotine, the prototypical agonist for neuronal nAChR, non-selectively activates nAChRs but binds with a higher affinity to α4β2 nAChRs than to the α7 nAChR. High affinity binding to [^{3}H] nicotine is associated with α4β2 nAChRs (Flores *et al.*, 1992; Zoli *et al.*, 1998) whereas the increased binding to [^{125}I] αbtx reflects an increase in α7 nAChRs (Séguéla *et al.*, 1993; Barrantes *et al.*, 1995; Orr-Urtreger *et al.*, 1997). It should be noted though that the increase in [^{125}I] αbtx binding is weaker than that of high affinity binding to [^{3}H]

nicotine, and can only be achieved *in vivo* with much higher concentrations of chronic nicotine treatment – concentrations that might not be relevant to human smokers (Wonnacott, 1990; Pauly *et al.*, 1991). Despite this, chronic nicotine treatment is still able to increase [^{125}I] αbtx in various cell types *in vitro* e.g. primary hippocampal cultures and SH-SY5Y cells (Barrantes *et al.*, 1995; Peng *et al.*, 1997), as well as α7 nAChR-transfected cell lines such as GH4C1 rat pituitary clonal line and HEK 293 cells (Quik *et al.*, 1996; Molinari *et al.*, 1998).

The reason for the receptor upregulation remains unclear although it has been speculated that the receptor's rapid desensitization property and inactivation upon chronic exposure to an agonist leads to a deficit in cholinergic function that is counteracted by an increase in receptor number (Schwartz and Kellar, 1985). This is supported by the fact that chronic nicotine exposure of oocytes expressing α7 nAChRs results in the irreversible inactivation of the majority of α7 receptors, indicating multiple states of recovery from desensitization (Olale *et al.*, 1997; Reitstetter *et al.*, 1999). Desensitization and recovery or eventual inactivation and subsequent upregulation seem to be dependent on the nature of the agonist and the length of exposure to the particular agonist (Olale *et al.*, 1997; Rowell and Duggan, 1998; Reitstetter *et al.*, 1999). The chronic exposure of human α7 nAChR expressed in HEK 293 cells to agonists like epibatidine, anabaseine and 1,1-dimethyl-4-phenylpiperazinium (DMPP) resulted in an increase of [^{125}I] αbtx sites, an effect that was observed to be concentration dependent (Molinari *et al.*, 1998). Treatment of these cells with 100 μM nicotine resulted in a 2.5 fold increase of [^{125}I] αbtx sites in a concentration dependent manner.

The structural changes wrought by the chronic binding of an agonist leading to the irreversible inactivation of the receptor, is also thought to prevent the receptor from being removed from the cell surface (Peng *et al.*, 1994). The reduced turnover yet constant cellular production of receptors accounts for the observed increase in cell surface nicotinic acetylcholine receptors. This view is supported by the observations that no corresponding mRNA upregulation can be found during chronic nicotine treatment with the nAChR upregulation, thus implicating the involvement of a posttranscriptional mechanism (Marks *et al.*, 1992; Peng *et al.*, 1994; Bencherif *et al.*, 1995; Zhang *et al.*, 1995). Activity of certain nAChRs seems to be dependent on phosphorylation by protein kinase C (PKC). Inhibition of PKC by protein kinase A (PKA) leads to deactivation of the receptors by dephosphorylation of the PKC sites of the receptor protein (Madhok *et al.*, 1995; Gopalakrishnan *et al.*, 1997; Hsu *et al.*, 1997; Eilers *et al.*, 1997). It is thought that reactivation of the receptors require phosphorylation at these same PKC sites. This mechanism applies to α4 nAChRs but not α7 nAChRs, as the latter lacks a PKC phosphorylation site (Gault *et al.*, 1998).

As stated before, Chazot *et al.* (2002) reported a 12–15-fold increase in α7 nAChR protein following KCl-induced cortical spreading depression in the mouse cerebral cortex, with an unaltered expression of α4 nAChR subunit protein (Chazot *et al.*, 2002). Further *in vivo* support comes from epidemiological and clinical studies that have revealed that the amount of α7 subunit mRNA was significantly higher in the putamen of patients with multiple small infarcts (*status lacunaris*) compared with controls (Utsugisawa *et al.*, 1999). This is in contrast to findings on α4 and β2 subunit mRNA expression, which were lower in the putamen of patients with *status lacunaris* compared with controls (Tohgi *et al.*, 1998). The mechanism by which this upregulation occurs seems to differ

from that mediating agonist-induced α7 nAChR upregulation (De Koninck and Cooper, 1995; Ridley *et al.*, 2001). Contrary to the above mentioned mechanism, the upregulation of α7 nAChRs upon chronic KCl depolarization is accompanied by a corresponding increase of α7 nAChR mRNA (De Koninck and Cooper, 1995; Utsugisawa *et al.*, 1999; Ridley *et al.*, 2001; Chazot *et al.*, 2002). This upregulation is specific to the α7 subset of receptors, with α3, α4, α5, β2 and β4 subunit mRNA and protein expression levels remaining mostly unaffected (De Koninck and Cooper, 1995; Tohgi *et al.*, 1998; Utsugisawa *et al.*, 1999; Chazot *et al.*, 2002). Upregulation of α7 nAChR mRNA is thought to result from an influx of Ca^{2+} through L-type Ca^{2+} channels and activation of a Ca^{2+}-calmodulin dependent kinase (CaM-kinase II) pathway in a PKA/ PKC-independent manner (De Koninck and Cooper, 1995; Ridley *et al.*, 2001). KN-62, a CaM-kinase II inhibitor, not only blocks the upregulation of α7 nAChR mRNA upon KCl stimulation, but also the transcription of Egr-1, further lending evidence to the argument that Egr-1 is responsible for transcriptional activation of the α7 gene (De Koninck and Cooper, 1995; Enslen and Soderling, 1994).

Alpha 7 nicotinic acetylcholine receptors play an important role in neuroprotection. Co-incubating cells with nicotine has been demonstrated to be neuroprotective against various lethal insults: cultured striatal neurons against an excitotoxic NMDA insult (Marin *et al.*, 1994); mixed cortical neuronal and glial cultures against a glutamate insult (Donnelly-Roberts *et al* 1996); cultured hippocampal neurons against kainic acid-induced neurotoxicity (Semba *et al.*, 1996); pure hippocampal neuronal cultures against a NMDA stimulus (Dajas-Bailador *et al.*, 2000); PC12 cells against hypoxia (Tohgi *et al.*, 2000); and the protection of pure neuronal primary cortical cultures against a hypoxic insult (Hejmadi *et al.*, 2003). Alpa-bungarotoxin was able to block nicotine's neuroprotective

effect in each of these studies, clearly indicating that although nicotine has a higher affinity for α4β2 nAChRs, the neuroprotective effect of nicotine is mediated by homomeric α7 nAChRs through an as yet unknown Ca^{+2} dependent mechanism (Donnelly-Roberts *et al.*, 1996; Dajas-Bailador *et al.*, 2000; Tohgi *et al.*, 2000). In the Hejmadi *et al.* (2003) study, activation by nicotine of both the α7 nAChR and the α4β2 nAChR was required for protection against the hypoxic insult. Unfortunately, the role played by α4β2 nAChR was not investigated in the other studies, making it impossible to say whether or not α7 nAChR alone was responsible for mediating the nicotine-induced neuroprotection (Donnelly-Roberts *et al.*, 1996; Dajas-Bailador *et al.*, 2000; Tohgi *et al.*, 2000).

Chronic exposure of PC12 cells to nicotine is not only neuroprotective, but this treatment is also known to upregulate α7 nAChR subunit expression (Jonnala and Buccafusco 2001). Jonnala and Buccafusco (2001) analysed the neuroprotective profiles of different α7 nAChR agonists. The agonists' rank order of potencies (EC_{50}) for activating α7 receptors correlated well with their rank order of binding affinities (K_i) for the α7 receptor but lacked correlation with the agonists' ability to elicit a neuroprotective mechanism for cell survival. This is indicative of a mechanism involved in α7 mediated neuroprotection that requires more than simply activating α7 receptors and might be a balance between the acute agonist action and the protracted antagonist/ agonist desensitization action characteristic to each chemical (Meyer *et al.* 1998; Briggs and McKenna, 1998). The research group managed to link the attained level of neuroprotection to the level of α7 nAChRs upregulation, after drug treatment, on the cell surface in PC12 cells. More importantly, Jonnala and Buccafusco (2001) found that by increasing the amount of α7 nAChRs on the cell surface, they were able to increase

nicotine's attained level of neuroprotection. It is, however, important to note that treatment of cells with the α7-prefering antagonist methyllycaconitine (MLA) increases the population of functional α7 nAChRs but is not neuroprotective (Jonnala and Buccafusco 2001; Molinari *et al.*, 1998).

It is also possible to precondition cells *in vitro* with nicotine against a subsequent lethal insult. Akaike *et al.* (1994) preconditioned mixed cortical neurons and glial cells by the addition of 10 μM nicotine for two hours against an acute ten minute 1 mM glutamate insult. The nicotine treatment was also able to ameliorate NMDA insults but no protection was generated against AMPA or kainate exposure. It is unlikely that that a direct action of nicotine on the NMDA receptor-ion channel complex was the cause of the nicotine-induced neuroprotection since concomitant incubation of nicotine with the insult proved to be ineffectual. The short extent of the nicotine incubation makes it doubtful that the generated protection was due to *de novo* protein synthesis, although enough time was required for either cell signalling or receptor inactivation given that a thirty minute preconditioning period proved to be unsuccessful. Kaneko *et al.* (1997) also preconditioned mixed cortical neurons and glial cells with 10 μM nicotine but for 24 hours, not two hours as in the Akaike *et al.* (1994) study, before the acute glutamate insult. Concomitant treatment with either α7 nAChR agonist αbtx or α4β2 nAChR agonist dihydro-β-erythroidine (DHβE), resulted in a reduction but not the abolishment of the amount of protection generated by preconditioning with nicotine against the glutamatergic insult. This indicates that the preconditioning effect is due to the additive actions of α7 and α4β2 nAChRs (Kaneko *et al.*, 1997). The researchers in this study could not rule out the possibility that the observed protection was largely due to the inactivation of the nAChR following the long-term exposure to nicotine.

Controversial *in vivo* studies have shown that nicotine could be protective against neurodegeneration induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Maggio *et al.*, 1998), as well as quinolinic acid evoked neuronal death (O'Neill *et al.*, 1998) and systemic kainic acid toxicity (Borlongan *et al.*, 1995).

1.1.5. Alpha 7 Nicotinic Acetylcholine Receptor and Anxiety

Anxiety can be defined as a future-oriented state involving perceived uncontrollability and unpredictability over dangerous events or the individual's emotional response to those events (Barlow, 2002). There exists a well documented connection between anxiety disorders and cigarette smoking (Russel, 1970; Lasser *et al.*, 2000). Patients with an anxiety disorder without depression were found to be twice as likely to smoke as individuals without any psychiatric disorders (Breslau *et al.*, 1991). Individuals with generalized anxiety disorder were more likely to become lifetime smokers and found it harder to stop smoking, than individuals without this disorder (Covey *et al.*, 1993). Patients with a past history of major depression disorders and with a high anxiety sensitivity score have a higher tendency of relapsing within the first seven days of smoking cessation (Brown *et al.*, 2001). Anxiety is commonly implicated by smokers as a risk factor for the relapse to smoking (Shiffman, 1982; Brandon *et al.*, 1990). Patients with agoraphobia have a higher rate of smoking (Black *et al.*, 1999). Patients with panic disorders where the illness started after the usual age of onset have a higher rate of smoking but patients with a preadolescent onset of the illness essentially did not smoke (Pohl *et al.*, 1992). However, no correlation could be found between panic disorders and smoking cessation. Interesting enough, it has been found that daily smoking increases the risk for the first occurrence of panic attack and disorder, with the risk higher in active

than in past smokers (Breslau and Klein, 1999). It should also be noted that there exist conflicting reports concerning the relative anxiety levels experienced post smoking cessation. Some findings conclude that withdrawal can be anxiolytic, with higher levels of anxiety experienced pre-cessation, whereas other reports state it is anxiogenic in that the levels of anxiety experienced were the anxiety levels experienced were lower before the subjects stopped smoking (Gross and Stitzer, 1989; Hughes *et al.*, 1994; West and Hajek, 1997).

Nicotine was shown to be the active anxiolytic ingredient in smoking in experiments where subjects received varying yields or no nicotine in cigarettes before being subjected to a shock and their anxiety levels measured (Silverstein *et al.*, 1982; Pomerleau *et al.*, 1984; Kassel and Unrod, 2000; Juliano and Brandon, 2002). Nicotine and some, but not all, nicotinic receptor agonists have an anxiolytic effect in animal models as well as in humans (Pomerleau, 1986; Brioni *et al.*, 1993; Decker *et al.*, 1995; Potter *et al.*, 1999). Also, of note is the fact that although low doses of nicotine have been shown to be anxiolytic, the administration of higher doses in rats can be anxiogenic (File *et al.*, 1998). The differential behavioural profiles induced by the various nicotinic receptor agonists, as well as the concentration dependent manner in which nicotine affects behaviour, indicate that specific subtypes of nAChR configurations play a role in anxiety and the anxiolytic effect of nicotine.

The pivotal role of the α4 nAChR subunit in nicotine binding and anxiety has been well established. The α4 subunit in combination with the β2 subunit form the heteromeric α4β2 nAChR which has a greater affinity for nicotine than the homomeric α7 nAChR. The anxiolytic effect of a low dose of nicotine in the dorsal Raphé nucleus, the crucial

structure in mediating the anxiolytic effect of nicotine, can be blocked by the α4β2 high affinity antagonist dihydro-β-erythroidine (DHβE) (Cheeta *et al.*, 2001a; Cheeta *et al.*, 2001b). The α4-subunit knockout mice model show apparent increased basal levels of anxiety during the elevated plus-maze assay. In this test, the application of nicotine had little effect on the anxiety levels of the animals except in the later stages of the assay with unhabituated mutant animals (Ross *et al.*, 2000).

Contrary to α4β2 nAChR's role in mediating nicotine's anxiolytic effect, the α7 nAChR seems to be involved in mediating nicotine's anxiogenic behavioural effect. Alpha 7 nAChR subunit knockout mice showed an increase in the time spend in the centre of an open field platform, indicating a reduction in anxiety levels (Paylor *et al.*, 1998). From studies performed by File and colleagues, it would seem that α7 nAChRs and the binding of nicotine to this receptor subtype are involved with increased anxiety levels (File *et al.*, 1998; File *et al.*, 2000a; File *et al.*, 2000b; Tucci *et al.*, 2003). This receptor is highly expressed in the dorsal hippocampus (Fabian-Fine *et al.*, 2001), a region that has been shown to mediate nicotine's anxiogenic effect (File *et al.*, 1998; File *et al.*, 2000a; File *et al.*, 2000b). The co-administration of nicotine with methyllycaconitine, an antagonist at α7 and α3 nAChR subunits, to the dorsal hippocampal region blocked any anxiogenic effect the application of a high concentration of nicotine would have had, although co-administration with DHβE (an antagonist of α4β2 and α3β2 receptors) had no effect on nicotine's anxiogenicity (Tucci *et al.*, 2003). Alpha3 nAChRs have not been detected in the dorsal hippocampus and only low levels of mRNA, further eliminating the role that this subunit may play in nicotine's anxiogenic effect (Wada *et al.*, 1989; Whiteaker *et al.*, 2002). The anxiogenic effect of nicotine has been shown to be facilitated by the release of 5-hydroxytryptamine (5-HT) in the dorsal hippocampus which then binds to the 5-HT_{1A}

receptors (Kenny *et al.*, 2000; Cheeta *et al.*, 2000a; Cheeta *et al.*, 2000b; Cheeta *et al.*, 2001a). In nicotine-treated hippocampal slices methyllycaconitine succeeded in blocking the release of 5-HT, whereas DHβE failed, indicating that α7 nAChRs are responsible for the release of 5-HT (Tucci *et al.*, 2003).

It can be concluded that a possible explanation for the bimodal effect of nicotine in anxiety is that low concentrations of the drug first binds to the high-affinity α4β2 receptor thus leading to the observed anxiolytic behaviour but with higher concentrations and possible α4β2 receptor saturation, binding to the lower affinity α7 nAChRs occur more frequently leading to the observed anxiogenic behaviour.

1.2. Aims of the Investigation

1. Creation of a functional and reliable *in vitro* model for studying spreading depression preconditioning.

2. Determination whether α7 nicotinic acetylcholine receptor upregulation (nAChR) induced by preconditioning contributes effectively to the subsequent increase in the brain tolerance to ischaemia.

3. The generation of an anti-Ric3 antibody to study the relationship between Ric-3 and α7 nAChRs.

4. To determine whether increased expression of α7 nAChRs by itself, without pharmacological intervention, is sufficient to increase the brain tolerance to ischaemia to a level similar to that achieved with CSD preconditioning.

CHAPTER 2: Generation of an *In Vitro* Preconditioning Model and Analysing the Role Played by Alpha 7 Nicotinic Acetylcholine Receptors.

2.1. Introduction

Neuronal tissue culture have been important experimental models for the study of the cellular and molecular mechanisms underlying excitotoxicity leading to direct applications for the study of neurodegenerative processes *in vivo* (Choi *et al.*, 1987; Akaike *et al.*, 1994; Doble, 1999). The first aim of this study was to establish an *in vitro* model for the study of spreading depression-induced preconditioning that could protect against an excitotoxic insult. Such a model is not only of scientific importance but would also provide advantageous at various practical levels, e.g. the reduction of labour, time and cost; repeatability; ease of drug application. There is also the ethical impact that should also be considered, i.e. the reduction in animal use. In an *in vivo* experiment, a single animal leads to a single increase in *n*-numbers, whereas in an *in vitro* experiment a single animal could potentially lead to as many as three hundred *n*-numbers.

As has been clearly illustrated by the *in vivo* models, preconditioning is a fine balance between a minor insult that would be ineffectual, an excessive insult which would result in cellular death, or the appropriate insult that would engage the innate survival mechanisms (Horiguchi *et al.*, 2005). To date, little success has been attained in the creation of an *in vitro* model for KCl-dependant preconditioning. Grabb *et al.* (1999, 2002) produced a mixed neuronal and glial cortical culture that relied upon a 15 minute preconditioning period with 45 mM KCl protecting against a 10 minute 1 mM glutamate insult – 24 hours subsequent to the preconditioning event. Hester *et al.* (2007)

preconditioned the DRG/neuroblastoma hybrid cell line F-11 and cerebellar granule neurons isolated from 7- to 9- day-old mice for 90 minutes with 50 mM KCl, washed the cultures and then acutely applied the 3 hour 1 mM potassium cyanide insult; with viability being measured 24 hours later. The short KCl treatment period and the acute delivery of the insult in this latter study, makes this an acute phase preconditioning event where the observed protection is likely due to changes in the ion channel permeability, protein phosphorylation and other post-translational modifications (Perez-Pinzon *et al.*, 1997; Pérez-Pinzón and Born, 1999; Stagliano *et al.*, 1999; Lu *et al.*, 2005).

The creation of an *in vitro* preconditioning model is merely the first step in analyzing the importance of the Chazot *et al.* (2002) observation that an increase in α7 nAChRs might play an important role in *in vivo* cortical spreading depression-induced preconditioning. As discussed earlier, this receptor has been shown to play an important role in neuroprotection, possibly in conjunction with α4β2 nAChRs (Donnelly-Roberts *et al.*, 1996; Dajas-Bailador *et al.*, 2000; Tohgi *et al.*, 2000; Hejmadi *et al.*, 2003). Whether or not a simple change in activity of the α7 nAChRs is required without any need for an increase in receptor numbers remains unknown. Both nicotine and potassium chloride treatments have been shown to upregulate α7 nAChRs, albeit through different mechanisms (Olale *et al.*, 1997; Rowell and Duggan, 1998; Reitstetter *et al.*, 1999; De Koninck and Cooper, 1995; Utsugisawa *et al.*, 1999; Ridley *et al.*, 2001; Chazot *et al.*, 2002). This is another factor to be addressed within this chapter.

2.2. Materials and Methods

2.2.1. Neuronal Cortical Culture

Rat cortical regions were removed from day 17 embryonic Sprague Dawley rats (Charles River) and maintained at 4 °C in Hanks Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+} (5 mM KCl; 0.4 mM KH_2PO_4; 137 mM NaCl; 4 mM $NaHCO_3$; 0.3 mM Na_2HPO_4; 5 mM Glucose; 5 mM HEPES; 1 mM Sodium Pyruvate). Cortical neurons were dispersed by triturating (10x) with a fire-polished Pasteur pipette in 1 ml of the maintenance solution. 2x HBSS containing Ca^{2+} and Mg^{2+} (5 mM KCl; 0.4 mM KH_2PO_4; 137 mM NaCl; 4 mM $NaHCO_3$; 0.3 mM Na_2HPO_4; 1.25 mM $CaCl_2$; 0.5 mM $MgCl_2$; 0.85 mM $MgSO_4$; 5 mM Glucose; 5 mM HEPES; 1 mM Sodium Pyruvate) was added to the neuronal suspension. Non-dispersed tissue settled within 90 seconds and was removed before the solution was centrifuged for 1 minute at 300 rpm. The supernatant was removed and the cells were gently resuspended in 5 ml cold HBSS containing Ca^{2+} and Mg^{2+}.

The surface of Iwaki 24 well tissue culture treated microplates were coated with poly-D-Lysine (0.05 g/ l) for 1 hour before being washed with water and allowed to air-dry. For the mixed glial and neuronal cortical cultures, a cell suspension of 10^5 cells/ cm^2 was created in culture medium consisting of 80 % Eagle's Minimum Essential Medium (MEM), 10 % heat-inactivated fetal bovine serum (FBS), 10 % heat-inactivated horse serum, 25 mM glucose, 2 mM L-glutamine, 20 units/ ml penicillin and 20 µg/ ml streptomycin before plating them in 500 µl medium on poly-D-Lysine coated 24 well plates. The cultures were incubated at 37 °C in 5 % CO_2. The mixed culture population containing both neurons and glial cells were treated on day *in vitro* (DIV) 4 with 15 µg/

ml 5-fluoro-2'-deoxyuridine and 35 μg/ ml uridine to minimize glial growth. One half of the medium was replaced on DIV 6 with growth medium consisting of 90 % MEM and 10 % heat-inactivated horse serum.

Pure neuronal cortical cultures were created by seeding the plates with a concentration of 1500 cell/ mm^2 (unless otherwise stated) in 500 μl culture medium consisting of Neurobasal, 2 % B27 supplement, 20 units/ ml penicillin, 20 μg/ ml streptomycin, 2.5 μM L-glutamate and 0.5 mM Glutamax-1. The cultures were incubated at 37 °C in 5 % CO_2. On DIV 3 and DIV 7, 200 μl of the culture medium was removed and the cultures were fed with 300 μl feeding medium (Neurobasal, 2 % B27 supplement, 20 units/ ml penicillin, 20 μg/ ml streptomycin, 0.5 mM Glutamax-1).

2.2.2. Mixed Glial and Neuronal Cortical Culture Treatments

On DIV 14 culture medium was removed and the cultures were exposed to either 1 μM MK 801 for 30 minutes, 10 μM nicotine for 1 hour or 45 mM KCl for 15 minutes, all dissolved in Exposure Buffer Saline (EBS) (137 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 0.3 mM Na_2HPO_4, 0.3 mM KH_2PO_4, 4 mM $NaHCO_3$, 5.6 mM Glucose, 0.01 mM Glycine, 10 mM HEPES, pH 7.4). The EBS in which the KCl was dissolved only contained 97 mM NaCl to maintain osmotic pressure. Controls were exposed to drug-free EBS solution and received all the washing steps in conjunction with the treatments. This was followed by removal of the drug and three wash steps with EBS before the culture medium was reapplied and cultures incubated for either a 24 or a 48 hour time period. The lethal insult, consisting of the indicated concentration of N-Methyl-D-aspartic acid (NMDA) dissolved in EBS, was applied for a specific time after the medium was

removed once again. A subsequent three further washes with EBS preceded the reapplication of the culture medium (fresh MEM containing only 20 units/ ml penicillin and 20 μg/ ml streptomycin for LDH purposes), followed by a further 24 hours incubation period.

2.2.3. Pure Neuronal Cortical Treatments

On DIV 14 (unless stated otherwise) culture medium was removed and the cultures were exposed for the allotted time to 500 μl Exposure Buffer Saline (EBS) (137 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 0.3 mM Na_2HPO_4, 0.3 mM KH_2PO_4, 4 mM $NaHCO_3$, 5.6 mM Glucose, 0.01 mM Glycine, 10 mM HEPES, pH 7.4) containing the indicated doses of preconditioning drugs, or blank EBS solution for the controls, which were processed in parallel. For 24 hour incubation treatments, the drug was dissolved in culture medium. Dissolving the chemical KN-62 required the solvent dimethyl sulfoxide (final concentration 0.1 %), which was also added to control cells in this experiment. Treatment was followed by removal of the drug and three wash steps with EBS before the culture medium was reapplied and cultures incubated for the required time period. The lethal insult, consisting of either of 1 hour incubation with 1 mM or 200 μM NMDA, or 10 minutes with 1 mM glutamate dissolved in EBS, was applied after the medium was removed once again. A subsequent three wash steps with EBS preceded the reapplication of the used culture medium, followed by a further 24 hours incubation period and MTT cell viability assessment

2.2.4. Oxygen Glucose Deprivation

On DIV 8 pure neuronal cortical cultures were preconditioned as above. Twenty-four hours later the medium was removed inside of an Anaerobic Workstation (Whitley Scientific) where the atmosphere consisted of 5 % CO_2, 10 % H, 85 % N. Cultures were washed twice with a balanced salt solution lacking any glucose or oxygen (0.8 mM $MgSO_4$, 1 mM KH_2PO_4, 26 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 117 mM NaCl, 4.5 mM KCl, 10 mM HEPES, 0.01 mM Glycine). Subsequent to this, cultures were incubated in the chamber for the necessary amount of time before being removed, the normal culture medium reapplied, and incubated at 37 °C in 5 % CO_2. Control cultures received the same treatments although the balanced salt solution contained 5.6 mM glucose and were conducted in an aerobic environment. In addition to this, the control cultures were incubated in an aerobic environment for the same amount of time as the cultures in the Anaerobic Workstation. The MTT cell viable assay was performed 24 hours later.

2.2.5. Lactate Dehydrogenase Assay

Neuronal injury and death was assessed by the measurement of lactate dehydrogenase (LDH) efflux into fresh MEM 24 h after every lethal insult using a CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega). In brief; an equal volume of the culture MEM supernatant was incubated with reconstituted Substrate Mix for 30 minutes in the dark before and equal volume of Stop Solution was added. Absorbance was read at 490 nm. Normalization of the plates were achieved by freeze-thawing the cells in MEM after the removal of the initial MEM for LDH assay and calculating the percentage of the initial LDH release over the total LDH for every well.

2.2.6. MTT Assay

MTT (1-(4, 5-Dimethylthiazol-2-yl)-3,5-diphenylformazan) is a measure of cell viability by mitochondrial dehydrogenase. 50 µl Phosphate Buffered Saline (PBS) (136.9 mM NaCl, 2.68 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) containing 5 mg/ ml MTT was added to the cultures and incubated at 37 °C for 2.5 hours. The MTT-containing medium was removed, the surface of the wells were rinsed with 300 µl PBS before the application of 250 µl isopropanol. After the crystals dissolved, the optical density of 100 µl sample was spectrophotometrically read at 595 nm.

2.2.7. Chloroform/ methanol protein precipitation

Tissue or harvested cell cultures were homogenized in phosphate buffered saline (PBS) containing Protease Inhibitor Cocktail Set III (Calbiochem), 1/100 dilution. Protein samples for SDS-PAGE analyses were precipitated by the Chloroform/ methanol method (Wessel and Flügge, 1984). Four volumes of methanol was added to the protein samples (25 – 50 µg) followed by vortexing and 1 minute centrifugation at 12 000 rpm at room temperature. This was followed by the addition of one volume chloroform, vortexing and 1 minute centrifugation at 13000 rpm at room temperature. Three volumes of water were added to the mixtures before commencing with a repeat of the vortexing and centrifugation steps. Subsequent to the centrifugation step, the upper layer was removed and discarded without disturbing the intermediate phase containing the protein fraction. One volume of methanol was added to the remaining suspension, vortexed and centrifuged for 4 minutes. The supernatant was removed and the protein samples air-dried before the samples were resuspended in the correct amount of sample buffer. The

protein samples were resuspended in 6 µl H_2O before adding equal volumes of 2 x protein sample buffer (PSB) 0.125 M Tris-HCl pH 6.8, 4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol) and 1.5 µl 100 mM dithiothreitol (DTT). The samples were then denatured for 3 minutes at 95 °C and incubated for 1 min on ice before analysis by SDS-PAGE.

2.2.8. SDS-Polyacrylamide Gel Electrophoresis

A 6 % SDS-PAGE gels were made as described by Laemmli (1970). The separating gels consisted of 0.375 M Tris-HCl pH 8.8, 0.1 % SDS, 12 % acrylamide and 0.32 % bisacrylamide. Polymerisation of the gels occurred by the addition of 0.008 % (v/v) tetra-methyl-ethylene-diamine (TEMED) and 0.08 % (w/v) ammonium peroxysulfate. The stacking gel contained the same except for 0.125 M Tris-HCl pH 6.8 and 0.1 % SDS. TGS buffer (0.025 M Tris-HCl pH 8.3, 0.192 M glycine, 0.1 % SDS) was used for the protein electrophoresis which occurred using a Hoefer Mighty small II Vertical Slab Gel SE 250 unit (Hoefer Scientific Instruments). Electrophoresis was carried out for approximately 2 hours at 15 mA.

2.2.9 Western Blot Protein Analysis

The proteins were transferred from the SDS-PAGE gel (section 2.2.8) to a Hybond™-ECL™ Nitrocellulose membrane in a Hoefer TE series Transphor Tank (Hoefer Scientific Instruments) using transfer buffer consisting of 25 mM Tris, 192 mM glycine, pH 8.4, and 20 % (v/v) methanol. 50 volts was constantly passed through the tank for a period of 2.5 hours.

Following the protein transfer, the nitrocellulose membrane was briefly rinsed in Tris Buffer Saline (TBS) (50 mM Tris-HCl, 9 % w/v NaCl, pH 7.4) before being incubated for 1 hour at room temperature in blocking buffer consisting of 5 % (w/v) dried milk and 0.02 % (v/v) Tween-20 dissolved in TBS. After this blocking step, the appropriate primary antibody was added in an incubation solution consisting of 2.5 % (w/v) dried milk powder dissolved in TBS and incubated for 12 hours at 4 °C with gentle shaking.

Subsequent to the incubation with the primary antibody, the nitrocellulose membrane underwent four 10 minute washes with wash buffer containing TBS with 2.5 % (w/v) dried milk and 0.2 % (v/v) Tween-20. Depending on the host the primary antibody was raised in, the nitrocellulose membrane was incubated with either mouse, rabbit or goat horseradish peroxidase (HRP) labelled secondary antibody (1:2000) for 1 hour at room temperature with gentle shaking. The membrane underwent four more washes as before the 1 hour incubation step before being briefly rinsed in TBS. For the visualization of the chemiluminescent bands, the nitrocellulose membrane had to be incubated for 1 minute at room temperature with a development solution containing 68 mM p-couramic acid (100 µl), 1.25 mM luminol (10 ml) and 30 % H_2O_2 (6 µl). The fluorescent bands were exposed to Hyperfilm™ for various times and the film was developed in Kodak D-19 Developer before being fixed in Kodak Unifix for 5 minutes at room temperature. Band intensities were quantified with SigmaGel™ software from the autoradiographic records.

2.2.10. Alpha Bungarotoxin Chemiluminescence

Pure neuronal cortical cultures grown on glass coverslips coated with poly-D-Lysine (0.05 g/ l) were subjected to various treatments before undergoing staining with α-Bungarotoxin-FITC to detect functional levels of α7 nAChRs (Method is based on personal communication from Prof. Wonnocott, University of Bath). Medium was removed and cells on the coverslips were washed with PBS before being fixed in 2 % formaldehyde/ PBS solution for 30 minutes at room temperature in. This was followed by three 5 minute PBS washes and 20 minute incubation with 0.3 M glycine dissolved in PBS. Cells were washed twice for 5 minutes with PBS and incubated in 0.5 % Triton X-100 (Sigma)/ PBS solution for twenty minutes. This was followed by another double 5 minute wash with PBS before blocking with 0.5 % Bovine Serum Albumin (BSA) (Sigma)/ PBS solution for 30 minutes at room temperature. α-Bungarotoxin-FITC (Sigma) was made up to a final concentration of 10 nM in 0.5 % BSA and incubated with the cells for 30 minutes at room temperature before washing twice for 5 minutes with 0.5 % BSA/ PBS solution. For the negative control, cells were incubated first with 1 mM nicotine for 30 minutes before- and during the 30 minute incubation with α-Bungarotoxin-FITC. Cells were washed a final time for 5 minutes with PBS and coverslips were permanently fixed on a glass slide with Citifluor AF1 (Agar Scietific). Five pictures were taken with a Nikon digital camera DXM 1200 on a Nikon Eclipse E400 microscope at 10X magnification of every coverslip in an X configuration – a photograph at each point and one in the centre. The images were submitted to independent blind counting of the fluorescent signal.

2.2.11. Alpha 7 Nicotinic Acetylcholine Receptor Expression in Preconditioned Pure Neuronal Cortical Cultures

Pure neuronal cortical culture (sections 2.2.1 and 2.2.3) were seeded at 1500 cells/ mm^2 on poly-D-Lysine-coated 35 mm Iwaki tissue culture treated dishes and treated for 24 hours on DIV 14 with either 11.25 mM KCl dissolved in medium or just plain medium for the control group. Cells were harvested either acutely or chronically (3 wash steps and 24 hours after subsequent incubation) in 500 µl phosphate buffered saline (PBS) containing 1 in 100 dilution of Protease Inhibitor Cocktail Set III. The samples were sonicated four times for 30 seconds in a sonication bath with a 1 minute incubation period on ice in between every sonication pulse. 100 µl of the sample was chloroform/ methanol precipitated (section 2.2.7) before being applied to a 6 % SDS-polyacrylamide gel (section 2.2.8) and subjected to a Western blot analysis (section 2.2.9).

2.3. Results

2.3.1. Preconditioning of mixed cortical neuron and glial cell cultures

Tremblay *et al.* (2000) described a preconditioning model in which the application of the *N*-methyl-D-aspartate (NMDA) antagonist (1)-5-methyl-10, 11-dihydro-5H-dibenzo [a, d] cyclohepten-5, 10-imine maleate (MK801) was used to protect mixed cortical neurons and glial cells against a subsequent NMDA insult. A brief 30 min exposure to 0.1 μM MK801 was able to protect primary cortical neurons against a lethal insult applied up to 96 hours after the removal of the preconditioning agent (Tremblay *et al.*, 2000). This culture model was replicated and preconditioned with either MK801 (Tremblay *et al.*, 2000), 15 minute 45 mM KCl (Grabb *et al.*, 1999), or 1 hour with 1 μM nicotine (loosely based on the Akaike *et al.*, 1994 paper), with the 5 minute 50 μM NMDA insult 24 hours after preconditioning (Fig. 2.1). Due to the 'masking' effect generated by the presence of numerous glial cells, a viability test, such as the use of MTT, is insufficient to detect any changes caused by the NMDA insult. Tremblay and co-workers resorted to the time-consuming method of blind counting, whereas in this study the efflux of lactate dehydrogenase (LDH) from injured or lysed cells were used to determine the extent of the neuroprotection generated by the various chemicals (Tremblay *et al.*, 2000).

In this present study, a differential degree of preconditioning was achieved. It would appear that KCl has the greatest neuroprotective effect twenty-four hours after preconditioning, with only 25 % of the expected cell death actually occurring equating to 75 % obtained protection (Fig. 2.1A). Approximately sixty percent of the potentially lysed cells are protected by treatment with MK801 but only about fifteen percent could be protected by the one hour treatment with 1 μM nicotine.

Highly significant protection is achieved by both MK801 and KCl preconditioning when the 5 minute 50 μM NMDA insult is delayed until 48 hours after the preconditioning event (Fig. 2.1B). The neuroprotection offered by MK801 seems to increase with a longer time period between the sublethal and the lethal insult, whereas KCl's neuroprotection decreased over the same time period. Preconditioning was also attempted using 1 μM and 10 μM nicotine but no significant protection was achieved. There does, however, exist a definite trend towards protection with the cell death being reduced by 30 – 40 %. MK 801 seems to elicit the strongest protection with approximately only 20 % of the cells being damaged (80 % protection). Potassium chloride treatment achieves less neuroprotection with approximately 55 % of the cells protected. These different levels of protection, and the time period elapsed before maximum protection post-preconditioning are achieved, indicate separate molecular pathways activated by each treatment.

An investigation was initiated to optimize the efficacy of the NMDA insult by applying either 50 μM NMDA or 200 μM NMDA for 1 hour to the 48-hour post-preconditioned cultures (Fig. 2.2). It should be noted that plate normalization was not applied in this set of experiments and the results might be skewed as a result. Approximately 20 % fewer cells were killed with the lower dose of NMDA for each of the two preconditioning strategies employed in this study. The neuroprotection offered by the preconditioning agents were unable to combat the overwhelming cell loss caused by the higher concentration NMDA insult (Similar findings to other studies in the laboratory, Errington *et al.*, unpublished).

Of importance is the fact that the results for this section can be split into two groups: successful and unsuccessful protection. The 24 hour preconditioning experiments (Fig.

2.2) were performed in four sets of which 2 sets gave no significant protection (Data not shown). The data compiled for Figure 2.1 arose from 8 separate data sets (individual cultures). It should, however, be mentioned that an additional 7 data sets in support of this specific data compilation was attempted yet gave inconsistent results (Data not shown). The inconsistency of these cultures become apparent during the parallel processing of two identical culture plates – from the initial seeding of the neurons to the final LDH analysis – that yielded contradicting results. Attempts were made to stabilise and homogenise the cultures i.e. substituting glutamine with the more stable form of glutamax-1; reducing the unequal heat distribution and evaporation rates normally associated with 24-well tissue culture plates by the addition of preheated sterile water between the wells. Unfortunately all these additional attempts failed to improve the reliability of this tissue culture method. Visual inspection of mixed cortical neuronal and glial cell cultures on DIV 14 (Fig. 2.3A) show a mixture of the two cell types occurring in concentrated clusters throughout the sample. Pure neuronal cell cultures, discussed in the section 2.3.2, show evenly dispersed neuronal cells without any contaminating glial cells (Fig. 2.3B).

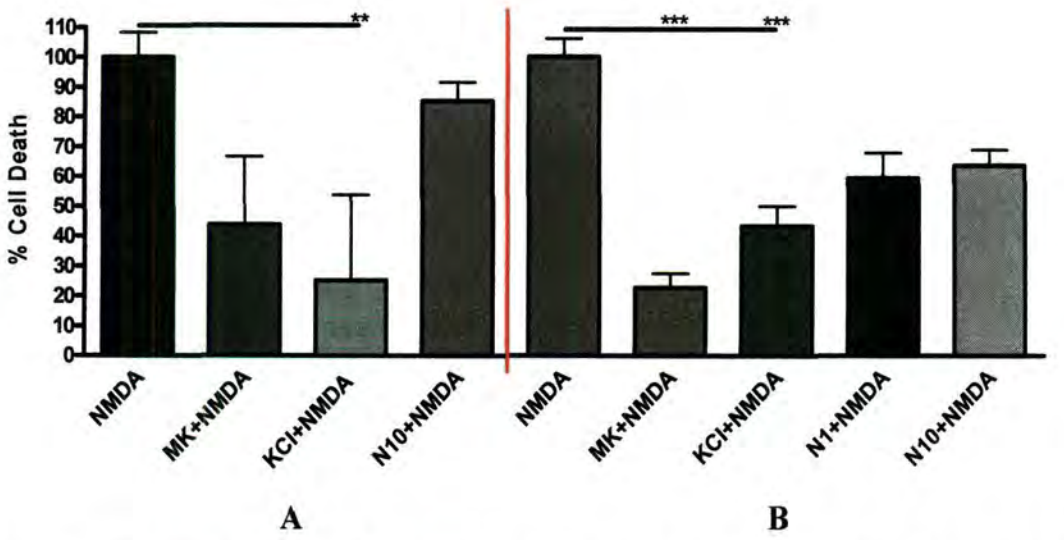


Figure 2.1. Comparison of treatments of mixed glial and cortical neuronal cultures treated with preconditioning agents (A) 24 hours and (B) 48 hours before the insult. The lethal insult consisted out of treatment for 5 minutes with 50 μM NMDA. LDH readings were taken 24 hours after the insults and values represent the mean LDH release/ absorbance ± S.E.M. Control cultures, which did not receive any insults, were subtracted from all the values before the values were expressed as a percentage of the lethal insult control [NMDA (A) n = 35; (B) n = 41]. MK+NMDA indicates 30 minutes of 1 μM MK801 treatment [(A) n = 12; (B) n = 35]; KCl is the 15 minute 45 mM KCl treatment [(A) n = 12; (B) n = 29]; and N1+NMDA is the treatment with 1 μM nicotine for 1 hour [(A) n = 8; (B) n = 6] and N10+NMDA represent treatment with 10 μM nicotine for 1 hour (n = 12) ** $P < 0.01$, *** $P < 0.001$.

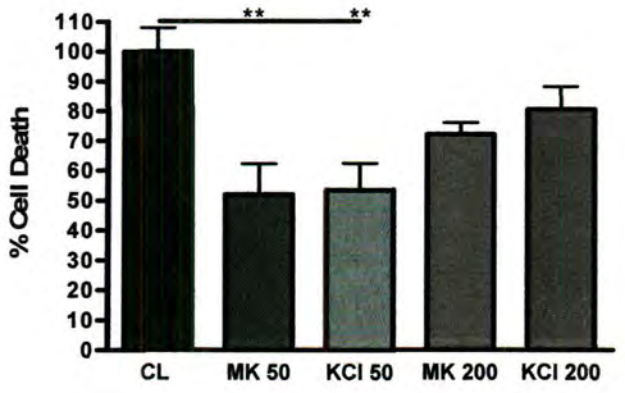
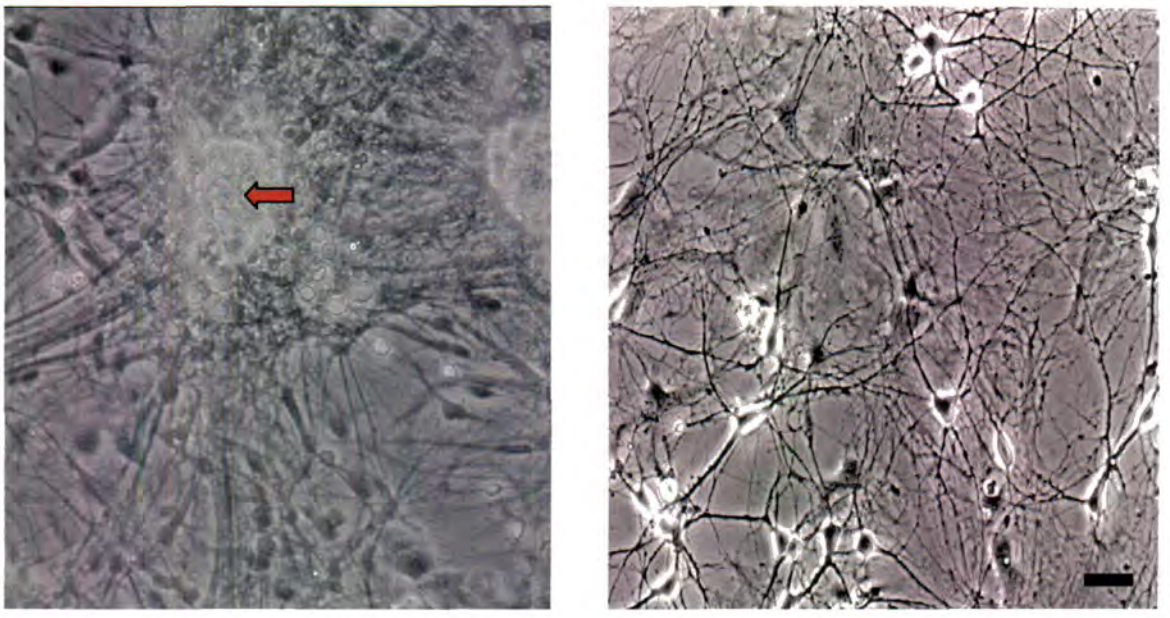


Figure 2.2. Comparison between lethal insults on mixed glial and cortical neuronal cultures that were preconditioned with either with 1 μM MK801 (MK) for 30 minutes or 45 mM KCl for 15 minutes, 48 hours before the insult. The lethal insult consisted out of either the application of 50 μM NMDA (50) for 1 hour or 200 μM NMDA (200) for 1 hour. LDH readings were taken 24 hours after the insult. Control cultures that did not receive any insults were subtracted from all the values before the values were divided by CL - the control that did received the insult. $n = 12$ ** $P < 0.01$.



A **B**

Figure 2.3. Visual comparison between DIV 14 mixed neuronal and glial cortical cell cultures (A), and DIV 14 pure neuronal cortical cell cultures from section 2.3.2 (B). The mixed neuronal and glial cortical cell cultures have clusters of glial cells (indicated by the red arrow), whereas in the pure neuronal cortical cell cultures, the cells are evenly spread without any clusters. Scale bar = 20 μm

2.3.2. Pure Neuronal Cortical Cultures

2.3.2.1. Excitotoxicity

The first step in the process of establishing a reliable *in vitro* preconditioning model was to determine the concentrations and optimal time periods required for the excitotoxic chemical which was to serve as lethal insult. Glutamate is thought to play an important role in the neurodegeneration subsequent to hypoxic-ischemic brain injury (Choi, 1988; Meldrum and Garthwaite, 1990). All subtypes of glutamate receptors can initiate excitotoxic processes (Leist and Nicotera, 1998) where an over stimulation of the receptors lead to an excessive increase in the intracellular Ca^{2+}, leading to impaired energy metabolism, mitochondrial dysfunction and oxidative stress, and finally neuronal cell death (Meldrum and Garthwaite, 1990; Leist and Nicotera, 1998). In cortical neurons it is generally accepted that the NMDA subtype of glutamate receptors play a crucial role in the glutamate-induced cytotoxicity since this effect can consistently be blocked by NMDA receptor antagonists (Meldrum and Garthwaite, 1990; Stuiver *et al.*, 1996). It was thought that NMDA would yield the maximum cell death when applied to the pure neuronal cortical cultures and would serve as the lethal insult. The initial insult level caused by the application of NMDA to the cultures was analysed by removing the medium, applying the different concentrations of NMDA for 1 hour to the cultures, followed by three washes with EBS, subsequent reapplication of the culture medium and incubation for 24 hours at 37 °C, after which cell viability was assayed with MTT. It seems that an NMDA insult of 70 μM and higher attain the maximum kill (approximately twenty percent of the cells) possible in these cultures (Fig. 2.4). The IC50 of 15 +/- 5 μM NMDA is consistent with the published affinity for NMDA (Erreger *et al.*, 2007). This homogeneous resistance to various high concentrations of NMDA could possibly be the

result of the neuron-selective pressure that took place at the creation of the cultures, especially the 2.5μM L-glutamate that was added at the start to the medium of the pure neuronal cortical cultures. To test this theory, cells were incubated with a single concentration of NMDA (200 μM) but for various times before receiving three EBS washes, a 24 hour incubation period, and being assayed with MTT (Fig. 2.5). Once again homogeneous resistance against the NMDA insult was observed, even when the cells were incubated for 24 hours with the 200 μM NMDA insult. Incubation for one or two hours with 200 μM NMDA killed approximately 10 % of the cultures whereas 24 hour incubation resulted in only a further cell loss of approximately 13 %.

In the Kaneko *et al.* (1997) study a more robust insult (1 mM glutamate for 10 minutes) was applied to mixed glial and cortical neurons. The insult was repeated on our pure neuronal cortical cultures (Fig. 2.6). Both 10 minute and 1 hour incubations with the excitotoxic glutamate resulted in an approximate 10 % loss in culture viability. The glutamate insults seemed to follow a similar trend as that of the NMDA insults, the only difference being that the 24 hours of incubation with glutamate resulted in a higher percentage cellular death (~ 27 %).

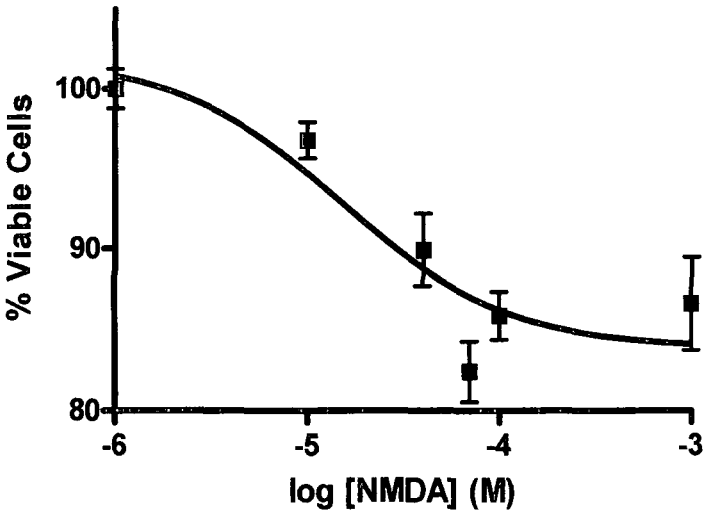


Figure 2.4. NMDA neurotoxicity optimization in pure neuronal cortical cultures to determine the most appropriate concentration of NMDA for optimum neuronal kill. DIV 14 cultures were treated with various concentrations of NMDA (Control, 10 μM, 40 μM, 70 μM, 100 μM, and 1 mM) for 1 hour. The control received no insult but blank EBS treatment with the same washes. After 24 hours of incubation; cell viability was assayed with MTT. The values represent the mean MTT produced/ absorbance. Data are expressed as percentage of controls with the bars representing the mean ± S.E.M. A non-parametric one-way ANOVA statistical analyses was done with significance indicated by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. $n = 8$.

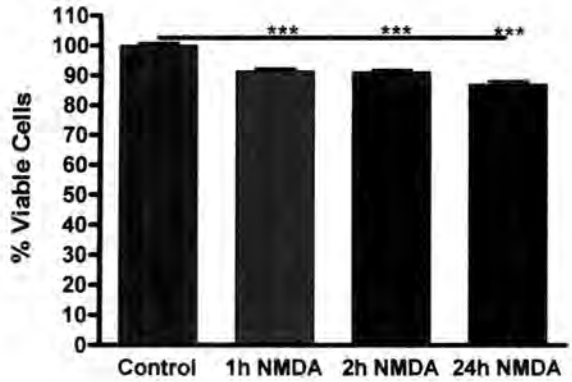


Figure 2.5. NMDA-mediated cytotoxicity optimization in pure neuronal cortical cultures to determine the ideal length of time for the lethal insult. DIV 14 cultures were treated with 200 μM NMDA for various periods of time. The control received no insult but blank EBS treatment with the same washes. After 24 hours of incubation; cell viability was assayed with MTT. The 24 hour glutamate treatment did not receive the subsequent 24 hour incubation but was rather assayed directly. The values represent the mean MTT produced/ absorbance and data are expressed as percentage of controls with the bars representing the mean ± S.E.M from at least two independent culture experiments. A non-parametric one-way ANOVA statistical analyses was done with significance indicated by *** $P < 0.001$. $n = 18$.

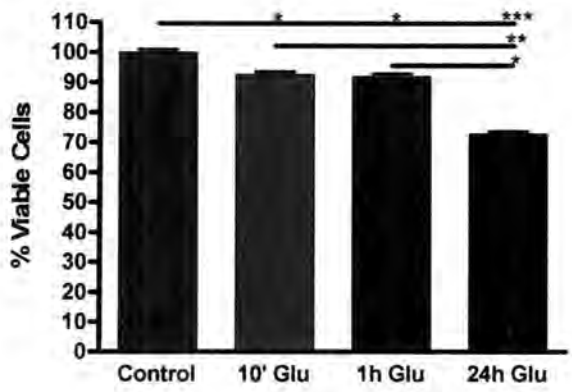
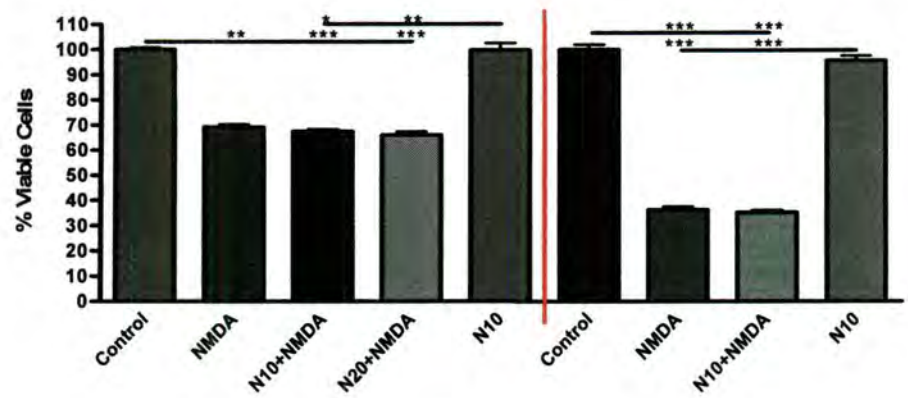


Figure 2.6. Glutamate cytotoxicity optimization in pure neuronal cortical cultures to determine the optimum length of time for the lethal insult. DIV 14 cultures were treated with 1 mM glutamate for various periods of time. The control received no insult but blank EBS treatment with the same washes. After 24 hours of incubation; cell viability was assayed with MTT. The 24 hour glutamate treatment did not receive the subsequent 24 hour incubation but was rather assayed directly. The values represent the mean MTT produced/ absorbance and data are expressed as percentage of controls with the bars representing the mean ± S.E.M from at least two independent culture experiments. A non-parametric one-way ANOVA statistical analyses was done with significance indicated by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. $n = 12$.

2.3.2.2. Nicotine Neuroprotection

The role of (–)-nicotine in neuroprotection is a well documented phenomenon *in vitro* (Marin *et al.*, 1994; Donnelly-Roberts *et al* 1996; Dajas-Bailador *et al.*, 2000; Tohgi *et al.*, 2000). Dajas-Bailador and co-workers (2000) reported neuroprotection where the co-incubation of 10 μM nicotine with 200 μM NMDA resulted in complete protection of the cells against the NMDA insult. This research group employed a high density pure hippocampal cell tissue culture model with less than 5 % glial cells present. This neuroprotective strategy was attempted in the pure neuronal cortical cultures but met with no success (Fig. 2.7A). Neither 10 μM nor 20 μM nicotine offered any protection when co-incubated for 1 hour with 200 μM NMDA. A refinement to the protocol was introduced by seeding the cells at a lower defined concentration of 10^5 cells per well and applying the lethal insult on DIV 9 – an attempt to see if the age and density of the neurons might have an effect on their survival. Neuroprotection by co-incubation with 10 μM nicotine was once again attempted but yielded no improvements in the achieved level of protection (Fig. 2.7B).

Of interest is the finding that the DIV 9 cells seem to be more sensitive to a one hour 200 μM NMDA insult than cells that were incubated longer until DIV 14, with DIV 14 cells having approximately 30 % and DIV 9 cells have approximately 60 % neuronal death (Fig. 2.7B). The younger age of the cells is probably the cause for this doubling in the neuronal death, although as noted before, the different seeding density might also have had an influence.



A B

Figure 2.7. Nicotine's neuroprotective properties against an NMDA insult was tested on (A) DIV 14 and (B) DIV 9 pure cortical cultures. The DIV 9 cultures were seeded at the lower defined concentration of 10^5 cells per well on a 24 well plate.10 μM nicotine (N10) was incubated for one hour without a lethal NMDA insult to test whether the nicotine treatment affected the cells. A NMDA insult consisting of 200 μM NMDA was applied for one hour to cultures containing no nicotine (NMDA). Either 10 μM nicotine (N10+NMDA; n = 12) or 20 μM nicotine (N20+NMDA) were co-applied with 200 μM NMDA for 1 hour before being removed, the cultures washed and subsequent incubation for 24 hours. MTT readings were taken 24 hours later and values represent the mean MTT produced/ absorbance. Data are expressed as percentage of controls and bars represents the mean ± S.E.M from at least two independent culture experiments. A non-parametric one-way ANOVA statistical analyses was done with significance indicated by ** $P < 0.01$; *** $P < 0.001$.

2.3.2.3. Nicotinic Preconditioning

Following upon the partial success of nicotine preconditioning in the mixed cortical neuron and glial cell cultures and despite the failure of this agonist to lend protection when co-incubated with NMDA, an attempt was made to precondition with nicotine in the pure neuronal cortical cultures. Pure neuronal cortical cultures were preconditioned for one hour with 10 μM nicotine (N10) 24 hours before receiving the 1 mM NMDA insult (Fig. 2.8). Both the nicotine pre-treated and the control NMDA-insult group had cell death of approximately thirty percent when compared to the control that did not receive any insult. No significant difference was observed between the nicotine preconditioned group and the control group, which received only EBS treatment before the NMDA insult (denoted as NMDA).

In accordance with the Kaneko *et al.* (1997) study, prolonged exposure to the preconditioning agent was attempted and the cultures were incubated for 24 hours with 10μM nicotine (Fig. 2.9). Prolonged exposure to the agent did not have any effect on the elicitation of a protective mechanism against the subsequent 1 hour 1mM NMDA (Fig. 2.9A), the chronic 10 minute 1 mM glutamate (Fig. 2.9B) or the acute 10 minute 1 mM glutamate (Fig. 2.9C) excitotoxic insults. Cell viability was reduced by twenty percent when the glutamate insult was applied directly after the 24 hours nicotine treatment (Fig. 2.9C), whereas a reduction of thirty percent was observed when the insult was applied 24 hours after the prolonged nicotine treatment (Fig. 2.9B). It is fair to assume that the cell densities were approximately the same since these two culture sets were produced at the same time from the same batch of dissociated neurons. This could possibly reflect the vulnerability of the cells to an NMDA insult directly after having been washed. Washes

with physiological saline are associated with a variety of brief insults, including osmotic, gluconic, thermal and ischemic insults (Stackpole *et al.*, 2007). Exposure of cultures to 24 hours of nicotine treatment did not have a detrimental effect on the cells; on the contrary, there seems to be an indication that the nicotinic treatment protected cells against the insults experienced during the wash steps. In both experiments where the cells were exposed for 24 hours without lethal insults to either fresh medium or nicotine-containing medium (Fig. 2.9B and C), the nicotinic groups yielded approximately five percent higher cell survival than their control counterparts.

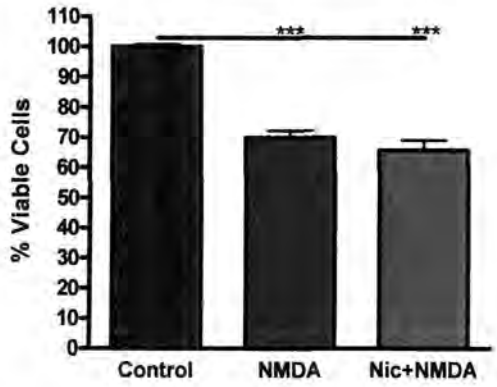


Figure 2.8. Pure neuronal cultures preconditioned on DIV 14 for one hour with 10 μM nicotine. Cultures were washed and incubated for 24 hours before receiving and insult consisting of 1 mM NMDA treatment for 1 hour. MTT readings were taken 24 hours after the insult and values represent the mean MTT produced/ absorbance. Control cultures did not receive a NMDA insult and data are expressed as percentage of controls; the bars represent the mean ± S.E.M from at least two independent culture experiments. A non-parametric one-way ANOVA statistical analyses was done with significance indicated by *** $P < 0.001$. Control $n = 12$, NMDA and Nic+NMDA $n = 16$.

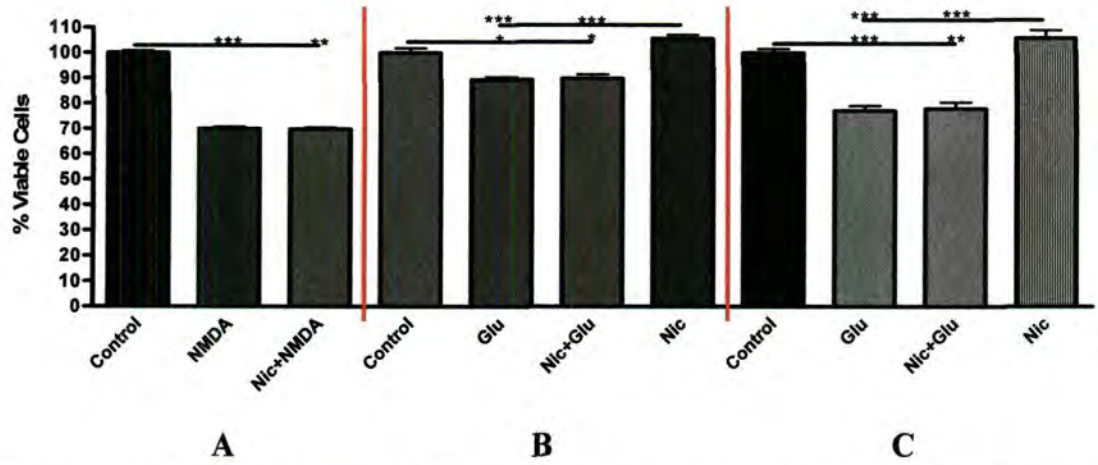


Figure 2.9. Prolonged exposure to 10 μM nicotine on DIV 14 was attempted by incubating pure neuronal cultures for 24 hours. Cultures were washed and incubated for 24 hours before receiving an insult consisting of (A) 1 mM NMDA treatment for 1 hour or (B) 1 mM glutamate (10 minutes). (C) 1 mM glutamate insult (10 minutes) but the insult was acutely i.e. directly after the 24 hour nicotine preconditioning, MTT readings were taken 24 hours after the insults and values represent the mean MTT produced/ absorbance. Control cultures in Figures (A), (B) and (C) and nicotine control cultures in Figures (B) and (C) did not receive an insult. The data are expressed as percentage of controls; the bars represent the mean ± S.E.M from at least two independent culture experiments in the case of (B) and (C), whereas a single culture experiment was performed in the case of (A). A non-parametric one-way ANOVA statistical analyses was done with significance indicated by * $P < 0.05$; *** $P < 0.001$. (A) $n = 6$; (B) and (C) $n = 12$.

2.3.2.4. Potassium Chloride Preconditioning

2.3.2.4.1. Potassium Chloride preconditioning of DIV 8 pure neuronal cultures

Pure neuronal cortical cultures, utilizing the exact same production methodology, were preconditioned by Kapinya and co-workers with the volatile anaesthetic isoflurane (Kapinya *et al.*, 2002). They achieved approximately 25 percent protection on DIV 8 cultures against an insult consisting of three hours of oxygen glucose deprivation (OGD). Notably, this experiment was successfully repeated and expanded upon in our lab using sevoflurane (Errington, van Rensburg, manuscript in preparation), indicating that preconditioning is indeed possible in the current tissue culture regime despite the failure of the nicotine preconditioning and neuroprotection experiments.

Building upon the success of the volatile anaesthetic experiments (results not shown), DIV 8 pure neuronal cortical cultures were preconditioned with 45 mM KCl for either 15 min (Fig. 2.10B and C) or 1 hour (Fig. 2.10A). Irrespective of the incubation time, the KCl treatment did not protect the cultures against the lethal insult (either 200 μM NMDA for 1 hour [(A) and (B)] or 50 μM NMDA for 5 min (C)), that was applied 24 hours later. The 1 hour NMDA insult resulted in a decrease in cell viability of approximately 35 – 45 percent. The less intensive 5 minute 50 μM NMDA insult, as used in the mixed cortical and glial cell cultures and utilised by Tremblay *et al.* (2000), killed less than ten percent of the cells, indicating that any preconditioning effect was not diminished by the magnitude of the lethal insult.

The Kapinya *et al.* (2002) experiments relied upon the application of an oxygen glucose deprivation (OGD) insult. This type of insult was also utilized in our replication of the

isoflurane preconditioning experiments. To dismiss the possibility that potassium chloride preconditioning was only effective against a certain type of insult in pure neuronal cortical cultures, DIV 8 neurons were preconditioned with 45 mM KCl for 1 hour 24 hours before receiving an OGD insult. Both a 4 hour and a 5.5 hour OGD insult was delivered to cells incubated in the OGD solution (Fig. 2.11). Both these insults reduced neuronal viability by approximately 45 percent, but with no apparent potassium chloride-induced protective effect induced.

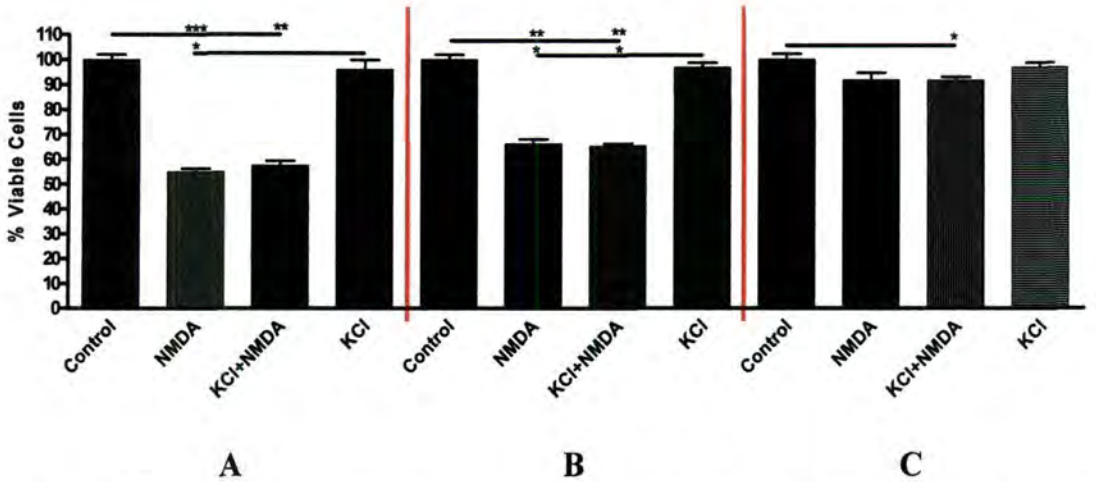


Figure 2.10. Pure neuronal cultures preconditioned on DIV 8 for either one hour (A) or fifteen minutes [(B) and (C)] with 45 mM KCl. Cultures were washed and incubated for 24 hours before receiving an insult consisting of 200 μM NMDA treatment for 1 hour [(A) and (B)] or 50 μM NMDA for 5 minutes (C). MTT readings were taken 24 hours after the insults and values represent the mean MTT produced/ absorbance. Neither the control nor the KCl group cultures received an insult. The data are expressed as percentage of controls; the bars represent the mean ± S.E.M. A non-parametric one-way ANOVA statistical analyses was done with significance indicated by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. $n = 8$.

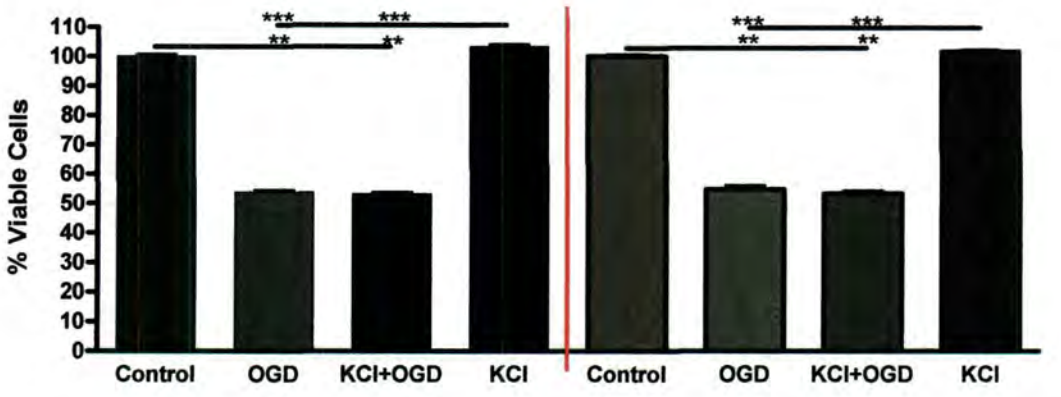


Figure 2.11. Pure neuronal cultures preconditioned on DIV 8 for 1 hour with 45 mM KCl. Cultures were washed and incubated for 24 hours before receiving an OGD insult for either 4 hours (A) or 5 hours 30 minutes (B). MTT readings were taken 24 hours after the insults and values represent the mean MTT produced/ absorbance. Neither the control nor the KCl group cultures received an insult. The data are expressed as percentage of controls; the bars represent the mean ± S.E.M. A non-parametric one-way ANOVA statistical analyses was done with significance indicated by ** $P < 0.01$; *** $P < 0.001$. $n = 12$.

2.3.2.4.2. Acute Potassium Chloride Treatment of DIV 14 Pure Neuronal Cultures

The failure of potassium chloride to precondition pure neuronal cortical cultures after eight days *in vitro* led to a study on DIV 14. The study of potassium chloride on DIV 14 pure neuronal cultures began by preconditioning the cultures with 45 mM KCl for 15 minutes, 24 hours before either a one hour 200 μM NMDA insult or an insult with 1 mM glutamate for 10 minutes (Fig. 2.12). Both these insults reduced cellular viability by approximately twenty percent, with no effect resulting from the potassium chloride treatment with or without the lethal insults. Following upon this, cultures were treated for one hour with various concentrations of potassium chloride 24 hours before receiving a 10 minute 1 mM glutamate insult (Fig. 2.13). Once again no protective effect against the excitotoxic glutamate insult was detected. Of note is the indication that 11.25 mM KCl treatment protects the cells against the insults associated with the washing of the cultures – as noted following the 24 hour nicotine treatment. The effect is modest (+ 4 % more viable cells) but consistent, and no such effect was detected with the 22.5 mM KCl treatment, while the 45 mM KCl treatment actually reduced neuronal viability by approximately 4 %. To delve further into this potential preconditioning effect elicited by 11.25 mM KCl, it was decided to look at the consequences of prolonged exposure to potassium chloride.

2.3.2.4.3. Chronic Potassium Chloride Treatment of DIV 14 Pure Neuronal Cultures

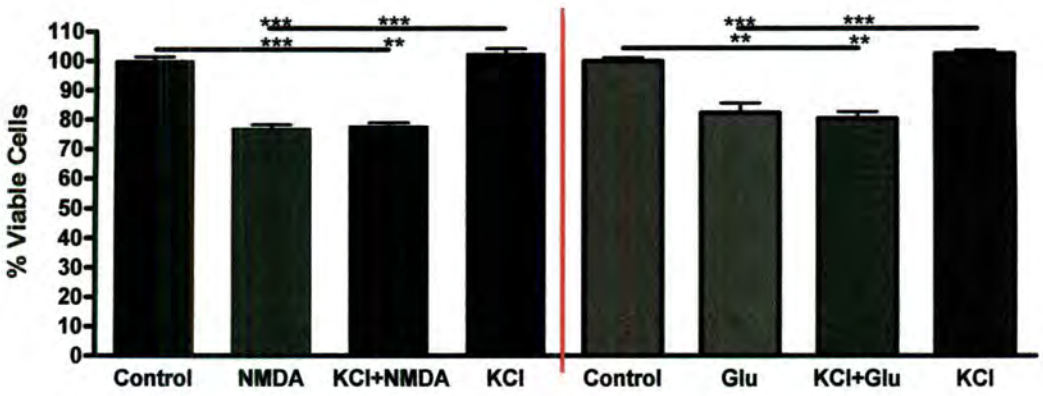
Subsequent to the observation in the previous section that 45 mM potassium chloride treatment reduced cell viability by approximately 4 % after only one hour of exposure, an assay was performed to establish the lowest concentration of KCl treatment that would

not reduce cell viability during 24 hours of incubation. Prolonged treatment with 45 mM KCl resulted in approximately a quarter of the cells dying, whereas 22.5 mM KCl reduced cell viability by approximately 13 % over the twenty four hour period (Fig. 2.14). In contrast, a minimal insignificant amount of neuronal death was caused by the twenty-four hour treatment with 11.25 mM KCl.

To fully investigate any potential preconditioning effect produced by the prolonged treatment with KCl, a 10 minute 1 mM glutamate insult was either delivered directly after the treatment (acute glutamate insult) or twenty-four hours after the treatment (chronic glutamate insult) (Fig. 2.15). Clear protection was achieved against either the acute (approximately 47 % protection) or the chronic (approximately 44 % protection) glutamate insult subsequent to prolonged KCl treatment. To further investigate this phenomenon cultures were created with a range of different cellular densities (Fig. 2.16). Glutamate insult efficacy seem to be dependent on the density of the cultures – the tendency being that denser cultures result in a higher cell death subsequent to the same insult probably due to the formation of a chain reaction where dead cells release their own glutamate and cytotoxins, exacerbating the effect (consistent with other findings in the laboratory). The preconditioning effect over the first three densities (1000 cells/ mm^2, 1500 cells/ mm^2, and 2000 cells/ mm^2) remained constant and approximately 6 – 7 % more viable cells. The highest seeded density (3000 cells/ mm^2) resulted in a doubling of the protected cells, approximately 12 % more viable cells (approximately 40 % protection).

The effect of prolonged potassium chloride exposure on the viability of the pure neuronal cultures and their resistance to an excitotoxic insult was investigated by treating DIV 14

cultures for 24 hours with 11.25 mM KCl but staggering the lethal glutamate insults (Fig. 2.17). After 48 hours cell death had increased from approximately 5 % at 24 hours post treatment, to approximately 11 %. At 72 hours post treatment, KCl-induced cell death rose to approximately 19 % where it stabilised and remained beyond 96 hours post KCl treatment. A lesser neuroprotective effect was observed in the KCl treated cells that also received the glutamate insult. Forty-eight hours after the potassium chloride treatment, an insignificant amount of cells (approximately 2 %) were still protected against the glutamate insult, whereas at time points 72 hours and 96 hours, the viability dropped below the untreated glutamate-insulted cells, by approximately three percent.



A B

Figure 2.12. Pure neuronal cultures preconditioned on DIV 14 for fifteen minutes with 45 mM KCl. Cultures were washed three times and incubated for 24 hours before receiving an insult consisting of 200 μM NMDA treatment for 1 hour (A) or 1 mM glutamate for ten minutes (B). MTT readings were taken 24 hours after the insult and values represent the mean MTT produced/ absorbance. Neither the control nor the KCl group cultures received an insult. Data is expressed as percentage of controls; the bars represent the mean ± S.E.M from two independent culture experiments. A non-parametric one-way ANOVA statistical analyses was done with significance indicated by ** $P < 0.01$; *** $P < 0.001$. $n = 12$.

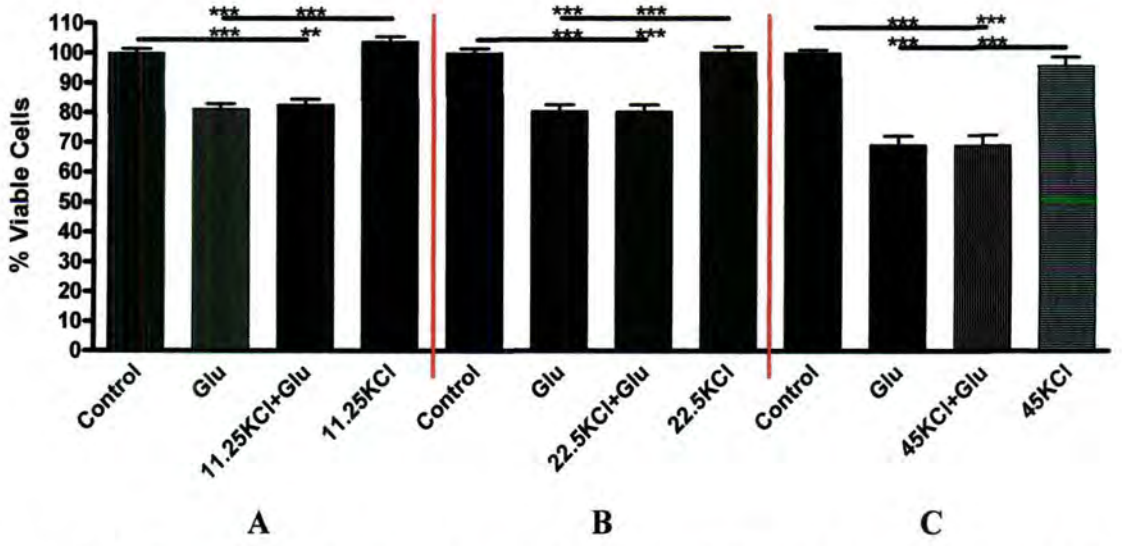


Figure 2.13. Pure neuronal cultures preconditioned on DIV 14 for one hour with either 11.25 mM KCl (A), 22.5 mM KCl (B), or 45 mM KCl (C). Cultures were washed thrice and incubated for 24 hours before receiving and insult consisting of 1 mM glutamate for ten minutes. MTT readings were taken 24 hours after the insult and values represent the mean MTT produced/ absorbance. Neither the control nor the KCl group cultures received an insult. Data are expressed as percentage of controls; the bars represent the mean ± S.E.M from two independent culture experiments. A non-parametric one-way ANOVA statistical analyses was done with significance indicated by ** $P < 0.01$; *** $P < 0.001$. $n = 12$.

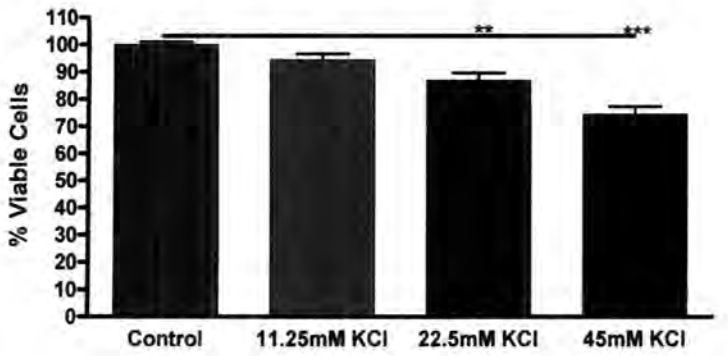


Figure 2.14. Pure neuronal cultures preconditioned on DIV 14 for 24 hours with either 11.25 mM, 22.5 mM, or 45 mM KCl. These cultures did not receive any excitotoxic insults. Cultures were washed thrice and incubated for 24 hours before MTT readings were taken. The values represent the mean MTT produced/ absorbance and the data is expressed as percentage of controls; the bars represent the mean ± S.E.M from at least two independent culture experiments. A non-parametric one-way ANOVA statistical analyses was done with significance indicated by ** $P < 0.01$; *** $P < 0.001$. $n = 18$.

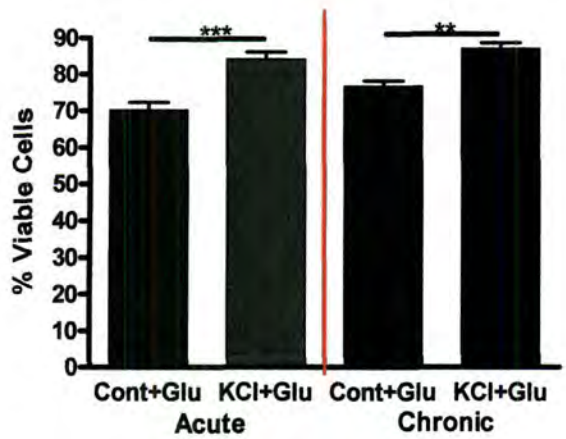


Figure 2.15. DIV 14 pure neuronal cortical cultures were treated for 24 hours by the addition of 11.25 mM KCl to the cell medium. Following the incubation period, cells were washed three times with EBS before, in the case of the acute study, receiving a 10 minute 1mM glutamate insult, three more EBS washes and a 24 hour incubation period before MTT analyses. For the chronic study, the KCl treated cells were washed with EBS and incubated in medium for an additional 24 hours before receiving the 10 minute 1 mM glutamate lethal insult, three more EBS washes and the 24 hour post-insult incubation before MTT analyses. In order the demonstrate the preconditioning effect, the lethal insult group is expressed as a percentage of the control whereas the KCl group that received a lethal insult is expressed as a percentage of the respective KCl treated cells that did not receive the glutamate insult. A *t*-test was used to compare the two resulting groups with the bars representing the mean ± S.E.M from two independent culture experiments. Significance indicated by ** $P < 0.01$; *** $P < 0.001$. $n = 12$.

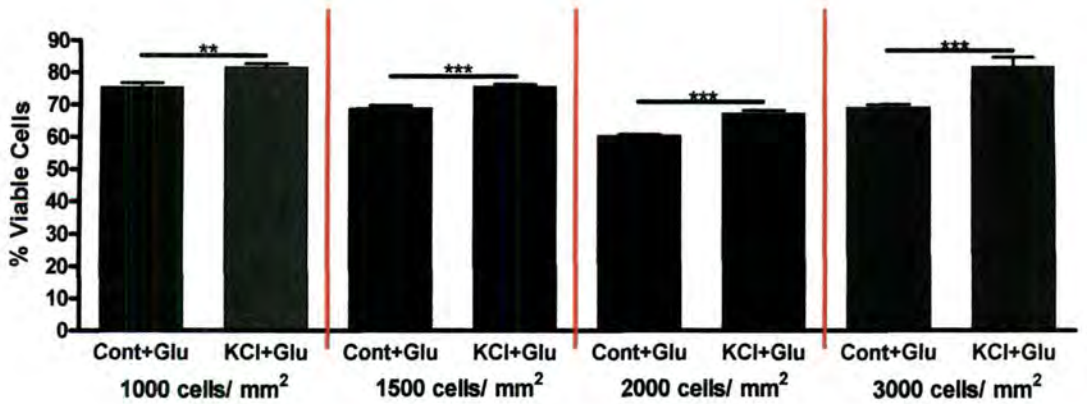


Figure 2.16. DIV 14 pure neuronal cortical cultures that were seeded at various cellular densities were treated for 24 hours by the addition of 11.25 mM KCl to the cell medium. Following the incubation period, cells were washed three times with EBS and incubated in medium for an additional 24 hours before receiving the 10 minute 1 mM glutamate lethal insult, three more EBS washes and the 24 hour post-insult incubation before MTT analyses. In order to demonstrate the preconditioning effect, the lethal insult group is expressed as a percentage of the control whereas the KCl group that received a lethal insult is expressed as a percentage of the respective KCl treated cells that did not receive the glutamate insult. A *t*-test was used to compare the two resulting groups with the bars representing the mean ± S.E.M from two independent culture experiments. Significance indicated by ** $P < 0.01$; *** $P < 0.001$. $n = 12$.

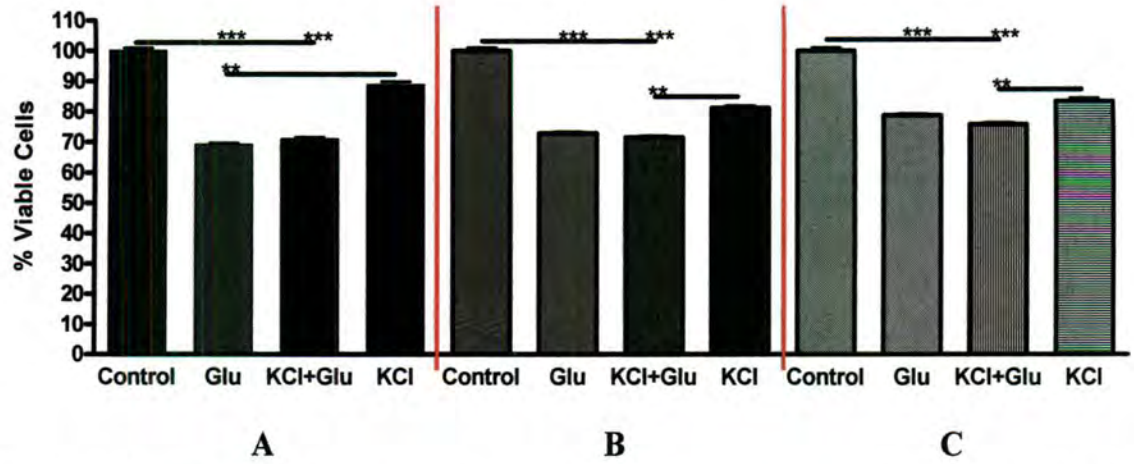


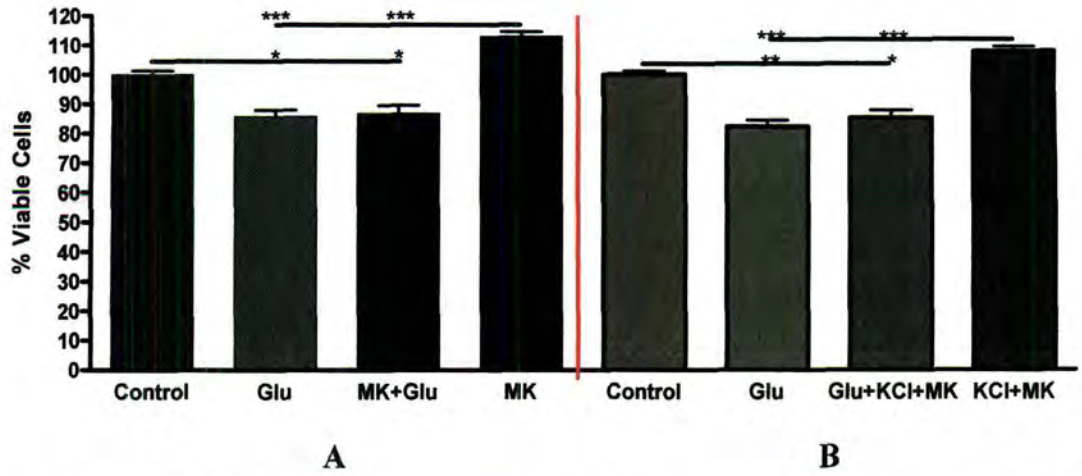
Figure 2.17. DIV 14 pure neuronal cortical cultures were treated for 24 hours by the addition of 11.25 mM KCl to the cell medium. Following the incubation period, cells were washed three times with EBS before but the 10 minute 1 mM glutamate excitotoxic insult was applied after incubating the cells for either 48 hours (A), 72 hours (B), or 96 hours (C). The cultures were incubated a further 24 hours before the MTT analyses. Neither the control nor the KCl group cultures received an insult. Data are expressed as percentage of controls; the bars represent the mean ± S.E.M from two independent culture experiments. A non-parametric one-way ANOVA statistical analyses was done with significance indicated by ** $P < 0.01$; *** $P < 0.001$. $n = 12$.

2.3.2.4.4. Confirmation of Spreading Depression Preconditioning Mechanism

Potassium chloride-induced cortical spreading depression *in vivo* can be blocked by the application of the N-methyl-D-aspartate (NMDA) channel antagonist MK801 (Kitagawa *et al.*, 1990; Kitagawa *et al.*, 1991; Kato *et al.*, 1992; Taga *et al.*, 1997). In order to confirm that the *in vitro* model relies upon a similar mechanism of activation despite the initial neuronal loss, experiments were preformed applying MK801 30 minutes before the initiation of the 24 hour KCl treatment of the pure neuronal cortical cultures (Fig. 2.18B). MK801 was also applied to cultures without the addition of any KCl to see if it is capable of preconditioning the pure neuronal cultures against an excitotoxic insult by MK801 itself as was done with the mixed glial and neuronal cell cultures in section 2.3.1 (Fig. 2.18A).

Prolonged exposure of pure neuronal cortical cultures to MK801 resulted in the loss of the KCl-induced preconditioning effect with no significant increase of neuronal viability observed. The absence of any protection against the 1 mM glutamate insult showed that as with *in vivo* CSD, the preconditioning effect of potassium chloride is mediated by NMDA receptors. Chronic exposure to MK801 by itself was also ineffective in producing any protection against a 10 minute 1 mM glutamate insult.

However, as with prolonged nicotine exposure in Figure 2.9, MK801 is able to elicit a neuroprotective effect against wash-related insults. Chronic exposure of cells to MK801 or MK801 co-incubated with KCl led to approximately 8 – 13 % overall increase in cell viability when compared to the control which also only received washes and no glutamate insult (Fig. 2.18).



A B

Figure 2.18. DIV 14 pure neuronal cortical cultures were treated for 24 h with either 1 μM MK801 alone (A) or 11.25 mM KCl and 1 μM MK801 with the MK801 being added to the culture 30 min before the KCl (B). Following the incubation period, cells were washed three times with EBS and incubated for 24 hours at 37 °C and 5 % CO_2 before the 10 minute 1 mM glutamate excitotoxic insult was applied. The cultures were incubated a further 24 hours before the MTT analyses. Control cultures did not receive an excitotoxic glutamate insult, nor did the KCl and the KCl/MK801 group. Data is expressed as percentage of controls; the bars represent the mean ± S.E.M from two independent experiments. A non-parametric one-way ANOVA statistical analyses was done with significance indicated by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. $n = 12$.

2.3.2.4.5. The Role of Alpha 7 Nicotinic Acetylcholine Receptors in Potassium Chloride Mediated Preconditioning.

With the successful establishment of a reliable *in vitro* KCl-dependent preconditioning model which reflects the *in vivo* cortical spreading depression mechanism, the role, if any, played by α7 nAChRs was investigated. Two strategies were employed; the first strategy blocked the potential upregulation of α7 nAChR expression during KCl preconditioning whereas the second strategy cancelled any potential protective effects provided by α7 nAChR against the glutamate insult subsequent to KCl preconditioning using a selective antagonist

KN-62, a Ca^{2+}-calmodulin dependent kinase II inhibitor (CaM-kinase II), has been shown to block the upregulation of α7 nAChR mRNA (De Koninck and Cooper, 1995). This inhibitor was applied to DIV 14 pure neuronal cortical cultures 30 minutes before the application of the 11.25 mM KCl and co-incubated for 24 hours before being removed. The application of the 10 minute glutamate insult followed 24 hours later in the absence of either KCl or KN-62. The second strategy relied upon the blocking of any functional homomeric α7 nAChRs by α-bungarotoxin (αbtx). Here, the DIV 14 neuronal cultures were preconditioned for 24 hours with 11.25 mM KCl alone. Directly after the removal of KCl and washing, the 200 nM αbtx was added to the medium and the cells incubated for 24 hours. The medium was removed and the lethal insult, consisting of a 10 minute exposure to 10 mM glutamate, was applied directly.

As can be seen in Figure 2.19, both the strategies effectively blocked the neuroprotective effect generated by prolonged preconditioning with KCl. An approximate 90 % reduction

of the KCl neuroprotective effect was observed when KN-62 was co-incubated during the preconditioning period. The possibility that both CaM-kinase II and Egr-I could lead to the activation of other neuroprotective genes, not necessarily α7 nAChR, necessitated the use of αbtx, an α7 nAChR-specific antagonist. The application of αbtx before the glutamate insult reduced the KCl-induced preconditioning effect by approximately 95 %, suggesting that this small protective effect is dependent upon the presence of functional α7 nAChRs.

In Figure 2.20, the preconditioning effect of either KN-62 or αbtx alone is analysed. Neither one elicited significant protection against the 10 minute 1 mM glutamate insult, although both treatments increased cell survival by approximately 3 %. KN-62 also did not have any effect on the survival of the cells against the wash-related insult with only a 1.3 % increase in viability being observed, whereas the αbtx treatment led to a significant increase of approximately 6 %. A summarised analysis of neuroprotection elicited by 24 hour treatment with various chemicals against the wash-related insult is represented in Figure 2.21. As can be seen, MK801 provides the highest amount of protection (approximately 13 % more viable cells), whereas nicotine and αbtx both also provide significant protection of approximately 6 % more viable cells. KN-62 is the only chemical which did not yield any significant protection against the wash related insult.

An analysis with α-Bungarotoxin-FITC of the functional expression of α7 nAChR after prolonged treatment with either potassium chloride or nicotine indicates a trend towards **down**-regulation of the receptor (Fig. 2.22). Nicotine treatment elicited minimal effects upon α-Bungarotoxin-FITC binding to α7 nAChRs, whereas depolarization with KCl decreased binding by approximately 18.82 %. Neither one of these treatments yielded a

statistically significant difference although a definite tendency for α7 nAChR down regulation upon KCl treatment was indicated. The negative control for the immunocytochemistry procedure used nicotine, which effectively blocked α-Bungarotoxin-FITC binding to α7 nAChRs. Confirmation by means of Western blot analyses was not possible. Experiments with the polyclonal anti-rabbit α7 nAChR from Santa Cruz Biotechnology directed at the C-terminal of the protein were too variable to make any conclusions (Results not shown). No results were attained with the anti-rabbit α7 nAChR from Abcam (ab23832) (1.5 µg/ ml), which is directed against a region within the first hundred amino acids of the N-terminal of the receptor (Results not shown).

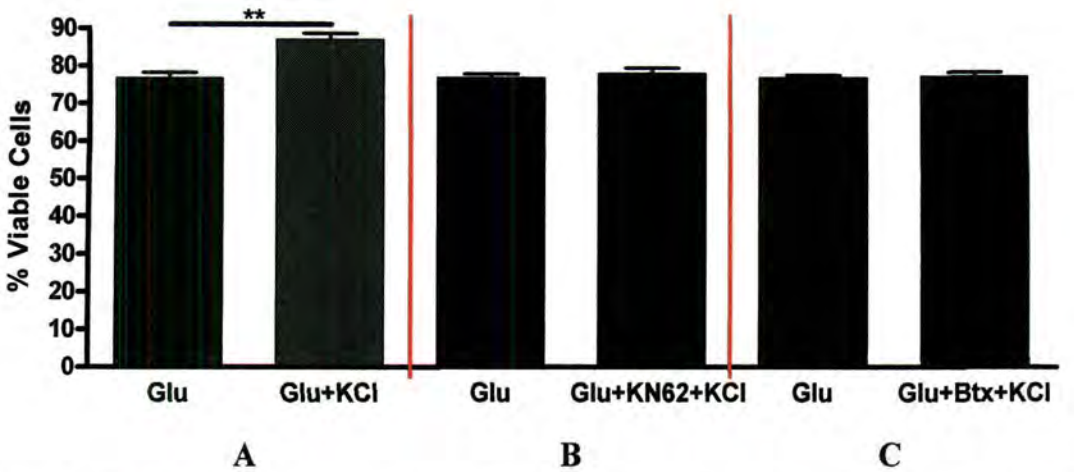


Figure 2.19. DIV 14 pure neuronal cortical cultures were treated for 24 hours with 11.25 mM KCl alone (A) or with 10 μM KN-62 (B), the KN-62 being added to the culture 30 min before the KCl. Following the incubation period, cells were washed three times with EBS and incubated for 24 hours before the 10 minute 1 mM glutamate excitotoxic insult was applied. Alternatively, cells were treated with 200 nM αbtx for 24 h, directly before the glutamate insult subsequent to the removal and washes of the KCl preconditioned cultures (C). Following the glutamate insult and three EBS washes, cultures were incubated a further 24 hours before the MTT analyses. Control, KCl or KCl/MK801 control cultures did not receive an excitotoxic glutamate insult. Data are expressed as percentage of controls; the bars represent the mean ± S.E.M from two independent culture experiments. A *t*-test statistical analyses was done with significance indicated by ** $P < 0.01$. $n = 12$.

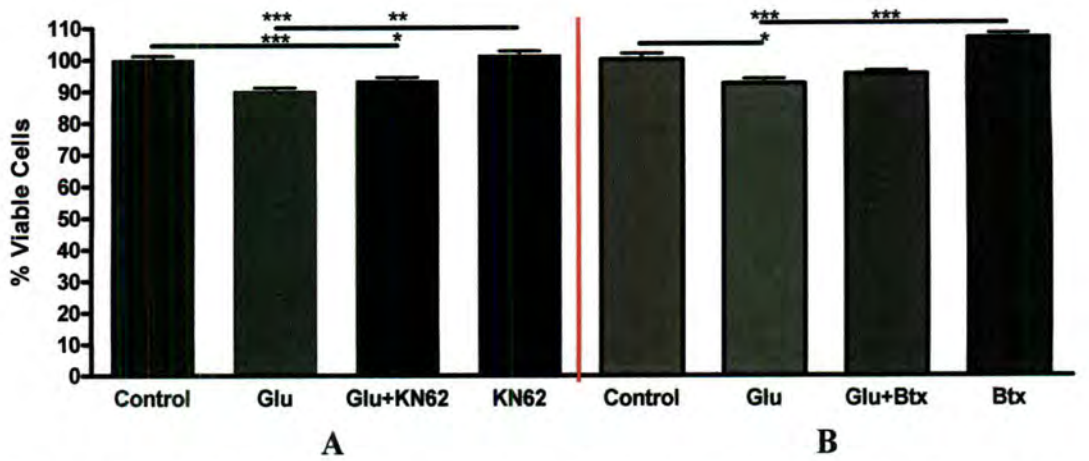


Figure 2.20. DIV 14 pure neuronal cortical cultures were treated for 24 hours with either 10 μM KN-62 24 hours before the glutamate insult (A) or 200 nM αbtx directly before the glutamate insult (B). Following the glutamate insult and three EBS washes, cultures were incubated a further 24 hours before the MTT analyses. Control, KN-62 or αbtx control cultures did not receive an excitotoxic glutamate insult. Data are expressed as percentage of controls; the bars represent the mean ± S.E.M from two independent culture experiments. A non-parametric one-way ANOVA statistical analyses was preformed with significance indicated by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. $n = 12$.

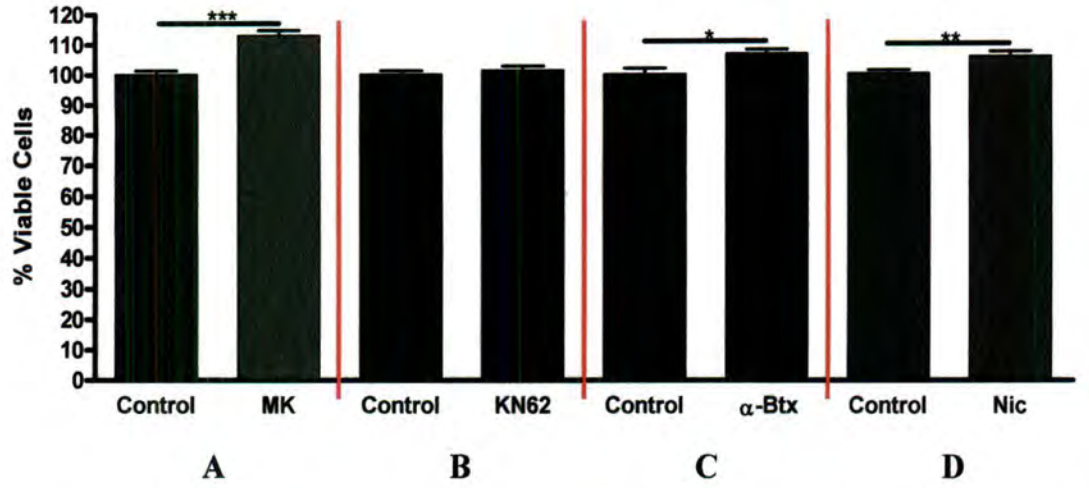


Figure 2.21. A summary of the neuroprotection generated against the wash insult by the treatment of pure neuronal cultures on DIV 14 for 24 hours with various chemicals. Either 1 μM MK801 (A), 10 μM KN-62 (B), 200 nM αbtx (C), or 10 μM nicotine (D) was incubated for 24 hours with the cells during the various preconditioning experiments. Data is expressed as percentage of controls; the bars represent the mean ± S.E.M from two independent cultures. A *t*-test statistical analyses was done on the MTT generated results and significance indicated by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. $n = 12$.

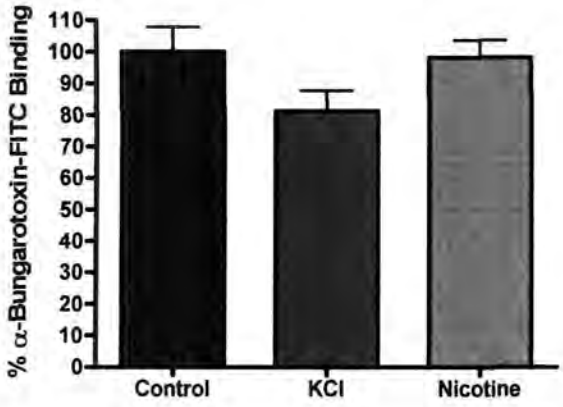
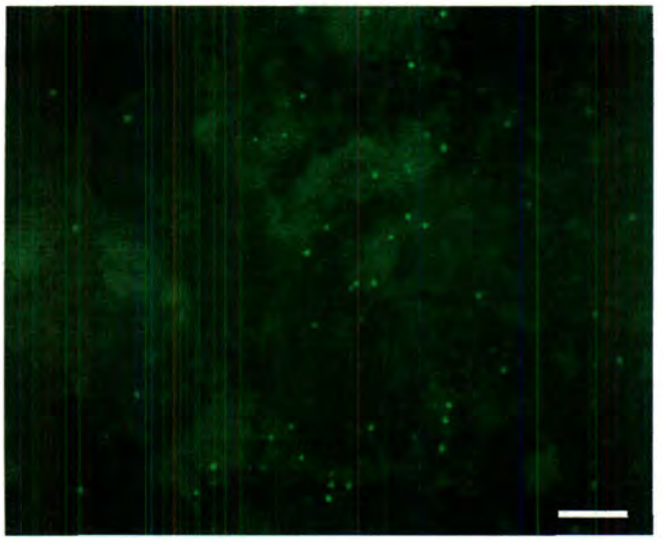


Figure 2.22. DIV 14 pure neuronal cortical cultures grown on glass coverslips were treated for 24 hours by the addition of medium containing either 11.25 mM KCl, 10 μM nicotine, or nothing in the case of the control. Following the incubation period, cells were washed with PBS before being labelled with α-Bungarotoxin-FITC. Five digital images were taken of each coverslip and submitted to independent blind counting. The average was calculated for each coverslip, each average representing a single *n*-number. Data are expressed as percentage of controls; the bars represent the mean ± S.E.M from two independent culture experiments. A non-parametric one-way ANOVA statistical analyses was done with $n = 12$.

A



B

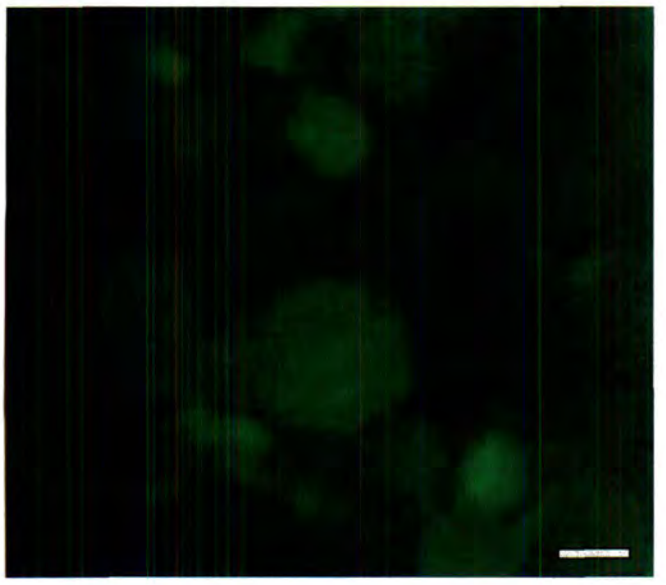


Figure 2.23. Visual representation of A) the α-Bungarotoxin-FITC bound to functional α7 nAChR subsequent to prolonged treatment with either potassium chloride or nicotine. B) A representation of the negative control which was incubated with 1 mM nicotine before-and during α-Bungarotoxin-FITC incubation to illustrate the non-specific staining with FITC. The white scale bar represents 10 μM.

2.4. Discussion

The aim of this chapter was to establish an *in vitro* model for the study of spreading depression (depolarisation)-induced preconditioning and to assess the role of α7 nAChRs in this neuroprotective mechanism. Taking into consideration that the CSD phenomenon is restricted to the brain and appears to involve both neuronal and glial cell populations (Smith *et al.*, 2006), it was thought that the creation of a mixed neuronal and glial cell culture would be less artificial and truer to an *in vivo* environment. Preconditioning with both MK801 (as reported by Tremblay *et al.*, 2000) and KCl (Grabb *et al.*, 1999; Grabb *et al.*, 2002), and to a lesser extend nicotine (Akaike *et al.*, 1994), was attainable in our model. The results also suggest that different molecular pathways are followed by MK801 and KCl treatments. The protection generated by MK801 seems to reach its maximum level after 48 hours incubation, whereas potassium chloride's maximum generated protection is reached much earlier, after only 24 hours and is already decreasing at 48 hours. Preconditioning remained sporadic and inconsistent, with repeated attempts at increasing the consistency being thwarted. Visual analysis of mixed glial and neuronal cell cultures revealed that the glial cells form clusters with an uneven cellular distribution and subsequent unequal treatment exposure. The failures, and the high variance levels attained during the 'successful' preconditioning experiments, are reported to illustrate the poor reliability of this particular tissue culture technique possibly owing to the variable presence of the clustered glial cells.

Pure neuronal cortical cultures were created in an attempt to overcome the shortcomings of the mixed neuronal glial cell cultures. The neurons in these cultures are evenly dispersed with a minimal, if any, presence of glial cells. This new culture technique led to

cultures with high reproducibility and low variance. There does, however, exist discrepancies between the glutamate and NMDA-induced cell death from cultures prepared on various dates due to variations in the respective cell densities. Tissue culture plates were consistently seeded at a density 1500 cells/ mm^2 or 300 000 cells per well, unless stated otherwise. The inconsistencies in cell densities are probably due to differences in the quality of the culture preparation i.e. the initial amount of haemorrhaging of the foetal brains; dissection time and precision; as well as the total duration of the culture preparation. As the α7 nAChR is one of the main focus points of this study, pure neuronal cultures has the additional advantage of possessing higher α7 nAChR expression than mixed neuronal and glial cell cultures. Jensen *et al.* (1997) found that the presence of glia in cortical cultures or the addition of glial-conditioned media to cultures, led to the reduced formation of functional α-bungarotoxin-binding α7 nAChRs in the neuronal population.

Taking into consideration the role of glutamatergic receptors, especially the NMDA receptor subtypes, in hypoxic-ischemic brain injury (Choi, 1988; Meldrum and Garthwaite, 1990; Stuiver *et al.*, 1996; Leist and Nicotera, 1998), an initial investigation was launched into the reduction in neuronal viability of DIV 14 pure neuronal cultures upon exposure to either glutamate or NMDA insults. A plateau of cellular death level was achieved, despite efforts to increase the cell death by either raising the concentration or extending the exposure time to the excitotoxic treatments. This is possibly due to the putative desensitization of the NMDA receptors by the prolonged exposure or the high concentrations of the agonists (Skolnick *et al.*, 1992; Shi *et al.*, 2001). In later experiments where pure neuronal cultures were exposed earlier at either DIV 8 or 9 to NMDA, preconditioning experiments with KCl and nicotine respectively, a greater reduction in cellular viability was achieved. This is in

contradiction to other studies where it was shown that susceptibility to glutamate or NMDA-induced cell death increases over time in cultured neurons from rats (Peterson *et al.*, 1989; Cheng *et al.*, 1999; Frandsen and Schousboe, 1990; Xia *et al.*, 1995) and mice (Frandsen and Schousboe, 1990; Mizuta *et al.*, 1998; Schubert and Piasecki, 2001). Fogal *et al.* (2005), comparing DIV 10 and DIV 14 neurons, ascribed the observed increased susceptibility of DIV 14 cells to the increased secondary release of glutamate from stimulated or depolarized cells; not from a difference in NMDA receptor expression or function. A possible explanation for the observed differences in this study is that at an early age of the culture a number of 'fragile' cells are still present in the culture, with only the more resilient neurons surviving in the older cultures. The presence of such 'fragile' cells would have the dual consequence of increasing the cell number with a subsequent higher glutamate release; and increasing the difference between the control group with its surviving 'fragile' cells and the insulted group that have lost the 'fragile' cells in addition to the loss of the cells normally associated with an NMDA excitotoxic insult. A simpler explanation might be that the increased cell death is due to cultures being seeded at a very high density which would yield an increased cell death, although this seems unlikely. As noted before, there are many factors involved in the early stages when creating a culture set that would affect cell density, although the variation seems to be limited between culture sessions and cell death of the magnitude observed in DIV 8 or DIV 9 cultures were never observed in DIV 14 cultures.

Nicotine neuroprotection, a well documented phenomenon in neuronal cultures (Marin *et al.*, 1994; Donnelly-Roberts *et al* 1996; Dajas-Bailador *et al.*, 2000; Tohgi *et al.*, 2000), remained elusive in the pure neuronal cortical cultures. The reason why nicotine succeeded to protect pure hippocampal neurons in the Dajas-Bailador *et al.* (2000) study

yet failed to protect the pure neuronal mixed cortical culture against the concurrent NMDA insult might be due to the differences in cell type between the two culture systems. In experiments of where neurons were deprived of oxygen, it was shown that the neuronal death depended not only on the magnitude of exposure (Banasiak *et al.*, 2000; Luhmann and Kral, 1997) but also on the CNS region affected (Jiang and Haddad, 1992; Nieber, 1999). This might be a similar case for the response of neurons to various stimuli, in that the response would depend on the neurons' region of origin. It could also be due to the differences in the culture regime that might generate different levels of neuroprotective proteins and cell surface receptor expression at various times. Preconditioning with nicotine also failed; independent of the nicotine concentration, the exposure length or the day *in vitro* upon which the cells were preconditioned. A conclusive reason for the failure of nicotine to elicit a neuroprotective effect was not reached, although it could probably, once again, be ascribed to the culture system.

Achieving neuroprotective preconditioning in pure neuronal cortical cultures seem to be more difficult than in mixed glial and neuronal cortical cultures, possibly owing to the stringent chemical selection necessary to achieve the pure neuronal culture quality. As stated, Kapinya and co-workers where able to precondition DIV 8 pure neuronal cortical cultures created in exactly the same way as employed in this study, with the volatile anaesthetic isoflurane against a subsequent insult consisting of oxygen glucose deprivation (OGD) (Kapinya *et al.*, 2002). Following successful replication of these experiments with isoflurane, an attempt was made to precondition DIV 8 pure neuronal cortical cultures with potassium chloride against either OGD or a NMDA insult. In the Grabb *et al.* (1999) study, 45 mM KCl treatment was used to precondition mixed neuronal and glial cells against a subsequent excitotoxic NMDA insult. These

experiments were also repeated with significant but irregular success, also using mixed cortical neuronal and glial cultures. A follow-up study by the same group once again successfully used potassium chloride induced depolarisation to precondition mixed neuronal and glial cells against an OGD insult (Grabb *et al.*, 2002). In pure neuronal cultures, KCl did not induce any neuroprotective mechanisms against various magnitudes of either NMDA or OGD insults.

Subsequent to the experiments on DIV 8 pure neuronal cortical cultures, it was decided to return to attempting KCl-induced preconditioning in more mature DIV 14 cultures. As stated above, Fogal *et al.* (2005) observed that DIV 14 cells have increased secondary release of glutamate from depolarized cells. It has been shown that *in vivo* cortical spreading depression is dependent on such a release, as indicated by the ability of an NMDA receptor antagonist MK801 to block the neuroprotective effect of CSD (Gill *et al.*, 1992; Iijima *et al.*, 1992; Obrenovitch *et al.*, 2002). Initial short term exposure of DIV 14 pure neuronal cortical cultures to various concentration of KCl for either 15 minutes or 1 hour proved to be ineffective against a subsequent NMDA or glutamate lethal insult. Chronic or prolonged exposure (24 hours) of the cultures to various concentrations of KCl led to a significant reduction in cell viability, except for the lowest concentration employed i.e. 11.25 mM KCl. This is due to the glutamate release associated with depolarization, as mentioned above (Fogal *et al.*, 2005). Similar damage is observed in *ex vivo* experiments, such as in isolated tissue slices, where continuous K^+– depolarisation was associated with neuronal injury (Jing *et al.*, 1991). During cortical spreading depression *in vivo*, a similar marked transient increase in extracellular glutamate has been detected (Fabricius *et al.*, 1993; Zilkha *et al.*, 1995; Basarsky *et al.*, 1999), although this release is not associated with neuronal injury in the normal brain (Nedergaard and

Hansen, 1988; Nedergaard and Hansen, 1993). The brain is able to accommodate the glutamate release by either re-uptake or carrying the glutamate away, emphasizing the effect of vascular clearance (Gardner-Medwin *et al.*, 1981; Gardner-Medwin, 1983). The release of glutamate within the confines of a tissue culture microplate well has a cumulative effect, leading to the reduced viability observed with chronic treatment of KCl.

Importantly, prolonged exposure of the pure neuronal cultures to 11.25 mM potassium chloride resulted in significant protection of the cells against a subsequent glutamate insult, reducing the kill by approximately 45 %. The preconditioning effect also seemed to increase with an increase in cellular density. The marked apparent increase in cell viability at a density of 3000 cells/ mm^2 is, however, an artefact of the statistical analysis. The cell death due to the prolonged KCl treatment in the KCl treated control group is approximately double that experienced by the other densities (±8 % versus ±4 %) and division by this lowered viability score resulted in an increased product. Attempts to accurately judge how long the elicited neuroprotective effect lasts were hampered by potassium chloride induced cell death. The decrease in the neuroprotection induced by the preconditioning with KCl between the acute and chronic glutamate insults could possibly be due to the decline in the cells neuroprotective response or simply a reduced viability of the cells due to the prolonged treatment with KCl. It can, however, be assumed that the preconditioning effect is not due to a lowered metabolic state of damaged cells since the MTT scores are subject to mitochondrial activity – an indicator a cell's metabolic status. The attainment of KCl-induced neuroprotection with prolonged treatment and not with acute treatment may reflect the number of depolarisations induced in the set period of time. As observed by Horiguchi *et al.*, (2005) and discussed in the literature review, achieving CSD-induced tolerance *in vivo* requires the correct number of

waves of spreading depression. This depends on the metabolic rate of the animal at the time of treatment, with a high metabolic rate leading to too many CSDs and no neuroprotection, whereas a low metabolic rate has too few CSDs, once again leading to no generation of tolerance. Factors including the concentration of the potassium chloride and the duration of KCl application can also be manipulated to achieve the correct number of cortical spreading depressions. Inducing depolarization *in vitro* has the additional complication of balancing the attained level of tolerance while limiting the damage from the KCl-induced glutamate release. It was confirmed that the induction of neuroprotection in the *in vitro* model follows a similar mechanism to that seen *in vivo*, with tolerance generation being dependent upon NMDA receptor function; blocking of the tolerance generation being possible with the NMDA receptor antagonist MK801 (Kitagawa *et al.*, 1990; Kitagawa *et al.*, 1991; Kato *et al.*, 1992; Taga *et al.*, 1997)

Chazot *et al.* (2002) noted a 12–15-fold increase in α7 nicotinic acetylcholine receptor (nAChR) protein following KCl-induced CSD in the mouse cerebral cortex, hypothesizing that this increase might contribute to the increased tolerance to ischaemia. The role of α7 nAChRs in the KCl-induced neuroprotective effect associated with CSD was analysed *in vitro*, first by inhibiting the activation of Ca^{2+}-calmodulin dependent kinase II with KN-62, and second by directly blocking any action of α7 nAChR with the receptor-specific antagonist α-bungarotoxin. KN-62 has been shown to block the upregulation of α7 nAChR messenger RNA, possibly by blocking its transcriptional activation by the Egr-1 protein (De Koninck and Cooper, 1995; Enslen and Soderling, 1994). In pure neuronal cortical cultures, KN-62 was able to negate the KCl-induced neuroprotective effect. This does not, however, imply that specifically α7 nAChRs are involved in obtaining the KCl-induced neuroprotective effect, since Ca^{2+}-calmodulin

dependent kinase II as well as Egr-1 lead to the activation of various genes with potentially neuroprotective functions. A more specific experiment was performed using α-bungarotoxin, a homomeric α7 nAChR-specific antagonist. Subsequent to preconditioning *in vitro* with KCl, the pure neuronal cortical cultures were treated with this antagonist, which effectively cancelled the activity of α7 nAChRs during the glutamate excitotoxic insult. This cancellation of α7 nAChR activity led to the loss of the KCl-induced neuroprotective effect, strongly supporting the contention that α7 nAChR activity is required for KCl-induced protection. In an attempt to quantify the change – if any – in α7 nAChR expression, KCl preconditioned and nicotine treated cultures were stained with α-Bungarotoxin-FITC. Prolonged treatment with nicotine (24 hours) had no effect upon the expression and formation of functional α7 nAChRs. Contrary to the findings of Chazot *et al.* (2002) and other various studies in which KCl treatment was shown to increase α7 nAChR protein expression (De Koninck and Cooper, 1995; Utsugisawa *et al.*, 1997; Ridley *et al.*, 2001), here it was found that there was no such change; if anything there is a trend towards a **decrease** in the formation of functional homomeric α7 nAChRs. The statistical non-significance can be attributed to the high variance due to the independent blind counting methodology employed, as well as the fact that the cultures were created on poly-D-Lysine coated glass coverslips to which the cells attach poorly.

Attempts at verifying the α-bungarotoxin-FITC binding result with a Western blot protein analysis was unsuccessful due to the anti-α7 nAChR antibodies employed. The Santa Cruz Biotechnology rabbit polyclonal antibody (sc-5544) gave inconsistent results. This antibody was raised against a recombinant protein corresponding to amino acids 367 – 502 mapping at the carboxy terminus of human α7 nAChR subunit. As mentioned in the

introduction, the α7 nAChR gene undergoes alternative splicing resulting in an array of mostly non-functional α7-like proteins being formed. In addition to this, a duplication of exons 5 – 10 of the α7 nAChR gene, corresponding to amino acids 386 – 502, can be found within 1 Mb and centromeric to the full-length α7 nAChR gene on chromosome 15 of the human genome proteins (Gault *et al.*, 1998; Garcia-Guzman *et al.*, 1995). Antibodies and genetic probes directed at the α7 nAChR subunit are notorious for their unreliability (Herber *et al.*, 2004; Jones and Wonnacott, 2005; Moser *et al.*, 2007). Attempts to probe the preconditioned samples with an anti-rabbit α7 nAChR antibody from Abcam (ab23832), directed against a region within the first hundred amino acids of the N-terminal of the receptor yielded no signal.

Taking into account the unreliability of the antibody probes for the α7 nAChR subunit, a closer look was taken at the critical studies in which α7 nAChR upregulation subsequent to exposure to KCl was reported. The Utsugisawa *et al.* (1999) study only looked at the α7 nAChRs on an mRNA level, using reverse transcription polymerase chain reaction (RT-PCR) primers amplifying nucleotides 684 – 1055, corresponding to amino acids 228 – 352 i.e. the duplicated genome region. Chazot *et al.* (2002) utilized two mouse anti-α7 antibodies, Mab306 and Mab319 (Cambridge Bioscience). Mab306 is a monoclonal mouse anti-α7 antibody that recognizes amino acids 380 – 400, whereas Mab 319 is another monoclonal anti-α7 antibody that recognizes amino acids 365 – 384. Both of these antibodies are targeted at regions in the C-terminus of the α7 nAChR subunit. The validity of studies on α7 nAChRs relying on either antibodies or genetic mRNA probes without looking at any functional changes by means of α-bungarotoxin binding are questionable (Herber *et al.*, 2004; Jones and Wonnacott, 2005; Moser *et al.*, 2007). It is, however, accepted that this current study does not absolutely refute these previous studies

since there exist large differences between the strategies and methodologies employed to study the α7 nAChR phenomenon.

Both the De Koninck and Cooper (1995) and the Ridley *et al.* (2001) studies relied upon the use of α-bungarotoxin binding to confirm their results that chronic depolarisation of neurons with KCl lead to an increase in α-bungarotoxin binding. De Koninck and Cooper (1995) applied 40 mM KCl to DIV 5 neonatal rat sympathetic neurons for two days, resulting in the detection of a three- to fivefold increase in α-bungarotoxin binding. Ridley *et al.* (2001) applied 20 mM KCl to DIV 3 rat primary hippocampal cultures for four days before analysing the functional α7 nAChR expression level, detecting an approximate 72 % increase in α-bungarotoxin binding. Neither of these studies looked at the preconditioning effect of KCl, which would have proved difficult taking into consideration the findings in the present study indicating the loss of neurons due to secondary glutamate release over prolonged periods of time. Of interest is the Barrantes *et al.* (1995) study in which it was shown that primary hippocampal cultures on average express five times as much functional α-bungarotoxin binding α7 nAChRs than primary cortical cultures, which might account for some of the differences observed between the present study and the literature. Utilizing similar cultures treatment methodology as the Ridley *et al.* (2001) study, it was also shown in the Barrantes *et al.* (1995) study that 24 hour treatment with 10 μM nicotine does not increase α7 nAChRs in hippocampal cultures. Only after four days of nicotinic treatment was an approximate 10 % increase in α-bungarotoxin binding detected.

In conclusion, this chapter has discussed the creation, optimization and the validation of a reliable *in vitro* KCl-induced preconditioning system using pure neuronal cortical

cultures. This chapter provided evidence that the model follows a similar mechanism as cortical spreading depression *in vivo*, despite the lack of glia. In contradiction to the prediction made in the Chazot *et al.* (2002) paper which served as basis for the current study, it was shown that functional α7 nAChRs are not upregulated upon chronic depolarisation with KCl, although the activity of this receptor subtype remained essential for the preconditioning mechanism. In addition, preliminary studies by Dr. G. Jackson have indicated that the same samples used in the Chazot *et al.* (2002) study do not have an increase in functional α-bungarotoxin binding to α7 nAChRs, further lending support for the present study and illustrating that any potential α7 nAChR protein expression changes previously reported does not extrapolate to increase in functional expression, but may in fact reflect a loss of function.

The next chapter is concerned with studying the putative obligatory α7 nAChRs trafficking protein, Ric3 and its potential role in controlling the functional expression of α7 nAChRs *in vitro*. This next stage required the development, characterisation and use of the first anti-mouse Ric3 selective antibody.

CHAPTER 3: Molecular Characterisation of the Putative Alpha 7 nAChR Trafficking Protein, Ric3

3.1. Introduction

The regulation of functional expression of the α7 nAChR has significant implications for this study. Ric3 has recently been identified as an obligatory trafficking protein for the α7 nAChR in heterologous expression studies *in vitro* (Halevi *et al.*, 2002) – a link that remains to be confirmed *in vivo*. In addition to this, Ric3 has been shown to have diverse effects on other ligand-gated ion channel expression, i.e. *hric-3* decreases the whole-cell current amplitude when co-expressed with α4β2 and α3β4 receptors in oocytes (Halevi *et al.*, 2003) but leads to an increase in these receptors when expression occurs in homologous mammalian cells (Lansdell *et al.*, 2005). Halevi *et al.* (2003) reported mRNA transcription of *ric3* gene isoforms as detected by Northern blot analyses in various tissues, including brain, heart, kidney, lung, liver, muscle, and pancreatic tissue. The functions and targets of all these isoforms are still unknown, although the diverse effects of this protein on ligand-gated ion channel expression and the varied tissue expression pattern necessitates a detailed *in vivo* Ric3 distribution analysis.

In an attempt to analyze the distribution of *ric3* expression within the mouse brain, Halevi *et al.* (2003) performed an *in situ* analysis using an mRNA probe based on the sequence of the second membrane domain and the coiled-coil domain of the partial mouse sequence to which they had access at the time. Taking into consideration the varied isoforms of the mRNA detected with their Northern blot analysis of various tissues,

it would be a leap to conclude that they all translate into protein – not to mention functional protein. To address this problem, we will attempt to design a specific anti-mouse Ric3 antibody to identify Ric3 protein expression in the mouse brain.

Within this chapter, the methods are described for structurally analysing the mouse Ric3 protein for the design of the antibody peptide, preparing the peptides for immunization, the immunization procedure, antibody purification and the experiments performed to analyse the antibody's specificity.

3.2. Materials and Methods

3.2.1. Sequence Homology Analyses and Secondary Structure Prediction

Nucleotide and amino acid sequences of the human and mouse *ric3* were aligned and compared using the CLUSTAL X (1.81) program (Higgins and Sharp, 1988; Higgins *et al.*, 1996). Secondary structure consensus prediction was performed using the Network Protein Sequence Analysis program (NPS@) (Combet *et al.*, 2000), which relied on the average of the following predictive methods:

SOPMA (Geourjon and Deléage, 1995)

HNN (Guermeur, 1997)

MLRC (Guermeur *et al.*, 1999)

DPM (Deléage and Roux, 1987)

GOR III (Gibrat *et al.*, 1987)

GOR IV (Garnier *et al.*, 1996)

PHD (Rost and Sander, 1993)

SIMPA96 (Levin, 1997)

SOPM (Geourjon and Deléage, 1994) parameters: Window width = 17; Similarity threshold = 8; Number of states = 4.

GOR I (Garnier *et al.*, 1978) parameters: Decision constant for: helix = -100; sheet = -88; turn = 0; coil = 0.

PREDATOR (Frishman and Argos, 1996) parameters: Secondary structure data = dssp

Each one of these secondary structure predictive methods is loosely based on the principal that the parameters used for the prediction were determined from a subset of x-

amount of proteins with known three dimensional structures to form an algorithm (using the theory of information) with conditional probabilities being calculated.

The prediction of transmembranous regions is based on hydrophobicity analyses and the positive inside rule that indicates that the cytoplasmic side of a membrane is rather positively charged whereas the external side tends to be more negatively charged. This was preformed using Von Heijne (1992) transmembrane predictive method of the ANTHEPROT package (Geourjon *et al.*, 1991; Geourjon and Deleage, 1995).

The NetNGlyc 1.0 Server (Gupta *et al.*, 2004) predicts N-glycosylation sites in human proteins using artificial neural networks that examine the sequence context for the presence of an Asn-Xaa-Ser/Thr motif, where X stands for any amino acid. A consensus prediction for the presence of possible phosphorylation sites was derived from KinasePhos (Huang *et al.*, 2005a; Huang *et al.*, 2005b) and NetPhosK 1.0 Server (Blom *et al.*, 2004).

3.2.2. Peptide Design and Creation

The ANTHEPROT package utilizing the Hopp and Woods predictive method (Hopp and Woods, 1981; Hopp and Woods, 1983) were used to calculate the hydropathy plot of the mouse Ric3 protein. The same package used the antigenicity predictive method as described by Parker *et al.* (1986) for identifying the strongest antibody generating regions within the Ric3 protein. The choice of the peptide sequence relied upon these two factors as well as the potential peptide's position relative to the carboxyl terminal. Another important factor is how unique the chosen peptide sequence is within the genome of the

organism for which the sequence was compared to all known protein sequences in National Centre for Biotechnology Information (NCBI) records system..

A cysteine residue was added to the carboxyl-terminal of the sequence enabling the peptide to be coupled to the thyroglobulin carrier protein and to the sepharose beads for the antibody purification (see below). The peptide itself was produced by Cambridge Research Biochemicals Ltd. (UK).

3.2.3. Coupling of the Peptide to the Carrier Protein

The 15 amino acid peptide is too small to stimulate an immune response and has to be coupled to a large immunogenic carrier protein. The 3-maleimimdobenzoic acid N-hydroxysuccinimide ester (MBS) method, as described by Duggan *et al.* (1991), was used to conjugate the peptide to the thyroglobulin carrier protein by means of a cysteine residue that was incorporated at the carboxyl-terminal of the peptide

Thyroglobulin was dissolved to a final concentration of 20 mg/ ml in a buffer (10 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.2) before being dialysed against 500 ml of the same buffer for 12 hours at 4 °C. 4 mg (200 µl) of the dialysed carrier protein was further diluted by the addition of 50 µl of the buffer prior to the activation of the carrier protein by dropwise addition of 85 µl stock MBS (3 mg/ ml MBS in N, N-dimethylformamide). The solution was incubated at room temperature for 30 minutes with shaking. The activated thyroglobulin was separated from free MBS by dialysis against 1 litre of a second buffer consisting of 50 mM KH_2PO_4, 50 mM Na_2HPO_4, pH 6.0, for 2 hours at room temperature with 2 x 1 litre solution changes. 4 mg of peptide was dissolved in 1 ml

of 10 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.2 before being added to the dialysed activated carrier protein and incubated for 12 hours at room temperature with gentle mixing. The peptide-carrier protein conjugate was separated from the uncoupled peptide by dialysis against phosphate buffered saline (PBS) for a total of 4 hours at 4 °C, with 4 x 500 ml solution changes. The dialysed peptide protein conjugate was made up to a final concentration of 1 mg/ ml with PBS and stored in 100 µl aliquots at -20 °C.

3.2.4. Inoculation procedure

A 100 µl (100 µg) of freshly thawed peptide-carrier protein conjugate was mixed with 200 µl sterile PBS and emulsified with an equal volume of complete Freund's Adjuvant. This preparation was injected intramuscularly into both hind legs of a Dutch rabbit. Secondary immunisations at 1 month intervals were prepared using incomplete Freund's Adjuvant. 10 – 15 ml of blood was collected from the marginal ear vein of the animal, 7 – 10 days subsequent to every secondary or booster injection. The blood sample was allowed to stand at room temperature for 2 hours, and then clot contraction was allowed to occur over 16 hours at 4 °C. The serum was collected by centrifugation at 12000g for 10 minutes at 4 °C, and was stored in 1ml aliquots at -20 °C. All procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 (PPL 602/657).

3.2.5. Coupling of Peptides to Sepharose Beads via Sulphydryl Groups

For the purification of anti-peptide polyclonal antibodies, the antigen peptide had to be coupled to thiol-sepharose beads via the peptides C-terminal cysteine residue. The coupling of the peptide to the activated sepharose beads was carried out according to the method described by Stephenson and Duggan (1989).

0.35 g of activated thiol-sepharose was allowed to swell in 100 ml ddH_2O at room temperature for 15 minutes. The swollen sepharose was added to a 15 ml column with a scintered filter at the bottom and the water allowed to flow through the column under gravity flow. The swollen sepharose was washed with a 100 ml of a Solution A consisting of 0.1 M Tris-HCl, 0.3 M NaCl and 1 mM EDTA, pH 8.0. 1 ml of Solution A containing 5 mg of the dissolved peptide was added to the sepharose. Subsequent to a one hour incubation period, the reaction was terminated by washing the sepharose with 25 ml of Solution A, followed by a second wash consisting of 10 ml of Solution B consisting of 100 mM citric acid at pH 4.5. Any remaining unbound thiol groups were blocked by incubating the sepharose with 3 ml of 1 mM β-mercaptoethanol dissolved in Solution B for 45 minutes at room temperature, with shaking. The blocking reaction was terminated by washing the sepharose with 25 ml of Solution B. The sepharose was equilibrated with 25 ml PBS before being stored in 10 ml PBS containing 0.02 % (w/v) sodium azide, and the column at 4 °C until use.

3.2.6. Peptide Affinity Purification of Antibodies

The sepharose column linked to the appropriate peptide (section 3.2.5) was equilibrated by passing a 100 ml of PBS through the column. Five millilitres of the immune serum (section 3.2.4) was added to the sepharose and incubated for twelve hours at 4 °C with gentle shaking. The unbound immune serum was allowed to pass through the column under gravity flow before the column was washed with 100 ml PBS. The bound antibody was released from the sepharose and eluted drop-wise from the column by the application of 10 ml of 50 mM glycine/ HCl, pH 2.3. 10 x 1 ml fractions were sequentially collected in eppendorf tubes before being equilibrated by the addition of 20 µl of 1 M Tris to give a final pH 7.4. The optical density (O.D.) readings of the antibody fractions were taken at λ = 280 nm before the concentration of the antibodies was calculated using the Beer Lambert law,

$$C = \frac{A}{\varepsilon L}$$

Where 'C' represents the concentration of antibody, 'A' is the absorbance of the antibody at λ = 280 nm, 'ε' is the molar extinction coefficient, and 'L' is the path length. The fractions with concentrations above 0.1 mg/ ml protein were pooled and dialysed against 500 ml TBS for 12 hours at 4 °C. Subsequent to dialysis, the antibody was again subjected to spectral analyses at λ = 280 nm and the concentration recalculated. The purified antibody was then stored with the addition of 250 µM sodium azide at 4 °C until use.

The sepharose affinity column was regenerated with the application of 100 ml PBS and, once again, stored with 10 ml of PBS containing 250 μM sodium azide at 4 °C until later use.

3.2.7. Immunoblotting of Various Tissues

Brain, kidney, and lung tissue were harvested from adult CD1 mice (25 – 30 g; Charles River UK) and homogenized in phosphate buffered saline (PBS) containing 1 in 100 dilution of Protease Inhibitor Cocktail Set III with a dounce glass/glass homogenizer at a concentration of approximately 50 mg/ ml. In chapter two the methods are described for SDS page gel analyses (section 2.2.8), and Western blot protein analyses (section 2.2.9).

Adult male C57BL/6 mice (25–30 g; Harlan UK) were preconditioned by the elicitation of 10 full consecutive recurrent cortical spreading depressions (CSD) by the epidural application of 1 M KCl to the right occipital cortex, applying physiological saline to the sham-operated controls, as described by Godukhin and Obrenovitch (2001). The brains were extracted at various times post-preconditioning (24, 72, and 168 hours) and frozen in liquid N_2 before being stored at -20 °C. They were a kind gift from Dr. G. Jackson and Prof. T. P. Obrenovitch. Whole brain samples were homogenized, as above, at final concentration of 2 mg/ ml.

3.2.8. Ric3 Expression in Preconditioned Pure Neuronal Cortical Cultures

Pure neuronal cortical culture (sections 2.2.1 and 2.2.3) were seeded at 1500 cells/ mm^2 on poly-D-Lysine-coated 35 mm Iwaki tissue culture treated dishes and treated for 24 hours on DIV 14 with either 11.25 mM KCl dissolved in medium or just plain medium for the control group. Cells were harvested either acutely or chronically (3 wash steps and 24 hours after subsequent incubation) in 500 µl phosphate buffered saline (PBS) containing 1 in 100 dilution of Protease Inhibitor Cocktail Set III. The samples were sonicated four times for 30 seconds in a sonication bath with a 1 minute incubation period on ice in between every sonication pulse. 100 µl of the sample was chloroform/ methanol precipitated (section 2.2.7) before being run on a 6 % SDS-polyacrylamide gel (section 2.2.8) and a Western blot analysis (section 2.2.9).

3.2.9. Immunohistochemistry

Murine brain samples subjected to cortical spreading depression or sham treatment by Dr. G. Jackson and Prof. T. P. Obrenovitch were harvested at various times (24 h, 72 h, and 168 h) and subjected to immunohistochemical analyses. 25 µm coronal cryostat sections of the unfixed brains were collected onto Polysine™ glass slides (Agar Scientific, Ltd.) coated glass slides before being air dried and stored at -20 °C until use. The samples were fixed in 2 % (v/v) formaldehyde in Tris Buffer Saline (TBS) and incubated at 37 °C for 20 minutes followed by 30 minutes incubation in 2 % (v/v) formaldehyde but at room temperature. Samples were washed thrice for 5 minutes with TBS before incubating in a solution consisting of 10 % (v/v) methanol and 3 % (v/v) hydrogen peroxide dissolved in TBS for 10 minutes. This was followed by a 15 minute incubation with 0.2 % (w/v)

glycine and 0.2 % (v/v) Tween 20 in TBS and a subsequent incubation with 0.2 % (v/v) Triton-X-100 in TBS for 15 minutes. Samples were next incubated in 50 mM sodium citrate, pH 8.4 first at room temperature for 30 minutes then at 80 °C for another 30 minutes. This was followed by a 15 minute incubation in 0.2 % (v/v) Triton-X-100 in TBS followed by the application of the blocking solution consisting of 2 % (v/v) fetal calf serum (FCS) and 0.2 % (v/v) Tween 20 in TBS for1 hour. The anti-mRic3 primary antibody (2 μg/ ml) was diluted in 1 % (v/v) FCS-containing TBS and applied to the tissue for 12 hours at 4 °C. For the peptide block experiment, the antibody was incubated with the original antigenic peptide (500 μg/ ml; Cambridge Research Biochemicals Ltd., UK) for a period of 12 hours before use in the immunohistochemistry procedure

The Vectastain ABC kit (Vector Laboratories, Inc.), a biotin-avidin-peroxidase system, was used to detect the bound primary antibody. The samples were washed thrice for two minutes with 2.5 % (w/v) dried milk in TBS before the application of 1 % (v/v) FCS containing a 1 in 100 dilution of the biotinylated secondary antibody for 20 minutes. Samples were washed again (3 x 2 minutes) with 2.5 % (w/v) dried milk in TBS before the 5 minute application of Vector ABC diluted in TBS (1 in 50). Samples were washed five times for 2 minutes in TBS and developed in DAB (3, 3'-diaminobenzidine) (10 ml TBS, 1 DAB tablet, 6 μl H_2O_2) for 20 minutes in the dark. Samples were washed a final time with water before being mounted with DPX Mountant for histology (Fluka). Images were taken with a COOLPIX 950 Digital Camera on a Nikon Eclipse E400 microscope at 10X magnification. The composite picture in Figure 3.9 was created by taking sequential photographs of the region of interest and aligning them in Microsoft® Word 2002.

3.3. Results

3.3.1. Ric3 Gene and Protein Sequence Analysis

At the time that Halevi and co-workers conducted their interspecies *ric3* sequence homology study (Halevi *et al.*, 2003), the complete mouse *ric3* gene (*mric3*) sequence was unavailable for comparison and they relied upon an extrapolation from the human *ric3* gene (*hric3*) sequence (NCBI accession number AY326435) to complete the mouse sequence. Subsequent to the Ric3 protein species alignment (Halevi *et al.*, 2003), several *mric3* isoforms cDNAs were submitted to NCBI, and the one with the highest homology was used in the following experiments (RIKEN Genome Exploration Research Group Phase II Team and the FANTOM Consortium; NCBI accession number AK053760). Other *mric3* isoforms (NCBI accession number NM_178780 and AK082275) consisting of equal or less nucleotides than the AK053760 isoform yet with lower homology, were also detected in the NCBI submission (results not shown). As can be seen from the sequence homology analysis using ClustalX version 1.81 in Figure 3.1, the 3' region of the mouse gene coding for the C-terminal of the Ric3 protein is distinctly different and considerably shorter (413 base pairs) than its human counterpart. The *mric3* consists of 687 base pairs (bp) coding for 228 amino acids (aa), whereas the human counterpart consists out of 1100 bp coding for a 369 aa protein. Only 87 bp of the 687 bp mouse *ric3* gene differ from the human gene, resulting in 87.33 % homology within this first stretch of DNA, although taking into account the much longer human gene results in the reduction of the homology to approximately 54.55 %.

A comparison between the predicted secondary structure of the human and mouse Ric3 proteins was also performed and is shown in Figure 3.2. There exists high homology

between the secondary structures with five common large alpha-helix motifs found at approximate amino acid positions 4 – 30, 66 – 81, 109 – 118, 143 – 168, and 181 – 201. Similarly, two common random coil regions or coil-coil regions are found at approximate amino acid positions 30 – 66 and 81 – 95, although only one of the three extended regions found in the human form of Ric3 is also located in the mouse version of the protein at approximate amino acid position 97 – 102. The extended regions in the first 228 aa of the human Ric3 protein accounts for 4.82 % of the structure, whereas only 2.19 % of the mouse homolog. The function of these regions in the Ric3 protein remain to be identified although the transmembrane predictive comparison in Figure 3.3 indicates that the first extended region at approximate 97 – 102 aa, which is common to both the human and mouse version, is in fact necessary for crossing the cell membrane (aa 99 – 112) from the extracellular domain to the cytoplasmic region. The transmembrane prediction also indicates an earlier crossing event at approximate aa position 11 – 22 which corresponds to an area within one of the large alpha-helix regions.

The amino acid sequence of both mouse and human Ric3 were analysed for N-glycosylation and phosphorylation sites. The NetNGlyc 1.0 Server detected no motifs in either the human or mouse versions of the Ric3 protein which would indicate the presence of a glycosylation site. The consensus phosphorylation prediction derived from the KinasePhos and NetPhosK 1.0 Server prediction programs revealed the presence of a putative protein kinase C (PKC) phosphorylation site at amino acid 178 of the human homolog, a protein kinase CK2 (previously known as casein kinase II) site at amino acid positions 180 and 334, and finally a potential phosphorylation site for either protein kinase CK1 or CK2 at amino acid position 273. The mouse Ric3 protein contains a single potential protein kinase CK2 phosphorylation site at aa position 179.

The mouse *ric3* gene sequence was further analysed using the ANTHEPROT package in an attempt to identify a possible region for the antibody generation. A hydrophilic region is required for the antigenic peptide since these are the regions most likely to be exposed on the surface of the mature folded protein and a short hydrophobic peptide might lead to aggregation. A hydropathy plot was drawn using the Hopp and Woods predictive method (Fig. 3.2 A), which depends on the hydrophilic character of each amino acid. This is based on the property of the amino acid's side chain to be more soluble in water than in an apolar solvent. As can be seen in Figure 3.2 A, Ric3 is an overall hydrophilic molecule with 49.1 % hydrophilic aa, 12.3 % ambivalent aa, and 38.6 hydrophobic aa, with a net hydropathy value of + 38. 6. It was decided that the peptide region from aa 208 to aa 222 with a net hydrophilic character of + 61 was ideally located close to the C-terminus. An antigenicity plot according to the predictive method described by Parker *et al.* (1986) was calculated and it was found that the region from aa 208 to aa 222 also has a high antigenic value (Fig. 3.2 B). In addition to the hydrophilicity character of the amino acids, the antigenicity predictive profile is based on the atomic flexibility and experimental HPLC retention times of synthetic peptides. A cysteine residue was attached the C-terminus to yield a final peptide with a sequence of H-Cys-Glu-Lys-Glu-Ala-Glu-Glu-Ala-Pro-Tyr-Met-Glu-Asp-Trp-Glu-Gly-NH_2.

```
M ric3      ATGGCGTACTCCACGGTGCAGAGAGTGGCGCTGGCCTCGGGGCTCGTCCTGGCCGTGTCG
M Ric3      M  A  Y  S  T  V  Q  R  V  A  L  A  S  G  L  V  L  A  V  S
H ric3      ATGGCGTACTCCACAGTGCAGAGAGTCGCTCTGGCTTCTGGGCTTGTCCTGGCTCTGTCG
H Ric3      M  A  Y  S  T  V  Q  R  V  A  L  A  S  G  L  V  L  A  L  S
DNA Homol   ********************** ********** ** ***** ** ***** ********  *****

M ric3      CTGCTGCTGCCCAAGGCCTTCTTGTCTCGCGGGAAGCGACCGGAGCCGCCGCCGGGCCCG
M Ric3      L  L  L  P  K  A  F  L  S  R  G  K  R  P  E  P  P  P  G  P
H ric3      CTGCTGCTGCCCAAGGCCTTCCTGTCCCGCGGGAAGCGGCAGGAGCCGCCGCCGACACCT
H Ric3      L  L  L  P  K  A  F  L  S  R  G  K  R  Q  E  P  P  P  T  P
DNA Homol   ********************** **** ********** * *************   **

M ric3      GAAGGAAAATTGGACCGATTTCCACCTATGATGCATCACCACTCGGCACCCTCAGATGGT
M Ric3      E  G  K  L  D  R  F  P  P  M  M  H  H  H  S  A  P  S  D  G
H ric3      GAAGGAAAATTGGGCCGATTTCCACCTATGATGCATCATCACCAGGCACCCTCAGATGGC
H Ric3      E  G  K  L  G  R  F  P  P  M  M  H  H  H  Q  A  P  S  D  G
DNA Homol   ************* ************************ ***  ***************

M ric3      CAGACACCAGGGGCTCGTTTCCAGAGGTCTCACCTTGCAGAGGCCTTTGCAAAGGCCAAG
M Ric3      Q  T  P  G  A  R  F  Q  R  S  H  L  A  E  A  F  A  K  A  K
H ric3      CAGACTCCTGGGGCTCGTTTCCARAGGTCTCACCTTGCCGAGGCATTTGCAAAGGCCAAA
H Ric3      Q  T  P  G  A  R  F  Q  R  S  H  L  A  E  A  F  A  K  A  K
DNA Homol   ***** ** ************** ************** ***** **************

M ric3      GGAGCAGGTGGAGGTGCTGGAGGAGGGGGTAGTGGAAGAGGACTGATGGGCCAGATCATT
M Ric3      G  A  G  G  G  A  G  G  G  G  S  G  R  G  L  M  G  Q  I  I
H ric3      GGATCAGGTGGAGGTGCTGGAGGAGGAGGTAGTGGAAGAGGTCTGATGGGGCAGATTATT
H Ric3      G  S  G  G  G  A  G  G  G  G  S  G  R  G  L  M  G  Q  I  I
DNA Homol   *** ********************** ************** ******** ***** ***

M ric3      CCAATCTATGGCTTTGGGATCTTTTTGTACATACTGTACATTTTGTTTAAGCTTTCAAAG
M Ric3      P  I  Y  G  F  G  I  F  L  Y  I  L  Y  I  L  F  K  L  S  K
H ric3      CCAATCTACGGTTTTGGGATTTTTTTATATATACTGTACATTCTATTTAAGCTCTCAAAG
H Ric3      P  I  Y  G  F  G  I  F  L  Y  I  L  Y  I  L  F  K  L  S  K
DNA Homol   ******** ** ******** ***** ** ************ * ******** ******

M ric3      GGGAAAACT---GCAGAGGATCGGAACTGCTCCACTGCCCCACCTGGAAACGCCCACAGG
M Ric3      G  K  T  -  A  E  D  R  N  C  S  T  A  P  P  G  N  A  H  R
H ric3      GGGAAAACAACTGCAGAGGATGGGAAATGCTATACTGCCATGCCTGGAAACACCCACAGG
H Ric3      G  K  T  T  A  E  D  G  K  C  Y  T  A  M  P  G  N  T  H  R
DNA Homol   ********    ********* **** ****  ******   ********* ********

M ric3      AAGATTACCAACTTTGAGCTTGTTCAACTACAAGAAAAACTAAAAGAGACAGAAGAAGCC
M Ric3      K  I  T  N  F  E  L  V  Q  L  Q  E  K  L  K  E  T  E  E  A
H ric3      AAAATTACCAGTTTTGAGCTTGCTCAACTGCAAGAAAAACTGAAGGAGACAGAAGCAGCC
H Ric3      K  I  T  S  F  E  L  A  Q  L  Q  E  K  L  K  E  T  E  A  A
DNA Homol   ** *******  ********** ****** *********** ** ********** ****

M ric3      ATGGAAAAATTAATCAACAGAGTTGGACCTAATGGTGAGAGCAGAGCACAGGCTGTGACT
M Ric3      M  E  K  L  I  N  R  V  G  P  N  G  E  S  R  A  Q  A  V  T
H ric3      ATGGAAAAATTAATCAACAGAGTGGGACCTAATGGTGAGAGCAGAGCACAGACTGTGACT
H Ric3      M  E  K  L  I  N  R  V  G  P  N  G  E  S  R  A  Q  T  V  T
DNA Homol   ********************** ************************** ********

M ric3      TCTGACCAAGAGAAACGATTACTGCATCAGCTCCGAGAAATCACCAGGGTCATGAAAGAA
M Ric3      S  D  Q  E  K  R  L  L  H  Q  L  R  E  I  T  R  V  M  K  E
H ric3      TCTGACCAAGAGAAACGGTTGCTACATCAGCTCCGAGAAATCACCAGGGTCATGAAAGAA
H Ric3      S  D  Q  E  K  R  L  L  H  Q  L  R  E  I  T  R  V  M  K  E
DNA Homol   ***************** ** ** ***********************************

M ric3      GGCAAGTTCATCGACACA---TCTCCAGAGAAGGAAGCTGAGGAAGCCCCATACATGGAG
```

```
M Ric3      G  K  F  I  D  T  -  S  P  E  K  E  A  E  E  A  P  Y  M  E
H ric3      GGAAAATTCATTGACAGATTTTCTCCAGAGAAAGAAGCTGAGGAGGCCCCTTACATGGAG
H Ric3      G  K  F  I  D  R  F  S  P  E  K  E  A  E  E  A  P  Y  M  E
DNA Homol   ** ** ***** **** *  ********** ********** ***** *********

M ric3      GACTGGGAAGGTAAA-----ATGCCCCTTCCTTGTTAG----------------------
M Ric3      D  W  E  G  K  -  -  M  P  L  P  C  Stop  -  -  -  -  -  -  -
H ric3      GACTGGGAAGGTTACCCTGAAGAGACTTACCCAATTTATGACCTTTCAGACTGTATCAAG
H Ric3      D  W  E  G  Y  P  E  E  T  Y  P  I  Y  D  L  S  D  C  I  K
DNA Homol   ********** *     *    * * **   **

M ric3      ------------------------------------------------------------
M Ric3      -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
H ric3      CGTAGGCAAGAAACAATCTTGGTGGATTACCCTGACCCAAAAGAACTTTCTGCTGAAGAA
H Ric3      R  R  Q  E  T  I  L  V  D  Y  P  D  P  K  E  L  S  A  E  E
DNA Homol

M ric3      ------------------------------------------------------------
M Ric3      -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
H ric3      ATAGCTGAAAGAATGGGAATGATAGAAGAGGAAGAATCAGATCATTTGGGTTGGGAAAGT
H Ric3      I  A  E  R  M  G  M  I  E  E  E  E  S  D  H  L  G  W  E  S
DNA Homol

M ric3      ------------------------------------------------------------
M Ric3      -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
H ric3      CTGCCCACTGACCCCAGAGCCCAGGAAGATAATTCTGTTACCTCGTGTGATCCAAAGCCA
H Ric3      L  P  T  D  P  R  A  Q  E  D  N  S  V  T  S  C  D  P  K  P
DNA Homol

M ric3      ------------------------------------------------------------
M Ric3      -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
H ric3      GAAACATGTTCCTGCTGTTTTCATGAAGACGAGGATCCTGCTGTCTTGGCAGAGAATGCT
H Ric3      E  T  C  S  C  C  F  H  E  D  E  D  P  A  V  L  A  E  N  A
DNA Homol

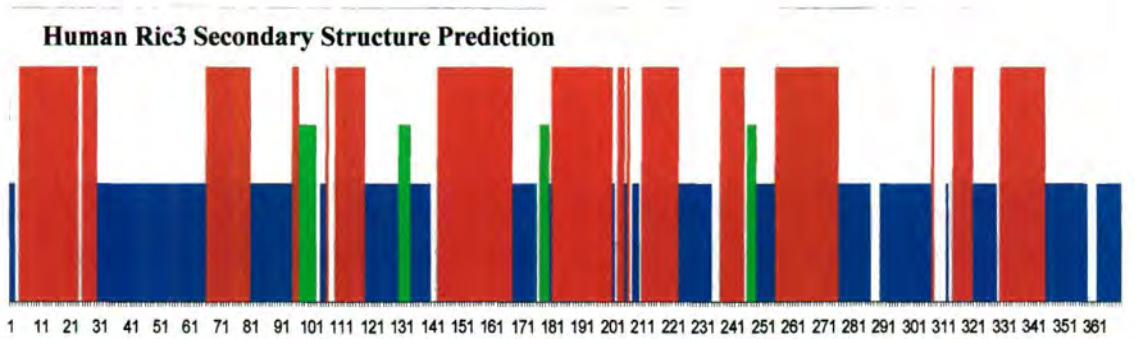
M ric3      ------------------------------------------------------------
M Ric3      -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
H ric3      GGATTCAGTGCAGATAGCTACCCTGAGCAAGAGGAAACCACCAAAGAAGAGTGGTCCCAA
H Ric3      G  F  S  A  D  S  Y  P  E  Q  E  E  T  T  K  E  E  W  S  Q
DNA Homol

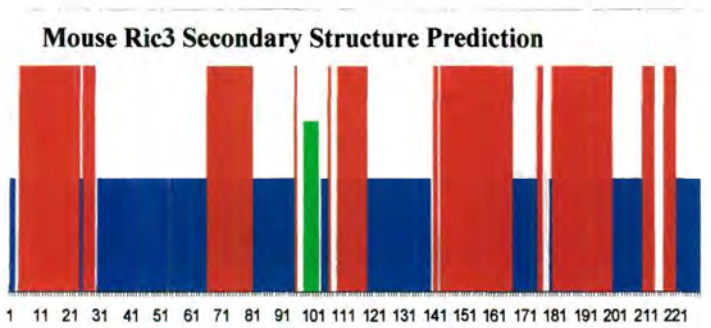
M ric3      ------------------------------------------------------------
M Ric3      -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
H ric3      GACTTTAAAGATGAAGGGTTGGGCATCAGCACCGATAAAGCATATACAGGCAGCATGCTG
H Ric3      D  F  K  D  E  G  L  G  I  S  T  D  K  A  Y  T  G  S  M  L
DNA Homol

M ric3      ----------------------------
M Ric3      -  -  -  -  -  -  -  -  -  -
H ric3      AGGAAGCGTAACCCCCAGGGTTTAGAGTGA
H Ric3      R  K  R  N  P  Q  G  L  E  Stop
DNA Homol
```

Figure 3.1. CLUSTAL X (1.81) multiple sequence homology alignment between the mouse (NCBI accession number AK053760) and human *ric3* (NCBI accession number AY326435) genes, accompanied by their respective amino acid sequences. DNA

homology is indicated by * whereas amino acid differences are highlighted in red in the human. The 15 blue amino acids represent the peptide chosen for antibody generation.





Secondary Structure Comparison			
Motif	**Mouse**	**Human First 228 aa**	**Full Length Human**
Alpha helix	**106 is 46.49%**	**109 is 47.81%**	**165 is 44.72%**
310 helix	0 is 0.00%	0 is 0.00%	0 is 0.00%
Pi helix	0 is 0.00%	0 is 0.00%	0 is 0.00%
Beta bridge	0 is 0.00%	0 is 0.00%	0 is 0.00%
Extended strand	**5 is 2.19%**	**11 is 4.82%**	**16 is 4.34%**
Beta turn	0 is 0.00%	0 is 0.00%	0 is 0.00%
Bend region	0 is 0.00%	0 is 0.00%	0 is 0.00%
Random coil	**103 is 45.18%**	**96 is 42.11%**	**161 is 43.63%**
Other states	0 is 0.00%	0 is 0.00%	0 is 0.00%

Figure 3.2. The secondary structure prediction of the mouse and human Ric3 proteins accompanied by a table containing an analysis of each motif's occurrence.

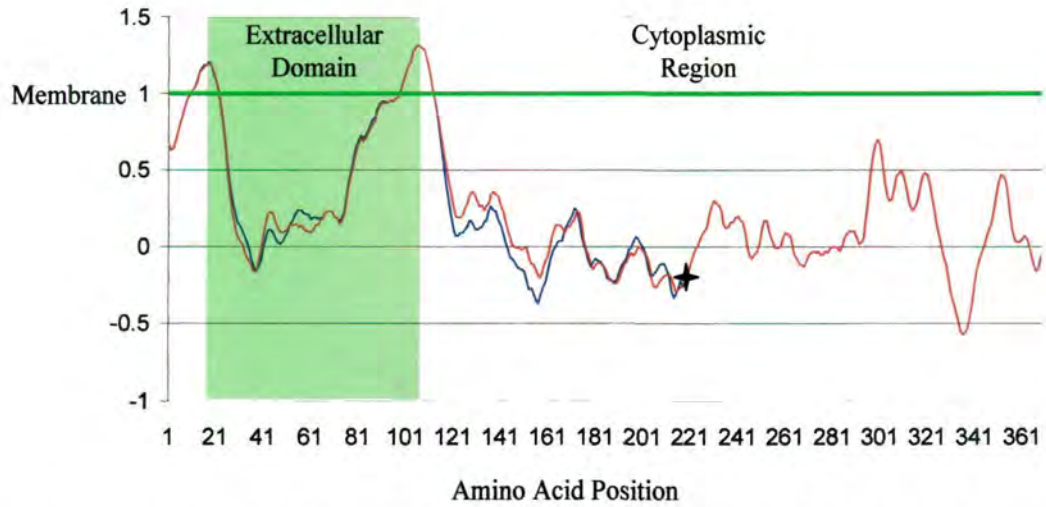


Figure 3.3. A prediction of the transmembrane regions indicates that both the mouse (blue) and the human (red) Ric3 proteins cross the membrane (green) twice as is depicted by the two peaks rising above the green line. ✦ indicates the end of the mouse Ric3 protein.

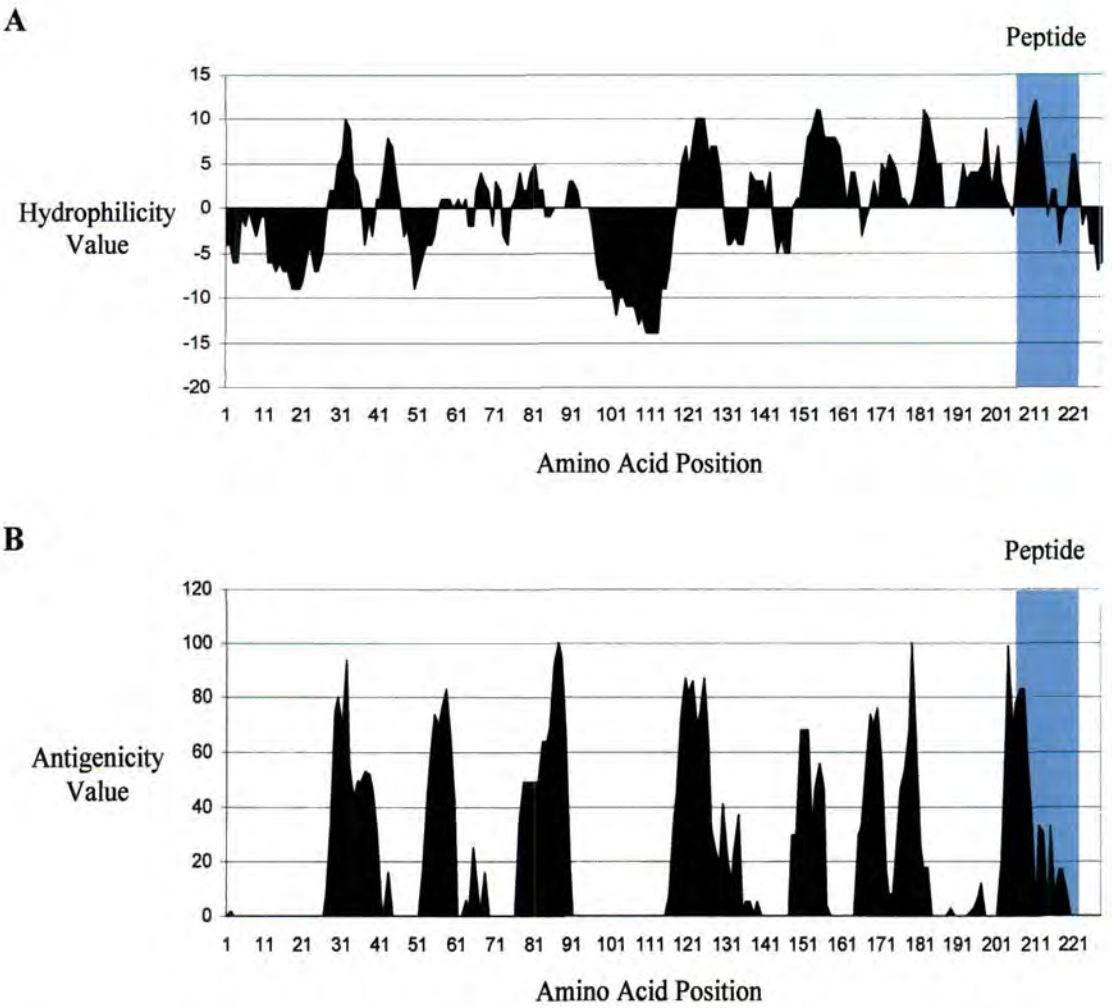


Figure 3.4. Hydrophilicity Plots of mouse Ric3 according to the Hopp and Woods Predictive method (Hopp and Woods, 1981; Hopp and Woods, 1983.) (A), and the antigenicity plot according to the predictive method described by Parker *et al.* (1986) (B).

3.3.2. Peptide Affinity Purification and Concentration Determination

The polyclonal antibody raised against the mRic3 protein is intermingled in the serum with animal's natural antibodies as well as antibodies against the thyroglobulin carrier protein. To remove the impurities that could possibly cause non-specific binding, the pure peptide was coupled to activated sepharose beads using a similar coupling strategy as that between the peptide and the carrier protein. The serum was then incubated with the peptide affinity column which bound the anti-mRic3 antibodies. Subsequent to washing, the purified anti-mRic3 antibody was eluted in 1 ml fractions and the absorbance of each fraction was measured spectrophotometrically at 280 nm. The fractions from each bleed with an optical density (O.D.) of approximately 0.1 or higher were grouped (Fig. 3.5). The first six fractions from the first bleed fulfilled this requirement and were grouped to yield a final absorbance of 0.103 which, according to the Beer Lambert law, equates to 0.0769 mg/ ml of anti-mRic3 antibody. The antibody concentrations from the second bleed were too low to be used but the concentrations from the third bleed yielded two groups with high enough concentrations: fractions 2 – 3 (O.D. = 0.146, thus 0.108 mg/ ml) and fractions 4 – 10 = 0.177, thus 0.132 mg/ ml. The first three fractions from the final terminal bleed were grouped to yield a final O.D. of 0.134, which equates to 0.1 mg/ ml anti-mRic3 antibody.

3.3.3. Immunoblotting Analysis of the Anti-mRic3 Antibody

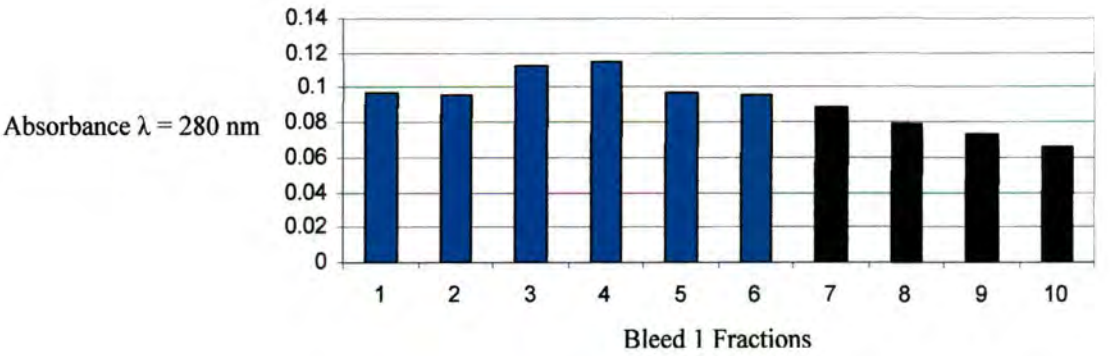
HEK 293 cells were infected with adenovirus expressing either enhanced green fluorescent protein, the α7 nAChR subunit, the 25 kDa isoform of Ric3, or both the α7 nAChR subunit and the 25 kDa Ric3 protein (See Chapter 4). Samples were analysed on a Western blot with 2 μg/ ml of the new anti-mRic3 antibody. The antibody only identified a single band at approximately 25 kDa in cells infected with an adenovirus expressing Ric3. In the absence of α7 nAChR subunits, an additional band was observed at approximately 37 kDa.

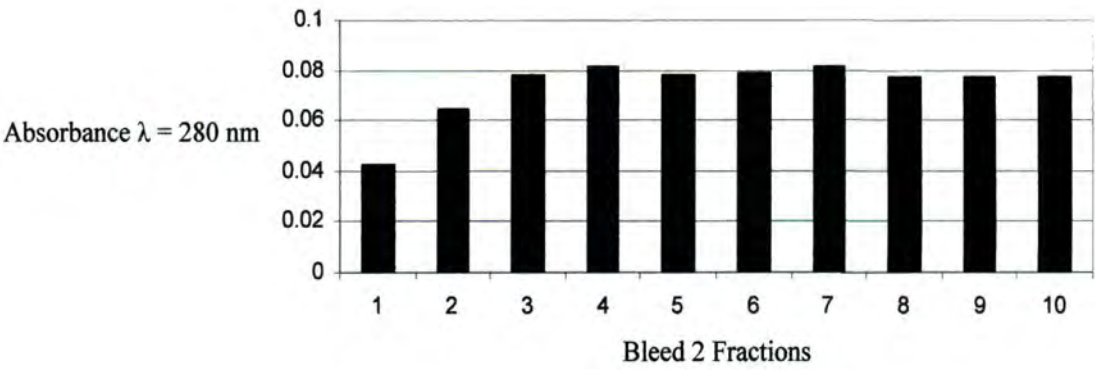
Various quantities of mouse brain (50 mg/ ml), kidney (75 mg/ ml) and lung (45 mg/ ml) samples were analyzed with 2 μg/ ml of the new anti-mRic3 antibody (Fig. 3.7). A range of immunoreactive bands were identified in all three tissue types with a major Mr ~ 56,000 species being identified in the mouse brain samples, which is absent or modestly expressed in lung and kidney samples, respectively. This species corresponds with the human size Ric3 protein (theoretical size 41 kDa) as observed by Halevi *et al.* (2003) and not with the mouse Ric3 protein, which has a theoretical molecular weight of approximately 25 kDa – at this size only a very faint band can be identified (indicated with an arrow in Figure 3.7). The 25 kDa species also seem to appear in the kidney tissue at a higher concentration than in the brain but is absent in mouse lung tissue. A range of bands are closely clustered around the Mr ~ 56 kDa species, indications of possible isoforms or various post-translational states. Both the mouse lung and kidney samples share a prominent species of Mr ~ 80 kDa – low levels of which is present in the brain samples. In addition to this, a species of lower prominence can be detected in the kidney samples at Mr ~ 40 kDa and a distinct species in the lung samples at Mr ~ 35 kDa. A

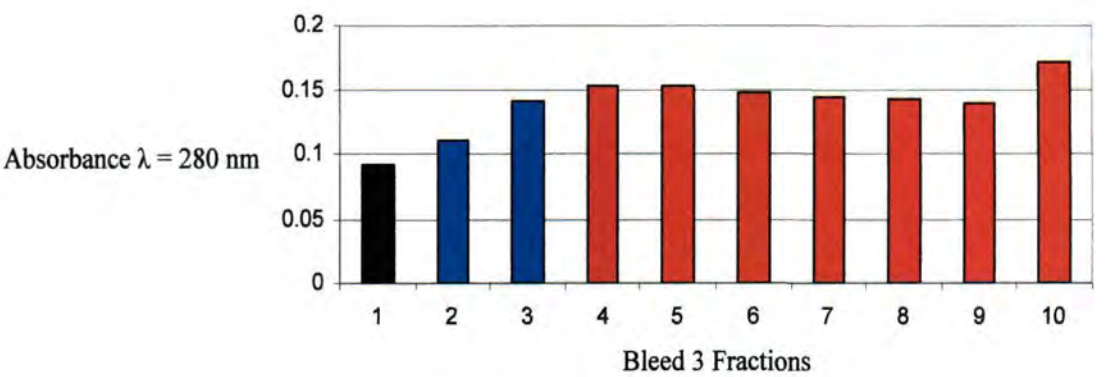
band of Mr ~ 180 kDa is found in both the brain and kidney samples at approximate equal intensity – the only band between the brain and any of the other two samples corresponding in both size and concentration.

The effect of prolonged exposure to potassium chloride on the levels of the Ric3 protein expression was investigated by exposing rat pure neuronal cortical cultures to 11.25 mM KCl for 24 hours (Fig. 3.8). Samples were either harvested acutely post-exposure or chronically, following a 24 hour treatment-free recovery period. Two main species of protein were detected by the anti-mRic3 antibody at Mr ~ 50 kDa and Mr ~ 25 kDa. The Mr ~ 25 kDa corresponds with the expected size of the mouse protein upon which the antibody was based whereas the Mr ~ 50 kDa species seems, once again, to correspond to the human size Ric3 protein as observed by Halevi *et al.* (2003). Normalization with β-actin revealed that the expression of the Ric3 protein remains unaltered subsequent to prolonged treatment with KCl, irrespective of when the samples were harvested. Another possible isoform of the Ric3 protein is visible marginally above the position of the Mr ~ 50 kDa species.

Murine brain samples harvested at 24 hours, 72 hours, or 168 hours subsequent to *in vivo* cortical spreading depression or sham treatment, were analysed with the new anti-mRic3 antibody (Fig. 3.9). CSD induced an approximate doubling in the amount of Ric3 species expressed at approximately 50 kDa for the 24 hour and 72 hour harvested samples. No increase in this species was detected in samples harvested 168 hours after the CSD event.







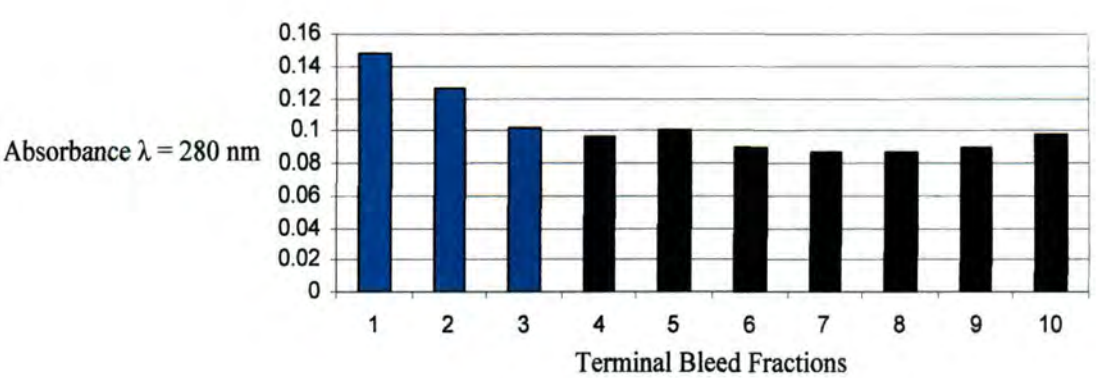


Figure 3.5. The optical density (O.D.) readings of the eluted antibody fractions for each bleed were taken at λ = 280 nm. Fractions that had readings of approximately 0.1 were grouped and are represented by blue bars, or blue and red bars for the two groups in Bleed 3. The black bars in the graphs represent fractions that were too low to be used.

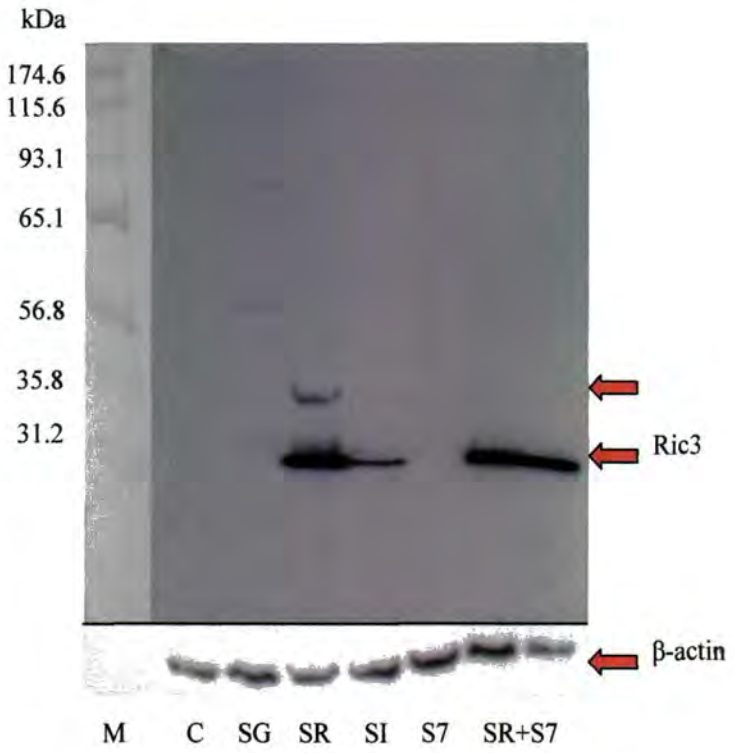


Figure 3.6. A copy of Figure 4.4A illustrating the effectiveness and specificity of the Ric3-antibody in identifying Ric3 transgene expression in recombinant adenoviral infected HEK 293 cells. 50 µl of the 1 ml harvested cell suspension was analysed on a 6 % SDS-PAGE gel. M represents the molecular weight marker used (Precision Plus Protein™ Standards, Bio-Rad), C the control uninfected HEK 293 cells, SG the SG-AdV infected cells expressing green fluorescent protein (GFP), SR the SR-AdV infected cells expressing Ric3, SI the bicystronic adenovirus SI-AdV infected cells expressing α7 nAChR subunit and Ric3, S7 the S7-AdV infected HEK cells expressing α7 nAChRs, and SR+S7 the HEK cells infected with both SR-AdV and S7-AdV simultaneously. The Ric3-antibody was used at a concentration of 2µg/ ml in combination with an anti-rabbit horseradish peroxidase linked secondary antibody (1 in 2000 dilution; ECL™). To normalize the protein expression, monoclonal anti-β-actin antibody (Sigma Aldrich) at a 1 in 2000 dilution was used with an anti-mouse horseradish peroxidase linked secondary antibody (ECL™) also at a 1 in 2000 dilution (inserted at the bottom).

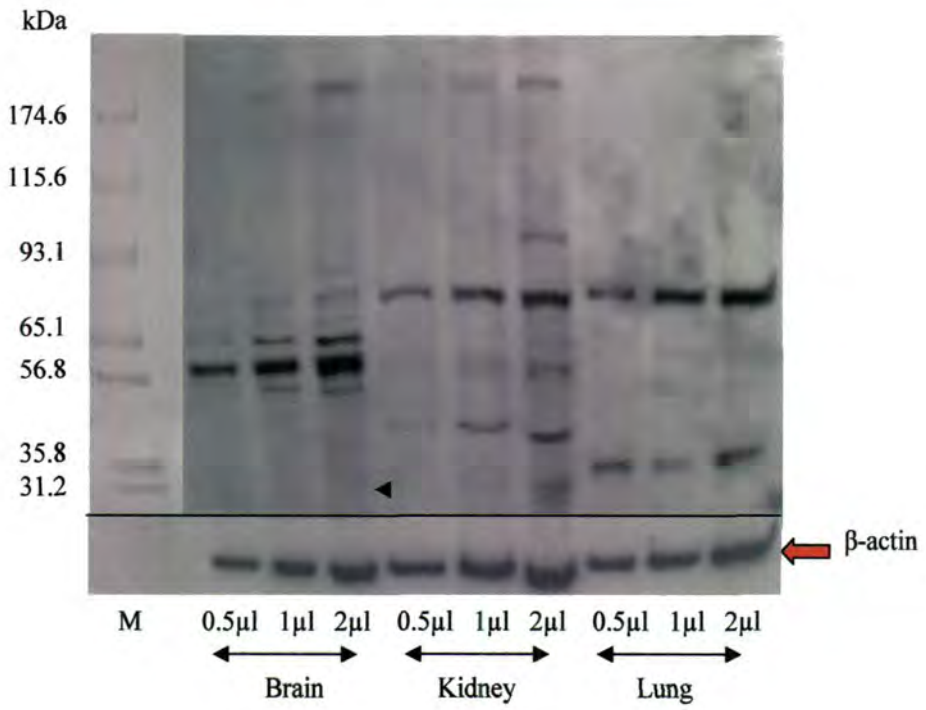


Figure 3.7. Various quantities of mouse brain (50 mg/ ml), kidney (75 mg/ ml) and lung (45 mg/ ml) samples were analyzed on a 6 % SDS polyacrylamide gel and immunostained with the new anti-mRic-3 antibody (2 µg/ ml) in conjugation with the anti-rabbit horseradish peroxidase linked secondary antibody (1 in 2000 dilution; ECL™). This was followed by immunostaining with a monoclonal anti-β-actin antibody (Sigma Aldrich) at a 1 in 2000 dilution in combination with an anti-mouse horseradish peroxidase linked secondary antibody (ECL™) to normalize the amounts of protein loaded unto the gel. M represents the molecular weight marker used (Precision Plus Protein™ Standards, Bio-Rad). The arrowhead indicates a faint 25 kDa band.

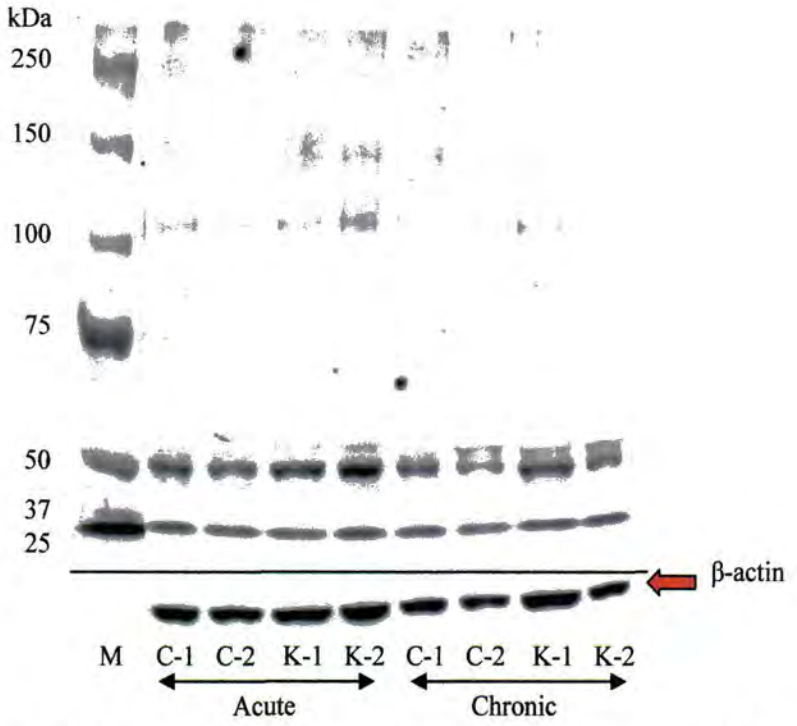


Figure 3.8. Rat pure neuronal cortical cultures were treated for 24 hours on DIV 14 with plain medium (C) or with 11.25 mM KCl (K) dissolved in medium. The acute group was harvested directly after the 24 hours treatment whereas the chronic group was washed thrice with EBS and incubated for another 24 hours in plain medium before being harvested. Samples were analysed on a 6 % SDS polyacrylamide gel and immunostained with the new anti-mRic-3 antibody (2 µg/ ml) in conjugation with the anti-rabbit secondary antibody (1 in 2000 dilution). This was followed by immunostaining with a monoclonal anti-β-actin antibody (1 in 2000 dilution) in combination with an anti-mouse secondary antibody to normalize the amounts of protein loaded unto the gel. M represents the molecular weight marker used (Precision Plus Protein^{TM} Standards, Bio-Rad).

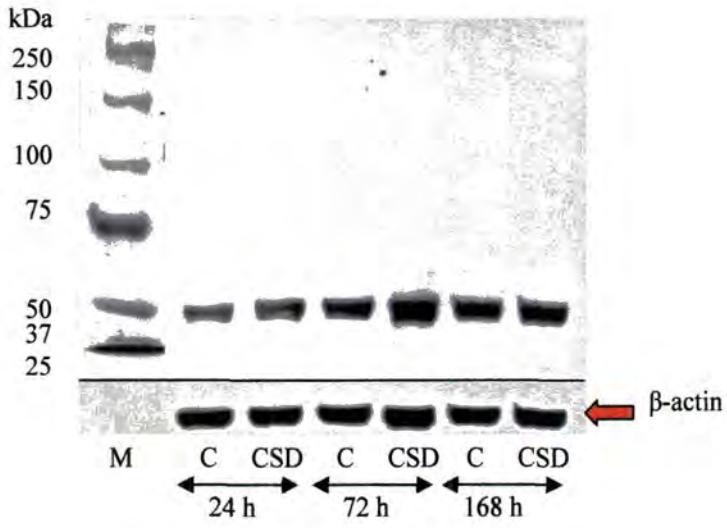


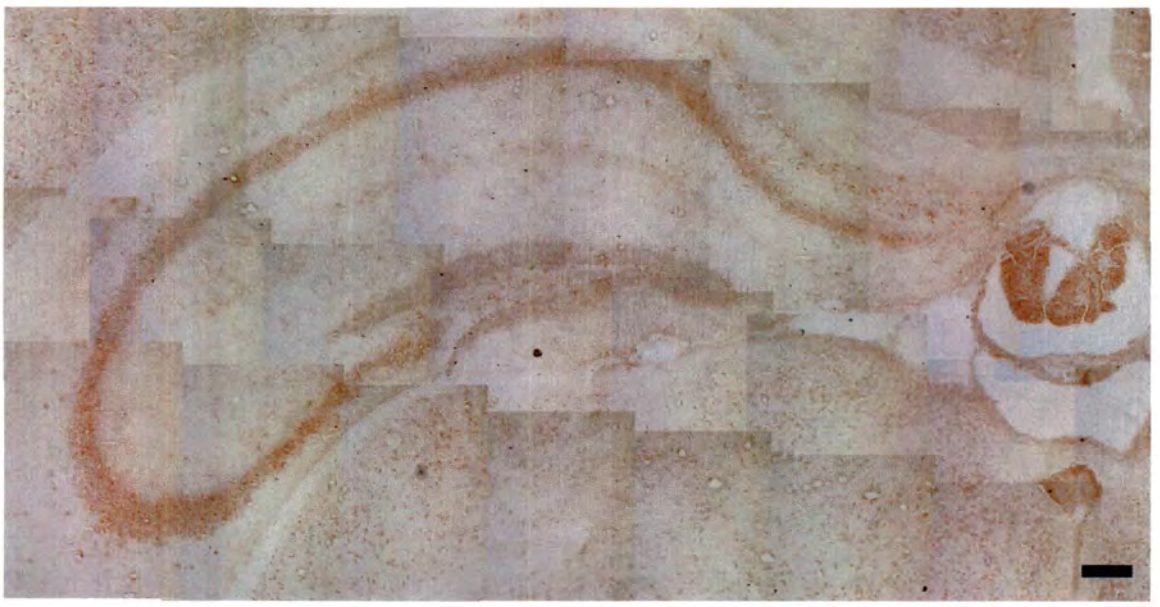
Figure 3.9. Mouse brains samples were harvested at 24 h, 72 h, or 168 h subsequent to *in vivo* cortical spreading depression (CSD) or sham treatment (C). 15 μg of murine whole brain samples were analysed on a 7.5 % SDS polyacrylamide gel and immunostained with the new anti-mRic-3 antibody (2 μg/ ml) in conjugation with the anti-rabbit secondary antibody (1 in 2000 dilution). This was followed by immunostaining with a monoclonal anti-β-actin antibody (1 in 2000 dilution) in combination with an anti-mouse secondary antibody to normalize the amounts of protein loaded unto the gel. M represents the molecular weight marker used (Precision Plus Protein™ Standards, Bio-Rad).

3.3.4. Immunohistochemistry

To identify the distribution of the Ric3 protein within the mouse brain, brain sections were subjected to immunohistochemical analyses using the new anti-mRic3 antibody. In addition, to confirm the results from the *in vitro* preconditioning experiment, the brains from mice subjected to 10 consecutive cortical spreading depressions were harvested at 24 hours, 72 hours, or 168 hours post-preconditioning. These samples were analysed with their respective sham-operated controls.

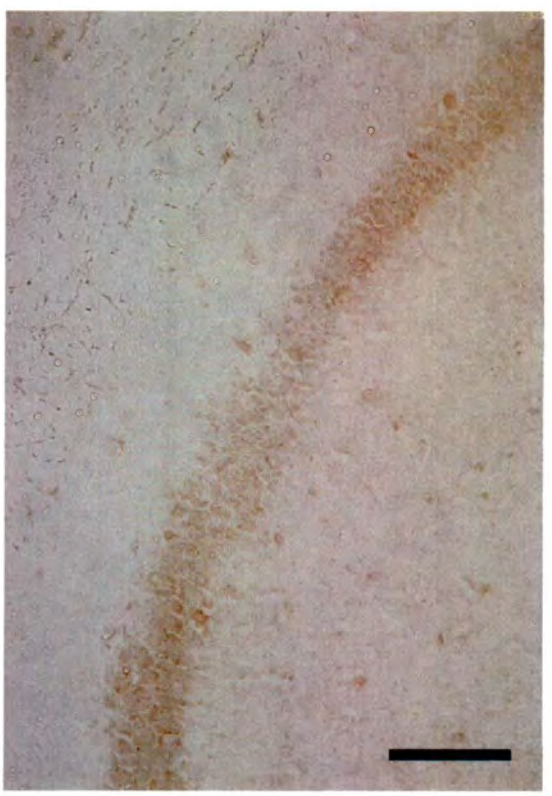
Figure 3.10 represent the images taken from a sham treated animal that was harvested 24 hours post treatment. Low levels of Ric3 protein expression was detected in most regions of the brain. A high expression signal was detected in the CA1 (Fig. 3.10B) and CA3 (Fig. 3.10D) regions of the hippocampus, whereas lower levels were detected in the CA2 (Fig. 3.10C) and CA4 (Fig. 3.10E) regions. High expression levels were also detected in the dentate gyrus (Fig. 3.10F), and the thalamic paraventricular nucleus (PVN) (Fig. 3.10H), Lower levels of Ric3 protein expression was detected in the cortex (Fig. 3.10J) but with an increase in the perirhinal cortex (Fig. 3.10K) stretching down to the piriform cortex region. The hypothalamic PVN (Fig. 3.10I) and surrounding areas also had higher levels of Ric3 protein expression. Of note is the high expression of Ric3 that was detected in the dorsal third ventricle ((Fig. 3.10G) and the other ventricles containing cells of the choroid plexus (results not shown).

No obvious changes in gene expression were detected in the potassium chloride treated animals, no matter at which time point the samples were harvested (results not shown).



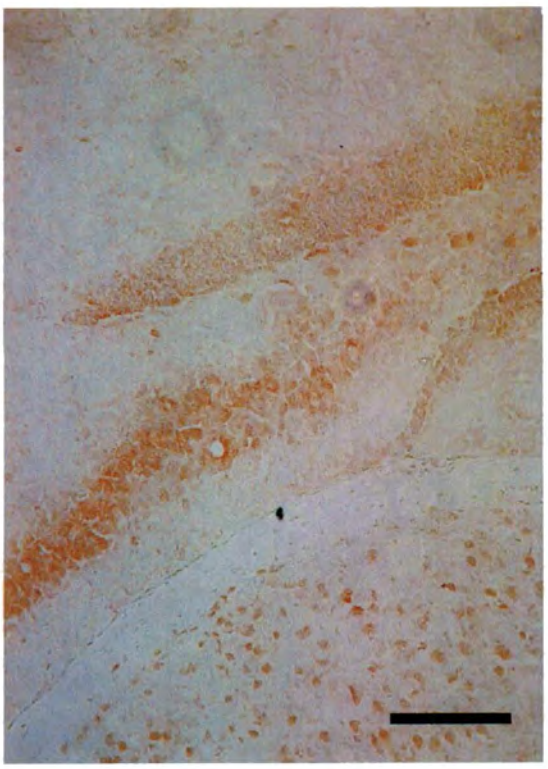
A) Composite figure spanning the hippocampal, dorsal third ventrical and thalamic paraventricular nucleus regions. Scale bar = 1 mm.

B) CA1

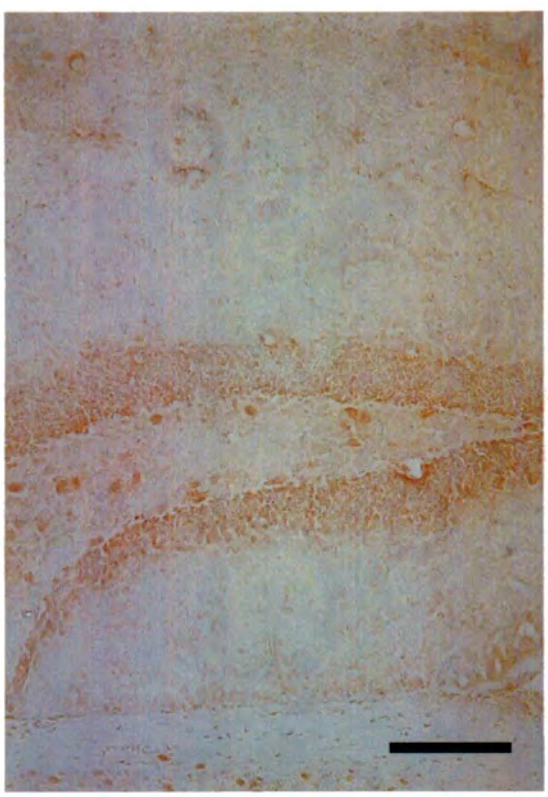


C) CA2

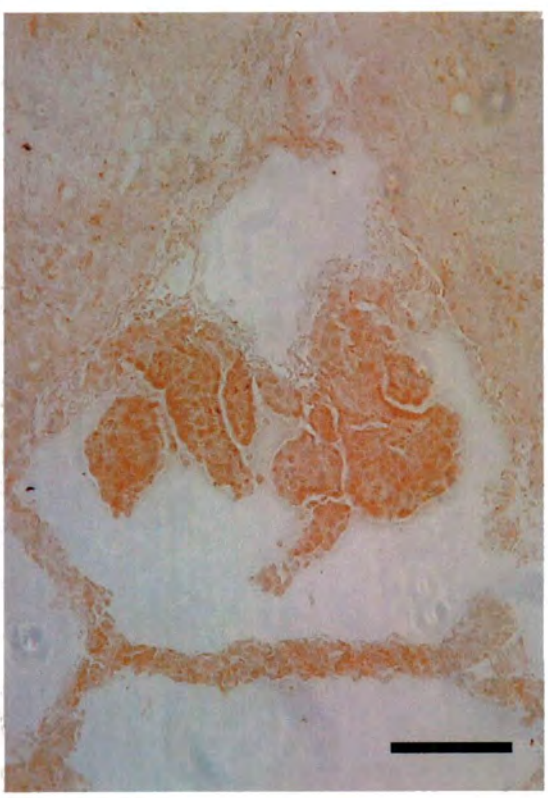
D) CA3



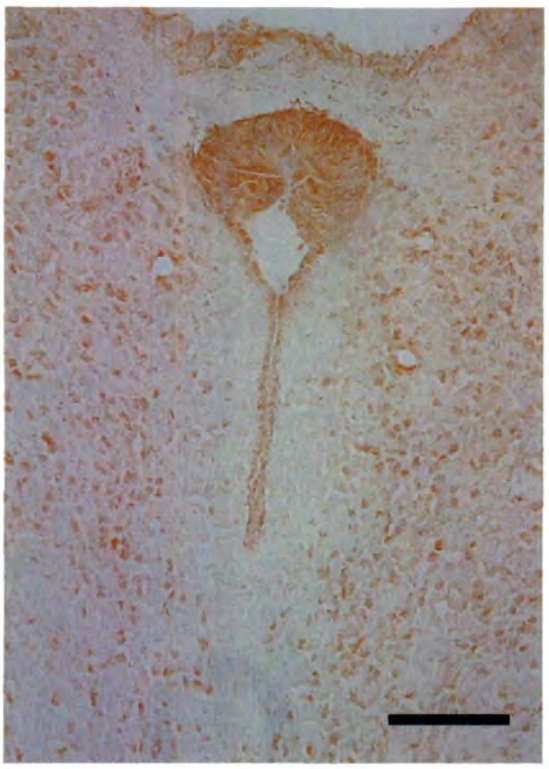
E) CA4



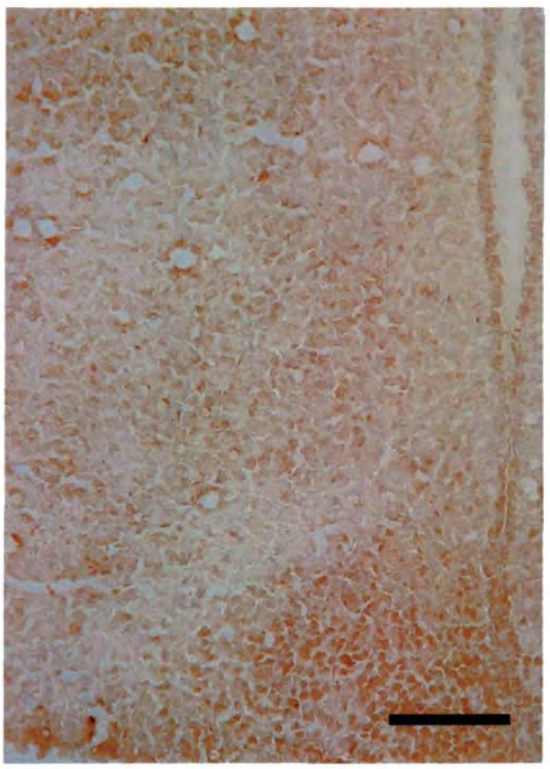
F) Dentate Gyrus



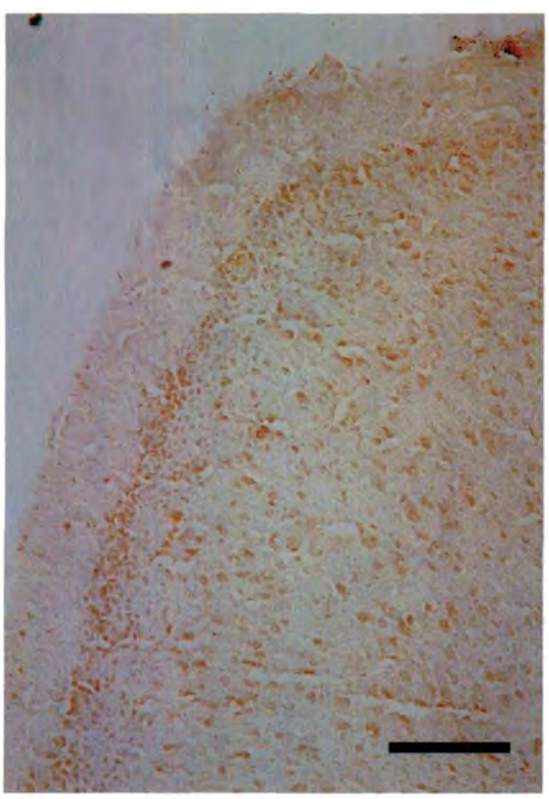
G) Dorsal Third Ventricle



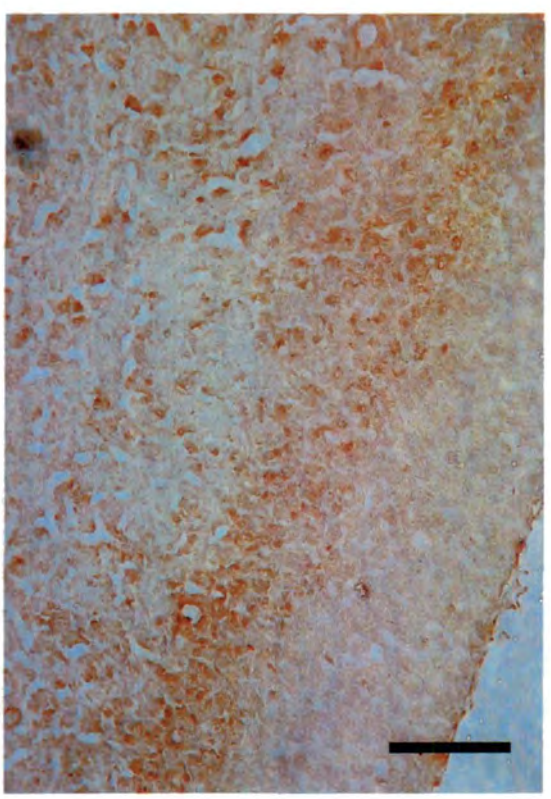
H) Thalamic Paraventricular Nucleus



I) Hypothalamic Paraventricular Nucleus



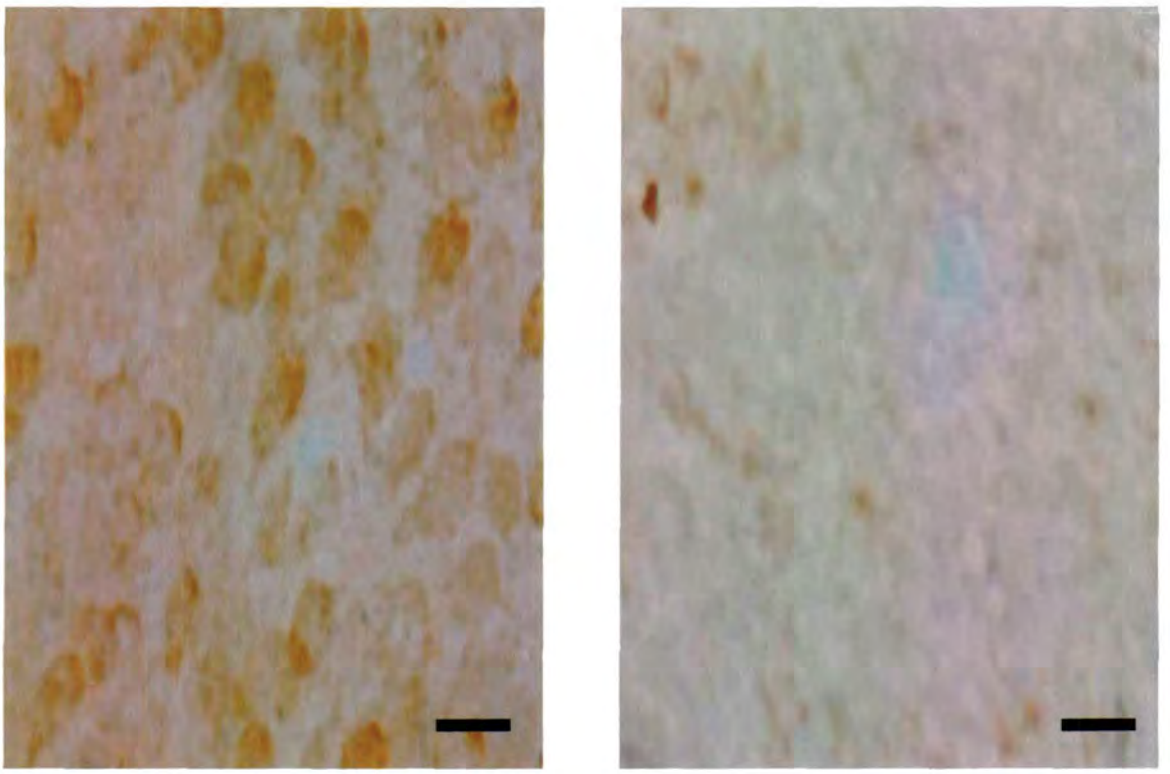
J) Cortex



K) Perirhinal Cortex

Figure 3.10. Immunohistochemistry of a 25 μm coronal mouse brain sections, which was harvest 24 hours after sham treatment. 2 μg/ ml of the anti-mRic2 antibody was used in

conjugation with a 1 in 100 dilution of the biotinylated secondary antibody from the Vectastain ABC kit. Scale bar = 1 mm.



A) Lamina II Cortex

B) Lamina II Cortex with peptide block

Figure 3.11. Immunohistochemistry of a 25 μm coronal mouse brain sections, which was harvest 24 hours after sham treatment. 2 μg/ ml of the anti-mRic2 antibody was used in conjugation with a 1 in 100 dilution of the biotinylated secondary antibody from the Vectastain ABC kit (A) and also in combination 500 μg/ ml peptide. Scale bar = 10 μm.

Brain Area	Immunoreactivity Intensity
Hippocampus	
CA1	+++
CA2	++
CA3	+++
CA4	++
Dentate gyrus	++
Cerebral cortex	
Lamina I	-
Lamina II	++
Lamina III	(+)
Lamina IV	(+)
Lamina V	(+)
Lamina VI	(+)
Perirhinal cortex	++
Piriform cortex	++
Thalamic paraventricular nucleus	++
Hypothalamic paraventricular nucleus	++
Ventricles	
Dorsal third ventricle	+++
Lateral ventricle	+++

Table 3.1. Distribution of the Ric3 protein in adult mouse brain where no staining is indicated by -; (+) indicates light diffuse or scattered staining; and +, ++, and +++ indicates increasing stained profiles.

3.3. Discussion

The aim of this chapter was to develop a novel antibody for the identification of the mouse Ric3 protein. Ric3 has been shown to be involved in the regulation of functional expression of α7 nAChR and belongs to an evolutionary conserved gene family (Halevi *et al.*, 2003). At least four isoforms of this protein has been detected for the human homolog. An NCBI homology search resulted in the detection of three complete mouse cDNA sequences, all much shorter than the human version but exhibiting a high sequence identity when compared with this homolog.

Structural analyses of the human and the mouse homolog with the highest identity revealed two very similar proteins. Both proteins are predicted to contain two transmembrane regions although it is highly probable that the first crossing event at aa position 11 – 22 is a signal peptide and not a transmembrane region. The structural and transmembrane predictive methods portray a protein which has its first hundred amino acids outside the cell with the rest of the protein present within the cytoplasm. This agrees with other known receptor regulatory proteins e.g. the prototypical stargazin protein γ-2, a transmembrane AMPA receptor regulatory protein (TARP) (Chen *et al.*, 2000). This four-pass transmembrane protein is essential for the surface trafficking and synaptic targeting of AMPA receptors by binding directly to AMPA receptors – the first extracellular loop of stargazin associates with the glutamate-binding module in AMPA receptors (Tomita *et al.*, 2007). Similarly, engagement of the α7 nAChR by the Ric3 protein is necessary for the functional expression of the receptor *in vitro*. Of great importance to this study is the recent paper by Ben-Ami *et al.* (2005) where it was shown that the effects of Ric3 on the functional expression and on receptor properties, like

TARPs, are mediated by the two transmembrane domains – the variable length random coil domain or coiled-coil domain of the C-terminal is not required. A truncated version of the human Ric3 protein consisting only of the two transmembrane regions was able to upregulate the functional expression of the *Caenorhabditis elegans* DEG-3/DES-2 nAChR in oocytes. Coincidentally, it was also observed in this study that Ric3 may preferentially promote maturation of DEG-3-rich receptors, leading to alterations to the kinetics and agonist affinity of the DEG-3/DES-2 nAChR in *C. elegans* (Ben-Ami *et al.*, 2005).

Like stargazin where phosphorylation regulate AMPA receptors in synaptic plasticity (Tomita *et al.*, 2005), analyses of the Ric3 homologs revealed a single phosphorylation site in the mouse homolog and four phosphorylation sites in the human homolog. The cytoplasmic region of the mouse homolog is much shorter than that of its human counterpart resulting in two of the human Ric3 phosphorylation sites at aa position 273 and 334 to fall outside the range of the mouse Ric3 protein. In addition to these two sites, the human Ric3 protein contains a PKC phosphorylation site at aa 178 and a protein kinase CK2 site at aa positions 180. The mouse Ric3 protein contains a single potential protein kinase CK2 phosphorylation site at aa position 179.

Subsequent to the structural, as well as hydrophilicity and antigenicity analyses of the chosen mouse Ric3 protein isoform's sequence, a peptide was chosen for the generation of a polyclonal anti-mRic3 antibody. Affinity column purification of the serum from the first, third and terminal bleeds resulted in modest concentrations of antibody being recovered. The reason for the recovery of low concentration of anti-mRic3 antibody from the second bleed is unknown. Subsequent inoculations resulted in the higher generation

of purified anti-Ric3 antibody, as can be seen from the concentrations of the third and the terminal bleed (Chazot *et al.*, 1992).

The next stage of this study was to demonstrate the specificity and the sensitivity of the newly generated anti-mRic3 antibody. HEK 293 cells were infected with recombinant adenoviruses expressing transgenes (Fig. 3.6, Also see Chapter 4). Ric3 protein was detected in HEK 293 cells infected with SR-AdV and SI-AdV at 25 kDa. An additional band was detected in cells infected with SR-AdV in the absence of any α7 nAChR protein expression. This protein species of approximately 37 kDa is too small to indicate a Ric3 dimer but might indicate the interaction of Ric3 with another endogenous protein.

Various concentrations of mouse brain, kidney and lung tissues were analyzed under reducing/ denaturing conditions. All three tissue types yielded a range of anti-mRic3 immunoreactive species. A single prominent band was detected in the mouse brain sample at Mr ~ 56 kDa, corresponding to the band detected in Halevi *et al.* (2003) study, although the predicted size of the human Ric3 protein is only 41 kDa. The shorter mouse homolog has a predicted size of 25 kDa. It is possible that the prominent band in the mouse brain sample is a dimer, as it is known that under the reducing/ denaturing conditions employed it is possible for structures to retain dimeric formations e.g. transthyretin and many G-protein-coupled receptors (Blake *et al.*, 1978; Kent *et al.*, 2007). This is, however, improbable considering that the human isoform of Ric3 is known for it various isoforms; the very low levels of the monomer at 25 kDa; and taking into account the range of bands surrounding the ~ 56 kDa species which may indicate possible posttranslational modifications of the major species. Comparing the *hric3* gene with the mouse genome identified a region on chromosome 7 that has 85 % identity with the

region spanning 669 bp to 1110 bp of the C-terminal of the *hric3* gene. This would indicate that although only the cDNA for the shorter isoform of Ric3 has been cloned, it is possible for the longer 56 kDa isoform to be transcribed from the mouse genome. Also, it is possible for receptor regulatory proteins to have isoforms that differ greatly in size as is observed with TARPs where at least eight isoforms of various sizes have been identified. It has been suggested that a role for these auxiliary subunits may lie in dictating the unique properties of receptors in specific brain regions (Kato *et al.*, 2007). Taking into account the presence of anti-mRic3 immunoreactivity in non-α7 nAChR expressing tissues, it is highly probable that the possible Ric3 isoforms regulate the expression of other proteins e.g. 5-HT_3 (Cheng *et al.*, 2005; Castillo *et al.*, 2005; Castillo *et al.*, 2006). Various barely detectable bands are also visible throughout the blot but despite these additional and seemingly non-specific bands, the newly created anti-mRic3 antibody is a far more informative probe when compared to the Northern blot performed by Halevi *et al.* (2003).

An investigation was launched into the effect of cortical spreading depression on the expression of the Ric3 protein. Brain samples, subjected to either cortical spreading depression or sham treatment, were harvested at various time points post treatment: 24 h, 72 h and 168 h. A prominent protein species was detected at approximately 50 kDa, taken to be a larger isoform of Ric3. Subjecting the mouse to CSD resulted in an approximate doubling of the Ric3 protein expression up to 72 hours subsequent to the CSD event. Expression levels returned to normal at 168 hours after CSD. This upregulation suggests that Ric3 might be required, either to directly facilitate cytoprotection or with the formation of proteins that do have a direct function on the protective mechanism. A Western blot analyses of day *in vitro* 14 rat pure neuronal

primary cortical cultures detected two prominent bands at Mr ~ 50 kDa and at Mr ~ 25 kDa. The ~ 50 kDa species correlates with prominent band observed in the treated mouse brain samples of ~ 50 kDa. The second species of Mr ~ 25 kDa compares favourably with the mouse isoform upon which the antibody is based. The prominence of this smaller band could be due to a number of reasons: i) this isoform is more highly expressed in rat than in mouse brain; ii) the artificial tissue culture environment induced the upregulation of this protein; or iii) the isoform has a higher expression level in primary neurons than in the adult brain. Treatment of the pure neuronal cells for 24 hours with potassium chloride had no apparent effect on the expression level of the either one of the Ric3 isoforms. This contradicts the *in vivo* results where an upregulation in protein expression was detected. The apparent lack of Ric3 to be upregulated in the culture system might, once again, be due to a number of reasons: i) there is an upregulation but it was too small to detect; ii) Ric3 is not only upregulated in the neuronal brain tissue but also other cells such as glia or other cell types that are not represented in the *in vitro* model; or iii) the preconditioning model does not elicit the same molecular changes in rat primary neurons as CSD does in the adult mouse brain.

In order to identify the distribution of Ric3 within the mouse brain and to confirm the *in vitro* preconditioning results, an immunohistochemical analysis was conducted on *in vivo* sham and preconditioned brains harvested at various times post treatment. Halevi *et al.* (2003) conducted an *in situ* analyses of the distribution of *ric-3* mRNA production in the mouse brain using a 0.6 kb mouse *ric3* probe, which indicated that low levels of transcription is present throughout the brain although stronger signals were detected in the CA1 – CA3 region in the hippocampus, the deep nuclei and the Purkinje cell layer in the cerebellum, and the superior colliculus. The Halevi *et al.* (2003) study correlates well

with the normal distribution of high α7 nAChR expression, the exception being the absence of *ric3* mRNA production in the dentate gyrus – a region normally associated with high α7 nAChR expression (Adams *et al.*, 2001; Gahring *et al.*, 2005). As with the *in situ* analysis, a high level of Ric3 protein expression was detected in the CA1 and CA3 regions of the hippocampus although a clearly lower amount was detected in the CA2 region. In this study it was observed that Ric-3 protein, in contrast to the mRNA, is strongly expressed in the dentate gyrus (granule and molecular layers, with clear evidence of interneuron labelling) in agreement with high α7 nAChR expression. This discrepancy between the two probing methods can be the result of either a flaw in one of the detection systems or the existence of a low but more stable form *ric3* mRNA being transcribed in the dentate gyrus leading to elevated protein expression, although this latter explanation seems less probable.

Aside from the hippocampal area, the anti-mRic3 antibody also identified high Ric3 protein expression in the thalamic paraventricular nucleus (PVN) and the hypothalamic PVN. In addition to their respective functions as a viscerosensory input to the amygdala and the maintainer of homeostasis, both these regions are involved with the adaptation to internal or external stressors (Bhatnagar and Dallman, 1999; Spencer *et al.*, 2004; Benarroch, 2005). The putative role of α7 nAChR in anxiety has been discussed in the introduction. It would be of interest to investigate further the role, if any, that Ric3 might play in anxiety. High levels of Ric3 expression was also detected in the perirhinal and the piriform cortex, regions where α7 nAChRs are known to play a role in visual memory (Hampton, 2005; Hasselmo, 2006). Of interest is the high expression of the Ric3 protein in choroid plexus lining of the dorsal third and lateral ventricles. It is known that although Ric3 is not required for the functional formation 5-HT_3A receptors, it does increase the

surface expression of this receptor subtype (Cheng *et al.*, 2005; Castillo *et al.*, 2005; Castillo *et al.*, 2006). Despite α7 nAChR expression not occurring in the choroid plexus, 5-HT_3 expression has been detected in this tissue (Johnson and Heinemann, 1995) – providing a possible function for Ric3 within this tissue type. The presence of Ric3 in the choroid plexus might also account for the difference in Ric3 changes observed between the *in vivo* and the *in vitro* results subsequent to exposure to KCl, since a tissue-specific change in the choroid plexus would not be detected in the pure neuronal cortical culture system. It has been noted that CSD is capable of changing the expression of choroid plexus proteins, as is observed with a more than doubling of transthyretin mRNA production, a protein solely expressed in the choroid plexus of the brain (Personal communication Obrenovitch and Chazot).

The second part of the immunohistochemical study attempted to analyse any changes in Ric3 distribution associated with the induction of cortical spreading depression *in vivo*, as the expression level of CSD treated brains showed an approximate doubling in expression level. Brain tissues harvested at 24, 72 and 168 hours post preconditioning showed no apparent difference in the distribution of Ric3 or any obvious changes in expression level, although it should be noted that immunohistochemistry is not a quantitative technique.

In conclusion, we have developed a new anti-mRic-3 polyclonal antibody, which we have validated and used to define the anatomy of ric-3 expression in the mammalian brain, as well as studying the effect that depolarization has on the level of Ric3 protein synthesis. The next chapter will describe the development of a series of novel recombinant adenoviral constructs to be used to manipulate the expression of α7 nAChR and Ric-3

both *in vitro* and *in vivo* to investigate the roles of these proteins in preconditioning and brain function.

CHAPTER 4: Generation of Recombinant Adenoviruses for the Artificial Upregulation of Alpha 7 Nicotinic Acetylcholine Receptors

4.1. Introduction

A microarray analysis of mixed cortical neurons and glial cells subjected to potassium chloride-mediated membrane depolarisation have indicated the differential expression of more than 500 activity-dependent regulated genes (Li *et al.*, 2004). Microarray analyses of both nicotine and ischemic treatments have yielded similar results (Zhang *et al.*, 2001; Trendelenburg *et al.*, 2001; Stenzel-Poore *et al.*, 2003). Studying the effect that the up – or down regulation of a specific protein has on the physiology and survival of a cell is complicated when using pharmacological means since any effect might be immersed or attenuated by the altered regulation of other genes.

Adenovirus vectors are the viral vectors of choice for gene therapy applications (Nadeau and Kamen, 2003). They are naturally benign viruses able to infect a large variety of dividing and non-dividing cells, including neurons (Hermens and Verhaagen, 1998). Adenoviruses belong to the parvovirideae family and the virion is a small, non-enveloped particle with a spiked icosahedral morphology (Trapnell, 1993). The wildtype genome consists of a linear double stranded DNA molecule of approximately 36 kb in length, which is highly stable and undergoes rearrangement at a very low rate. As the virion is restricted in the volume of DNA it can accommodate, the amount of transgenes that can be added to the wildtype genome of the adenovirus is approximately 5 % i.e. ~ 2 kb (Bett *et al.*, 1993). The first generation of adenoviruses developed for gene delivery have a deletion in their E1 (early gene 1) region, restricting their replication to cells naturally expressing this gene, e.g. HEK 293 cells. Adenoviruses retain the ability to replicate at

high titers in these E1-complementing cell lines. The E1 deletion has the added benefit of increasing the transgene accommodation size to approximately 5 kb (Graham and Prevec, 1995).

Adenoviruses have been used in various neuronal transgene studies *in vivo* and *in vitro* (Hermens and Verhaagen, 1998), including similar neuroprotective studies as was conducted in chapter 2. Tan *et al.* (2003) created an adenovirus containing the gene coding for tissue inhibitors of metalloproteinases under control of a cytomegalovirus (CMV) promoter. Primary hippocampal cultures infected 5 to 7 days after plating at a multiplicity of infection (MOI) of 100 were significantly protected against a 1 hour 300 μM glutamate insult, 48 hours subsequent to the infection. This experiment was independently duplicated by Pi *et al.* (2004) using DIV 12 primary cortical neuronal cultures infected at a MOI of 0.7 for three days before being submitted to a 24 hour 200 μM glutamate insult. Similar neuroprotective studies were also conducted by various other groups, each using the adenoviral vector system as an effective means to upregulate their gene of interest (Tsai *et al.*, 2007; Hester *et al.*, 2007).

The aim of this chapter is to create an E1-deleted recombinant adenovirus vector expressing the α7 nAChR gene in neuronal cells. Any study aiming to look at the effect of increasing the functional expression of a protein would have to include the increase of any necessary function-dependent regulatory proteins. The formation of functional α7 nAChRs requires the chaperone protein Ric3 (Halevi *et al.*, 2002; Chapter 3). This dependency of functional α7 nAChRs on the presence of Ric3 necessitates the creation of an adenovirus for Ric3 protein production. Despite Ric3 being present in neuronal cells, the quantity of Ric3 present in these cells might prove to be a limiting factor for the

upregulation functional α7 nAChRs. For this reason and to further explore the function and nature of Ric3, a recombinant adenovirus expressing this protein will also be created.

4.2. Materials and Methods

4.2.1. Polymerase Chain Reaction

Polymerase chain reaction (PCR) was used to amplify the regions to be inserted into the genome of the adenoviral vector. All of these high fidelity reactions PCR reactions were carried out using TaKaRa Ex Taq™ polymerase (Life Technologies) and consisted of 10 ng template DNA, 100 pmol of each primer (table 4.1), 3 µl 2.5 mM Deoxynucleotide Solution Mix (New England Biolabs), 5 µl 10 x TaKaRa Ex Taq™ polymerase buffer (250 mM TAPS pH 9.3, 500 mM KCl, 20 mM $MgCl_2$, 10 mM 2-mercaptoethanol), 5 units (U) TaKaRa Ex Taq™ polymerase enzyme and made up to a final volume of 50 µl with ddH_2O. A MultiGene II thermal cycler (Labnet) was used for the PCR reactions.

The amplification conditions for both the reaction were as follows: 1 cycle at 94 °C for 2 minutes; 25 cycles 93 °C for 45 seconds, annealing temperature (provided in table 4.1 for each reaction) for 1 minute, 72 °C for 2 minutes; 1 final elongation cycle at 72 °C for 10 minutes.

Primer Name	Oligonucleotide Sequence and Restriction Enzymes	Tm °C
The mouse α7 nAChR gene was amplified from plasmid pCDNA5 mα7 for construction of pS7-AdV and pS7IR-AdV. Annealing temperature was set at 61 °C.		
Alpha7-5	5' TAGGTACC**ATG**TGCGGCCGGCGGGG 3' *Kpn*I **Start Codon**	62
Alpha7-3	5' CTTCTCGAG**TTA**AGCAAAGTCTTTGGACACAGCC 3' *Xho*I **Stop Codon**	59
pAAV-IRES-hrGFP was used as template for the amplification of the IRES region for the construction of pS7IR-AdV. Annealing temperature was set at 58 °C		
IRESfor	5' AACTCGAGCCCCTCTCCCTCCCC 3' *Xho*I	59
IRESrev	5' TGTGAATTCGGTTGTGGCCATTATCATCGTG 3' *Eco*RI	57
A RIKEN plasmid (AK053760) was used for the amplification of the *ric3* gene for the construction of pS7IR-AdV. Annealing temperature was set at 59 °C.		
Ric3for	5' ACAGAATTC**ATG**GCGTACTCCACGGTGCAG 3' *Eco*RI **Start Codon**	59
Ric3rev2	5' GTGGATCC**CTA**ACAAGGAAGGGGCATTTTACC 3' *Bam*HI **Stop Codon**	59
A RIKEN plasmid (AK053760) was used for the amplification of the *ric3* gene for the construction of pSR-AdV. Annealing temperature was set at 59 °C. Primer Ric3for was used as the forward primer.		
Ric3rev1	5' GTCTCGAG**CTA**ACAAGGAAGGGGCATTTTACC 3' *Xho*I **Stop Codon**	59

Table 4.1. Primer sequences and restriction enzymes employed in the amplification and cloning of the genes of interest.

4.2.2. Agarose Gel Electrophoresis

DNA samples were analyzed by the electrophoretic separation (Thermo EC Primo™ Horizontal Submarine Gel Systems) of DNA in agarose gels containing 0.5 μg/ ml ethidium bromide. A 1 % agarose gel was generally used depending on the sizes of the fragments to be separated, using 1 % UltraPure Agarose (Invitrogen) melted in TAE buffer (40 mM Tris-HCl, 20 mM NaAc, 1 mM EDTA, pH 8). The samples were mixed with a loading buffer (1 % bromophenol blue, 1 % xylene cyanol, 1xTAE buffer, 50 % glycerol) prior to loading on the set gel. DNA bands were separated by electrophoresis by placing the gel in TAE buffer and passing 100 mV through the system for approximately 20 minutes (Electrophoresis Power Supply – EPS301; Amersham Biosciences). Results were visualized by UV fluorescence.

4.2.3. Purification of Amplified DNA Fragments

DNA fragments of interest were purified after PCR and restriction enzyme digestions via the GFX PCR DNA And Gel Band Purification Kit (Amersham Biosciences).

4.2.4. Restriction Enzyme Digestion

The restriction endonucleases that were used in the cloning procedure during the preparation of DNA fragments or in the characterization and identification of recombinant DNA plasmids were used according to the specifications of the manufacturers (Roche Diagnostics).

4.2.5. Dephosphorylation

The digested composite expression cassette pXCXSynI-WPRE plasmid as well as the pS7-AdV plasmid were dephosphorylated by incubating the linearised DNA in a total reaction volume of 20 µl in the presence of 0.5 U of shrimp alkaline phosphatase (Roche Diagnostics) and 2 U of 10 x dephosphorylation buffer (0.5 M Tris-HCl, 1 mM EDTA, pH 8.5) at 37 °C for 20 min.

4.2.6. Ligation

Ligations were carried out at 16 °C for 12 h. A 10:1, ratio of insert: vector molecules was used in a total volume of 20 µl, which also contained 2 U of T4 DNA ligase (Roche Diagnostics) and 2 U of 10 x ligation buffer (660 mM Tris-HCl, 50 mM MgCl2, 10 mM dithio-erythritol, 10 mM ATP, pH 7.5).

4.2.7. Preparation of Competent Escherichia Coli Cells

The standard $CaCl_2$ method described by Cohen *et al* (1972) was used to prepare the *Escherichia coli (E. coli)* Xl1 Blue competent (Stratagene) cells used in all plasmid transformations. 1 ml of an overnight Xl1 Blue culture was used to inoculate 100 ml Terrific Broth medium (47.6 g Terrific Broth and 0.8 % glycerol in 1 litre ddH_2O). Cells were grown at 37 °C with shaking to logarithmic (log) phase (OD_{550} = 0.5). The cells were collected by centrifugation at 5000 rpm for 5 minutes at 4 °C. The pellet was then gently resuspended in 50 ml of freshly made ice-cold 50 mM $CaCl_2$, centrifuged again at the above-mentioned conditions and resuspended in 5 ml of 50 mM $CaCl_2$. The cells

were kept on ice for 1 h before sterile 15 % (v/v) glycerol was added and the cells frozen away at –80 °C.

4.2.8. Transformation of Competent Cells

Transformation of competent cells was performed as described by Dagert and Ehrlich (1979). The ligation mixture was added to 100 µl of competent cells and allowed to absorb for 30 min on ice. The mixture was then subjected to a 90 second heat shock at 42 °C and left to cool on ice for 2 min. 900 µl Terrific Broth medium was added, followed by an incubation period of 1 h at 37 °C with shaking. Aliquots of 100 µl were then plated out on Terrific Broth agar plates (1.2 % agar in Terrific Broth medium) containing 12.5 µg/ml tetracycline hydrochloride (tet) and 100 µg/ml ampicillin (amp). Plates were then incubated overnight at 37 °C.

4.2.9. Plasmid DNA Isolation and Purification

The Birnboim and Doly (1979) alkaline lysis method was used for the isolation of plasmid DNA. Selected colonies from the agar plates were inoculated into 5 ml of Terrific Broth medium, containing the appropriate antibiotics (amp and tet), and grown for 16 h at 37 °C with shaking. 3 ml of each culture was harvested by bench top centrifugation at 15000 rpm for 1 min after which the pellets were resuspended in 100 µl of solution 1 (50 mM glucose, 10 mM EDTA, 25 mM Tris, pH 8) and incubated at room temperature for 5 min. Cell lysis and chromosomal DNA denaturation were achieved by adding 200 µl of freshly prepared alkaline-SDS buffer (0.2 N NaOH, 1 % SDS), followed by a 5 min incubation on ice. 150 µl cold 3 M NaAc (pH 4.8) was added resulting in the

reannealing of the plasmid DNA and the precipitation of the genomic DNA, proteins and high molecular weight RNA. After a 10 min incubation period on ice, the supernatant, containing the plasmid DNA, was collected after centrifugation at 15000 rpm for 10 min at 4 °C. Two volumes of 96 % ethanol was added to the supernatants and incubated at – 20 °C for 30 min to precipitate the plasmid DNA. This was followed by 10 min centrifugation at 15000 rpm and subsequent washing of the DNA pellet with 80 % ethanol. The clean DNA pellet was resuspended in UHQ.

Alternatively, GenElute Plasmid Miniprep kit (Sigma-Aldrich) was used for the extraction of small volumes of high quality plasmid DNA or the QIAGEN® Plasmid Maxi kit (QIAGEN) for the extraction of large volumes of high quality plasmid DNA.

4.2.10. Nucleotide Sequence Determination

Nucleotide sequence determination was performed independently by Geneservice Ltd, UK. Due to the length of the α7 nAChR gene, three additional internal primers were required to enable the full sequence analysis of the cloned product (table 4.2)

Primer Name	Oligonucleotide Sequence	Tm °C
A7IntFw	5' ACCTGCGCTCAGCTCCACACTGG 3' Binds at 1100 – 1122 bp and elongates towards the 3' end	62
A7IntRev	5' CCTCTATAACAGTGCAGATGAACGC 3' Binds at 339 – 363 bp and elongates towards the 5' end	58
A7IntRev	5' CCAGTGTGGAGCTGAGCGCAGGT 3' Binds at 1100 – 1122 bp and elongates towards the 5' end	62

Table 4.2. Additional primers required for the full sequencing of the mouse α7 nAChR gene insert of the newly created pS7-AdV.

4.2.11. Sequence Homology Analyses

Nucleotide sequences were aligned and compared using the CLUSTAL X (1.81) program (Higgins and Sharp, 1988; Higgins *et al.*, 1996).

4.2.13. Subculturing of HEK 293 Cells

Low passage (<40) human embryonic kidney (HEK) 293 cells (Microbix Biosystems Inc., Toronto, Canada) were grown at 37 °C in 5 % CO_2 using 75 cm^2 Greiner culture flasks. The medium consisted of 80 % Dulbeccos's Minimum Eagle's Medium Nutrient Mixture F-12 (DMEM/F12), 10 % heat inactivated Fetal Calf Serum (FCS) (Biowest), 0.3 % (w/v) $NaHCO_3$, 2 % penicillin (500 IU/ ml)/ streptomycin (500 µg/ ml) solution, and 7.7 % ddH_2O, pH 7.6. Approximately every 4 days the cells reached 90 % confluence and had to be subcultured. The used medium was removed, and the cell monolayer was washed with 10 ml PBS before being incubated for one minute in 2 ml trypsin-EDTA (Sigma) at 37 °C. Cells were washed off the flask surface by the addition of 10 ml medium before being centrifuged for one minute at 1000 rpm. The cell pellet was resuspended in 12 ml medium and 2 ml of this suspension was added to 75 cm^2 culture flasks containing 10 ml medium, which was then incubated at 37 °C in 5 % CO_2.

4.2.14. Co-Transfection

Co-transfections to create the adenoviral particles were performed by Virapur, LLC (USA).

4.2.15. Overlaying

Methods for the purification of recombinant adenovirus vectors were adapted from (Graham and Prevec, 1995). Falcon 60 mm dishes (BD Biosciences) were coated with poly-D-lysine (0.05 g/ l) for 1 hour, washed with sterile ddH_2O and air dried. 1 ml of the HEK 293 cell suspension from section 4.2.13 was added to 5 ml media in the dishes and incubated at 37 °C in 5 % CO_2 for 24 hours. A 100 µl of a dilution series of either the transfection medium containing the new recombinant viruses (section 4.2.14.) or the PBS^{2+}/plaque (section 4.2.16) mixture was added to the cells that had reached approximately 80 – 90 % confluency. After incubation for an additional 12 hours at 37 °C in 5 % CO_2, the virus-containing medium was removed from the dishes. A sterile 37 °C agarose/ media mixture was prepared beforehand, consisting of 5 ml 10x DMEM, 5 ml heat inactivated FCS, 0.5 ml penicillin (500 IU/ ml)/ streptomycin (500 µg/ ml), 0.5 ml glutamine (stock = 200mM in ddH_2O), 11.5 ml ddH_2O, 2.5 ml 7.5% $NaHCO_3$ and finally 25 ml of 1.6 % agarose/ ddH_2O (SeaPlaque® Agarose, Cambrex). Each dish was carefully overlayed with 10 ml of this mixture and the agarose allowed to polymerise at room temperature, before being incubated at 37 °C in 5 % CO_2 for seven to ten days. Cytolytic viral plaques as observed by eye / microscopy would typically start to appear by day 8 post-infection.

4.2.16. Plaque Purification

A shortened yellow pipette tip was used to select and remove a plug of agarose surrounding an isolated viral plaque. The agarose plug, which contains the plaque-forming virus, was placed in an eppendorf tube before the addition of 1 ml of sterile

PBS^{2+} (136.9 mM NaCl, 2.68 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.68 mM $CaCl_2.2H_2O$, 0.5 mM $MgCl_2.6H_2O$, pH 7.4). The cells were lysed by freezing the suspension in liquid nitrogen and then rapidly thawing at 37 °C in a hot water bath. This step was repeated three times, vortexing the solution for 1 minute in between each turn. Low passage HEK cells were grown in 60 mm dishes and allowed to reach 80 – 90 % confluency. The cells were infected with either 10 µl of the PBS^{2+}/plaque mixture to be overlayed as described in Section 4.2.15 for another round of plaque purification, or 100 µl of the PBS^{2+}/plaque mixture and incubated in the 5 ml cell medium at 37 °C in CO_2 until full cytopathic effect (CPE) was reached.

4.2.17. Viral DNA Isolation

DNA was isolated from 100 µl of the virus-containing CPE medium (section 4.2.16) to confirm plaque identity, ensuring that only recombinant adenoviruses carrying the genes of interest were used for the next round of plaque purification. ViralXpress™ Nucleic Acid Extraction Kit (Chemicon® International) was used to extract recombinant adenovirus genomic DNA from the medium-containing virus.

4.2.18. PCR Analysis of Viral Recombinants

In order to confirm that the virus obtained was recombinant and contained the correct transgene of interest subsequent to every round of plaque purification, PCR analysis was performed. Each plaque underwent two PCR screening reactions. The first general PCR screening reaction was designed to detect the presence of the WPRE motif, eliminating the presence of any wildtype adenovirus plaques that might have formed. The second

reaction was specific to the transgene of interest, with genetic primers used for either α7 nAChR, *ric3*, IRES (table 4.1) or EGFP (table 4.3).

A MultiGene II thermal cycler (Labnet) with the appropriate heating/ cooling program was used for the PCR reactions which consisted of 1 μg of viral recombinant DNA (section 4.2.17), 100 pmol of each primer (table 4.1 and 4.3), 3 μl 2.5 mM Deoxynucleotide Solution Mix, 5 units (U) Taq DNA Polymerase in Storage Buffer A (Promega), 5 μl 10x Taq reaction buffer (Promega), 3 μl 250mM $MgCl_2$ (Promega), and made up to a final volume of 50 μl with ddH_2O. The amplification conditions for both the reactions were as follows: 1 cycle at 94 °C for 2 minutes; 25 cycles 93 °C for 45 seconds, annealing temperature (provided in table 4.1 and 4.2 for each reaction) for 1 minute, 72 °C for 2 minutes; 1 final elongation cycle at 72 °C for 10 minutes. Each PCR mix was electrophoresed on a 1 % agarose gel to assess the size of band produced (section 4.2.2).

Primer Name	**Oligonucleotide Sequence and Restriction Enzymes**	**Tm °C**
The adenoviral plaque DNA were tested for the presence of the WPRE sequence. Annealing temperature was set at 50 °C.		
WPREFor	5' GGGAGATCCGACTCGTCTGAGG 3'	55
WPRERev	5' GTGAAAGATTGACTGGTATTC 3'	43
The presence of the EGFP transgene of the SG-AdV plaques were identified with the following primers. Annealing temperature was set at 62 °C		
GFPFor1	5' CCGCATGGTGAGCAAGGGCGAGGAGC 3'	62
GFPRev	5' CTTCTCGAGCTTTACTTGTACAGCTCGTCCATGCC 3'	62

Table 4.3. Additional primer sequences required for confirming the identity of the recombinant adenoviruses and their transgenes.

4.2.19. Large Scale Adenovirus Amplification and Purification

Low passage HEK 293 cells were seeded into a 75 cm^2 tissue-culture flask (section 4.2.13) and allowed to reach 80 % confluency at 37 °C, 5 % CO_2. 500 µl of the plaque purified PBS^{2+}/plaque mix supernatant of the recombinant adenovirus that had gone through three sequential rounds of plaque purification and confirmation, was used to infect the flask. Full CPE was reached in approximately five days. Virus was liberated from HEK 293 cells by a freeze / thaw cycle where the cells were rapidly frozen with liquid nitrogen and thawed at 37 °C. Five 175 cm^2 tissue-culture flasks (Greiner), each containing HEK 293 cells at 80 % confluency, was infected with 2 ml virus-containing medium from the 75 cm^2 tissue-culture flask. Incubation lasted for approximately three days, until the cells had a swelled appearance but were not yet virally lysed. The supernatant and the cells were harvested and centrifuged at 1500 rpm for 10 minutes. The viral-infected cells were resuspended in 20 ml of supernatant before undergoing three freeze/ thaw cycles. Both the 150 ml supernatant and the 20 ml cellular released virus were used in the subsequent viral purification step utilizing the Vivapure® AdenoPACK™ 100 (Vivascience, Sartorius Group, USA).

4.2.20. Recombinant Adenovirus Titration by Plaque Assay

HEK 293 cells were grown in 60 mm dishes to approximately 80% confluency before being infected with a viral serial dilution (10 µl Ad suspension in 990 µl PBS^{2+}) to 10^{-12} of the original volume. Like a normal plaque overlay, the infection was incubated for 12 hours in medium before being overlayed with agarose, allowed to set and before, once

again, being incubated at 37 °C in CO_2 (section 4.2.15). Plaques usually started to appear at day 7 and were counted by eye or by microscopy over a 6-14 day period.

4.4.21. Recombinant Adenoviral Gene Expression in HEK 293 Cells

HEK 293 cells that were grown to approximately 80 % confluency in 60 mm dishes were infected with 10^7 viral particles and incubated at 37 °C in 5 % CO_2 for 48 hours. The cell medium was removed and the cells were harvested in 1 ml TBS with inhibitor and sonicated four times for 30 seconds in a sonication bath with a 1 minute incubation period on ice in between every sonication pulse. 100 µl of the sample was chloroform/ methanol precipitated before a Western blot analysis was performed on a 6 % SDS-polyacrylamide gel (section 2.2.7 to section 2.2.9).

4.4.22. Recombinant Adenoviral Gene Expression in Pure Neuronal Cortical Cultures

Day *in vitro* (DIV) 14 pure neuronal cortical cultures were prepared as in section 2.2.1. Cultures were infected during the DIV 7 medium change at either a multiplicity of infection (MOI) of 1 or 100. The MOI of 1 infections were performed on cultures that were grown on glass coverslips coated with poly-D-Lysine (0.05 g/ l). Cultures were also prepared in poly-D-Lysine-coated 35 mm Iwaki tissue culture treated dishes at the same density as the 24 well microplates. These dishes were infected on DIV 7 at a MOI of one for Western blot protein analyses (section 2.2.7 to section 2.2.9).

Pure neuronal cortical cultures grown on glass coverslips and infected on DIV 7 with recombinant adenovirus at a MOI of 1 (SR-AdV, SI-AdV, and S7-AdV), were also subjected to α-Bungarotoxin-FITC chemiluminescent staining on DIV 14, as described in section 2.2.10, to analyse the functional expression of α7 nAChR expression.

4.3. Results

An adenovirus expressing the mouse alpha 7 nicotinic acetylcholine receptor (α7 nAChR) (pS7-AdV) was created to study, in isolation, the importance of upregulating this protein for the survival of neurons without disturbing the regulation of other proteins. The adenovirus was created from the composite expression cassette (pXCXSynI-WPRE) received from Dr. James Uney (Glover *et al.*, 2002). The cassette contains a neuron-specific 5' human synapsin-1 (Syn1) promoter ahead of the multiple cloning site (MCS) into which the gene of interest is to be inserted. The expression of any gene cloned into the MCS region would also benefit from amplified mRNA translation due to the presence of the 3' woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) before the poly-A tail. This enhancer motif has been shown to increase protein production by a factor of three, without the loss of promoter specificity (Glover *et al.*, 2002). The promoter, the MCS, the enhancer element and the poly-A tail are all located between two homologous recombination sites, which are necessary for the whole region to be transposed into the adenoviral genome after any necessary modifications have been made.

4.3.1. pS7-AdV

The α7 nAChR gene was amplified using the pCDNA5 mα7 plasmid as template, a kind gift from Professor Lucia Sivilotti (UCL, London) (National Centre for Biotechnology Information accession number 790853). The gene consists of 1509 bp and was amplified with the primers given in table 4.1 at an annealing temperature of 61 °C. The primers (Alpha7-5 and Alpha7-3) were designed to incorporate a *Kpn*I restriction enzyme site directly before the ATG start codon, and a *Xho*I restriction enzyme site directly following

the translational stop codon. The multiple cloning site of pXCXSynI-WPRE was also cut with these two enzymes and dephosphorylated before the alpha 7 gene was directionally ligated into the plasmid, thereby completing the pS7-AdV vector required for the formation of the recombinant S7-AdV adenovirus. Competent *E. coli* Xl1 Blue cells were transformed with the ligation mixture and the colony forming bacterial cultures were screened for the presence of the recombinant plasmid by means of restriction enzyme digestion and agarose gel electrophoresis. The newly created plasmid construct was then submitted to nucleotide sequenced analysis. As can be seen in appendix A, no point mutations were created in the inserted gene.

4.3.2. pSR-AdV

The 684 bp mouse *ric3* gene was amplified from a plasmid received from the RIKEN Genome Exploration Research Group Phase II Team and the FANTOM Consortium (NCBI accession number AK053760). The PCR used the Ric3for and the Ric3rev1 primers at an annealing temperature of 59 °C (table 4.1). An *Eco*RI restriction enzyme site was included in the forward primer and a *Xho*I restriction enzyme site in the reverse primer. The amplified fragment was digested with these two enzymes. The composite expression cassette pXCXSynI-WPRE was also digested with these two enzymes and submitted to a subsequent dephosphorylation reaction before being ligated to the processed *ric3* gene. The newly created recombinant plasmid, pSR-AdV, underwent the same processing and screening as the pS7-AdV, and the homologous sequence comparison is reported in appendix B.

4.3.3. pSI-AdV

A bicystronic adenoviral construct was made expressing both the α7 nAChR and the *ric3* genes. This was made possible by the insertion of an internal ribosome entry site (IRES) between the two genes, enabling the translation of two genes from a single mRNA molecule. The 586 bp internal ribosome entry site (IRES) region was amplified using the pAAV-IRES-hrGFP plasmid vector (Stratagene) as template. The PCR utilized the IRESfor and the IRESrev primers at an annealing temperature of 58 °C (table 4.1). The IRESfor primer bound to start of the IRES motif of the pAAV-IRES-hrGFP plasmid at position 1528 and added a *Xho*I restriction enzyme site to the 5' end of the IRES fragment. The IRESrev primer bound to the end of the IRES motif at position 2114 of the plasmid and added an *Eco*RI restriction enzyme site to its 3' end. A second *ric3* gene was amplified with primers Ric3for containing the *Eco*RI site, and Ric3rev2 containing a *Bam*HI restriction enzyme in a PCR with an annealing temperature of 59 °C. The two fragments were digested with their respective enzymes before being simultaneously ligated to the newly created pS7-AdV plasmid vector that was digested with *Xho*I and *Bam*HI before being dephosphorylated. This ligation reaction resulted in a single plasmid denoted as pSI-AdV that contained the following sequence of motifs: SynI – α7nAChR – IRES – *ric3* – WPRE. Positive bacterial colonies carrying this full plasmid were selected, and the plasmid DNA submitted for nucleotide sequence determination of all three genes of interest (Appendix C).

4.3.4. pSG-AdV

Plasmid DNA and the virus for an enhanced green fluorescent protein (EGFP) (pSG-AdV) cloned into the pXCXSynI-WPRE plasmid vector were received as a kind gift from Dr. James Uney (Glover *et al.*, 2002), the sequence of which is reported in appendix D.

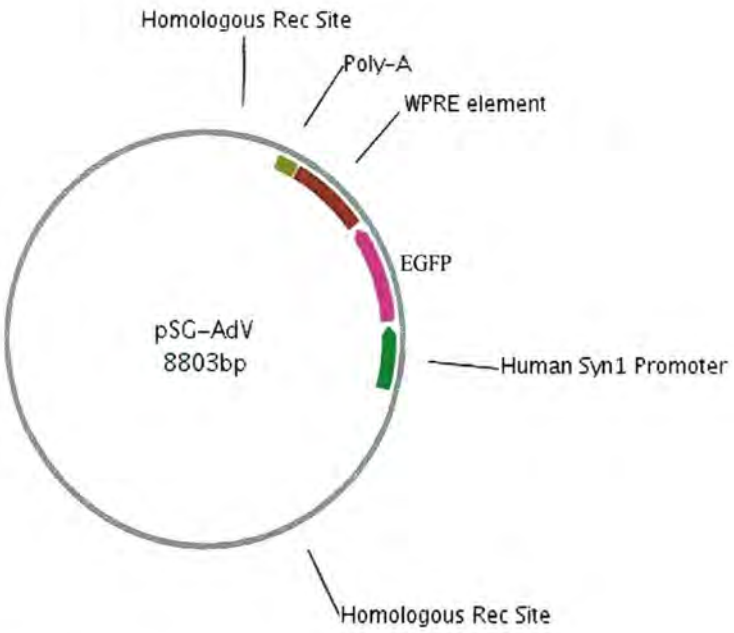
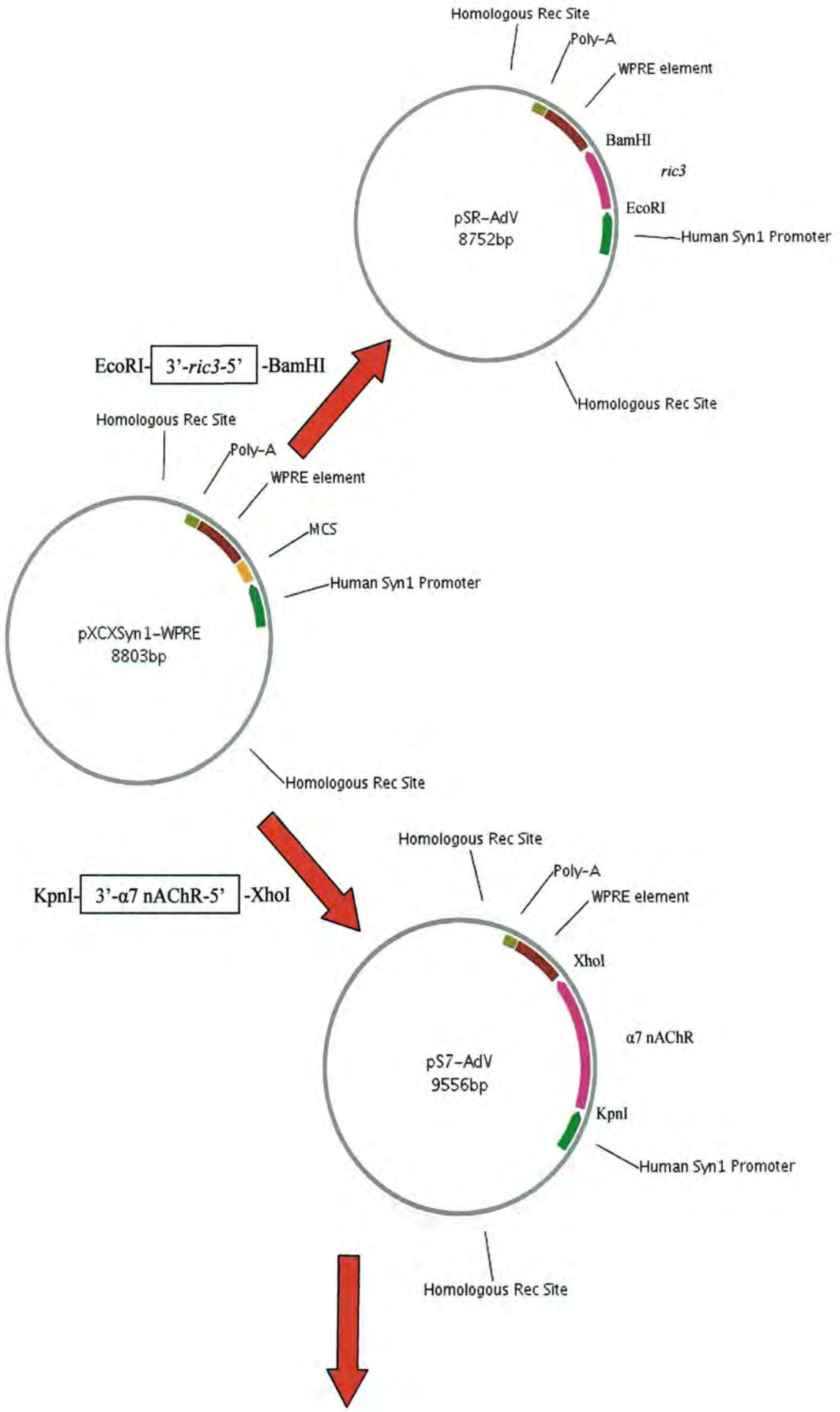


Figure 4.1. Plasmid map of the pXCXSynI-WPRE plasmid vector (Glover *et al.*, 2002).


Homologous Rec Site
Poly-A
WPRE element
BamHI
ric3
EcoRI
pSR-AdV
8752bp
Human Syn1 Promoter
Homologous Rec Site
EcoRI- 3'-ric3-5' -BamHI
Homologous Rec Site
Poly-A
WPRE element
MCS
Human Syn1 Promoter
pXCXSyn1-WPRE
8803bp
Homologous Rec Site
KpnI- 3'-α7 nAChR-5' -XhoI
Homologous Rec Site
Poly-A
WPRE element
XhoI
α7 nAChR
pS7-AdV
9556bp
KpnI
Human Syn1 Promoter
Homologous Rec Site

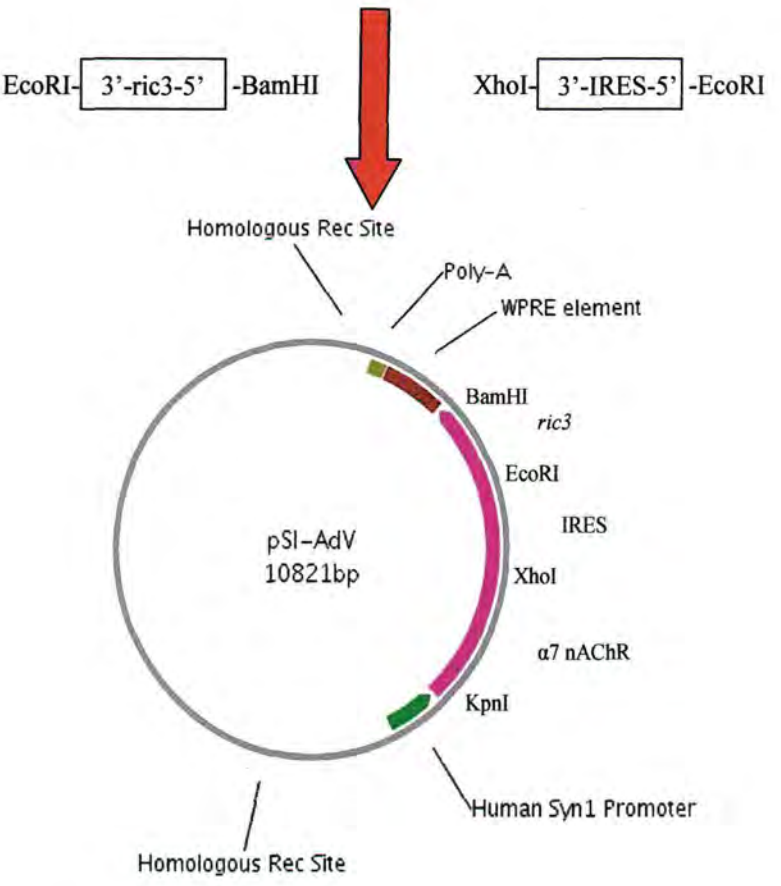


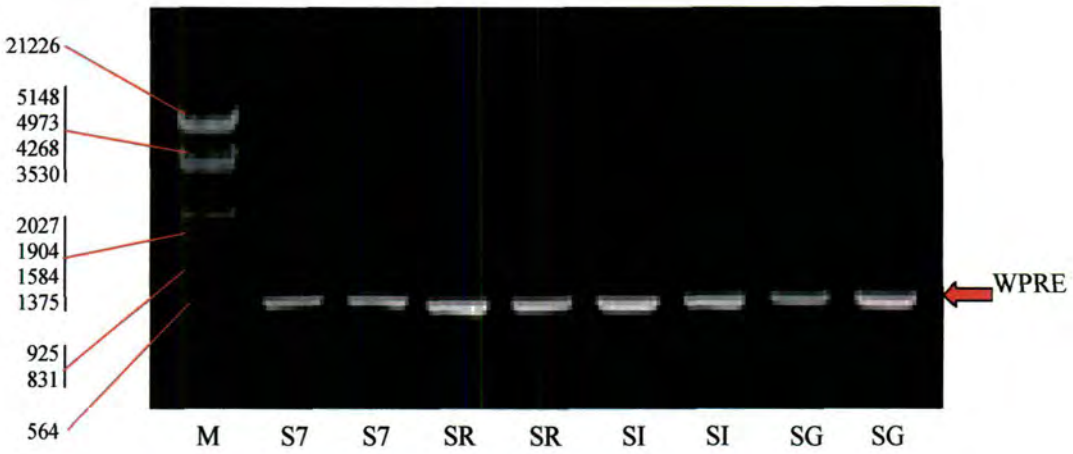
Figure 4.2. Plasmid map-based diagram depicting the construction of the various plasmid vectors using the pXCXSynI-WPRE plasmid vector (Glover *et al.*, 2002) as starting point.

4.3.5. Adenoviral Creation, Selection and Purification

Co-transfections to create the adenoviral particles were performed by Virapur, LLC. HEK 293 cells were co-transfected with the helper plasmid (pJM17) and either pS7-AdV, pS7IR-AdV or pSR-AdV DNA. The region between the two homologous recombination sites (the Syn1 promoter, the gene of interest, the WPRE element, and the poly-A tail) transposed to homologously recombine with the adenoviral genome contained in the JM17 plasmid. The expression of the viral genes form the proteins that encapsulate the recombined viral genomic DNA leading to the formation of the recombinant adenoviruses (S7-AdV, S7IR-AdV or SR-AdV).

The three recombinant viruses, as well as the recombinant adenovirus expressing EGFP (SG-AdV) were subjected to three rounds of viral plaque identification and purifications before a final large scale viral purification was done for each, which yielded viral concentrations of approximately 10^7 infective viral particles per μl. Viral DNA were screened after the large scale viral purification to confirm the identity of the recombinant adenoviruses.

A)


21226
5148
4973
4268
3530
2027
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1584
1375
925
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WPRE
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S7
S7
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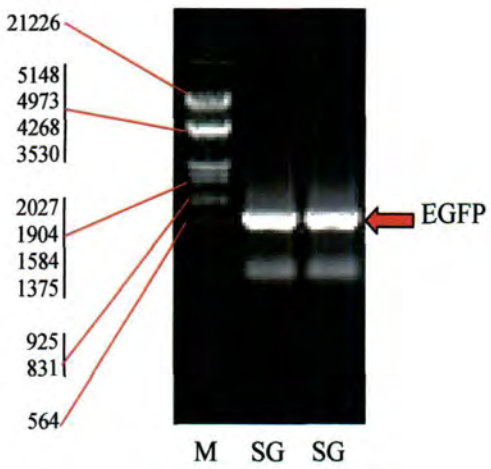
SR
SI
SI
SG
SG

B)


21226
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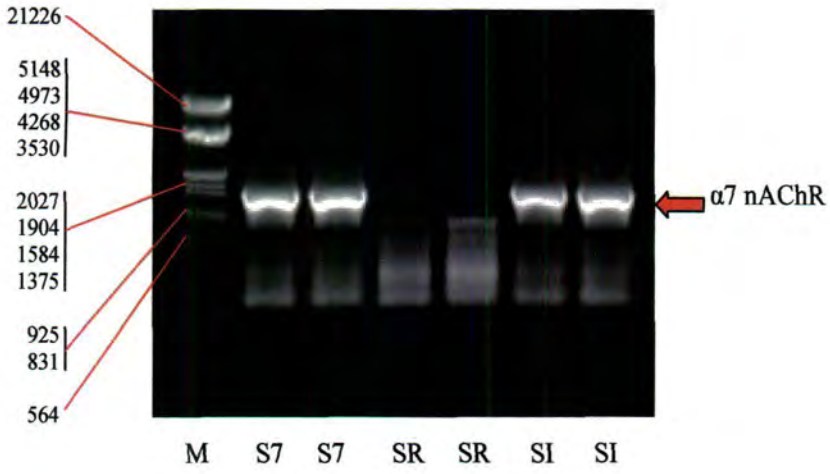
C)


21226
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SR
SR
SI
SI

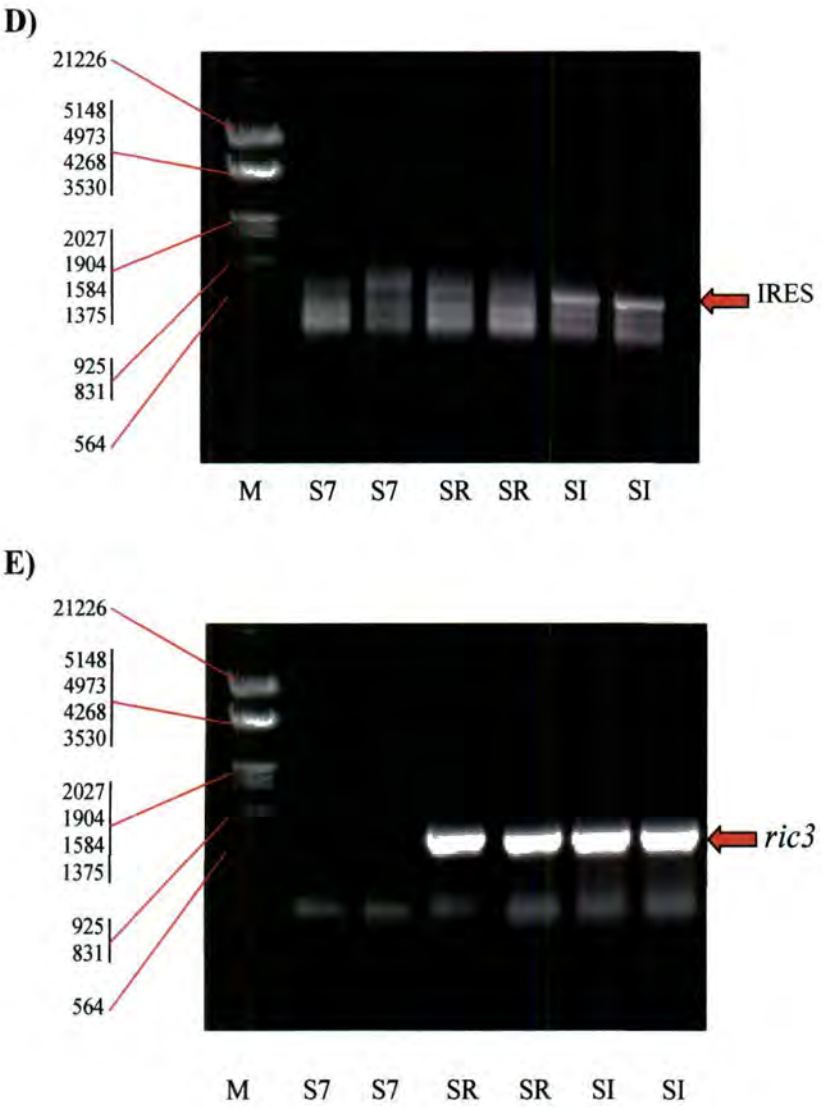


Figure 4.3. 1 % Agarose gel electrophoresis of plaque screening and confirmation PCRs. All extracted plaque viral DNA was first submitted to a PCR using the WPRE motif primers (table 4.3) in a general screening reaction, which resulted in the amplification of a 564 bp region (A). The SG-AdV construct's genomic DNA was also used in a further screening reaction for the confirmation of the presence of the EGFP gene (B). The GFPFor1 and the GFPRev primers (table 4.3) were used in this reaction to generate a 735 bp positive band. The S7-AdV, SR-AdV and SI-AdV recombinant adenoviruses were screened together using either the Alpha7-5 and Alpha7-3 primers to screen for the presence of the 1526 bp α7 nAChR gene (C), the IRESfor and IRESrev primers to screen for the presence of the 603 bp IRES fragment (D), or the Ric3for and Ric3rev2 for the amplification of a present 704 bp *ric3* gene (E). Lane 1 of all the gels contains the DNA Molecular Weight Marker III (Roche Diagnostics).

4.3.6. Adenoviral Infection of HEK 293 Cells and Western Blot Analysis

The human synapsin-1 (Syn1) promoter is a neuron-specific promoter, although low levels of non-specific expression can be detected in other cell types, e.g. HEK 293 cells. This trait was exploited to confirm and study the heterologous expression of both the α7 nAChR and Ric3 proteins in a non-native environment. HEK 293 cells were infected with either SG-AdV, SR-AdV, SI-AdV, S7-AdV, or SR-AdV and S7-AdV simultaneously. The cells were harvested 48 hours post-infection, sonicated to homogenize the protein suspension and analysed by 6 % SDS-PAGE. Western blots were performed (Fig. 4.4A and 4.4B).

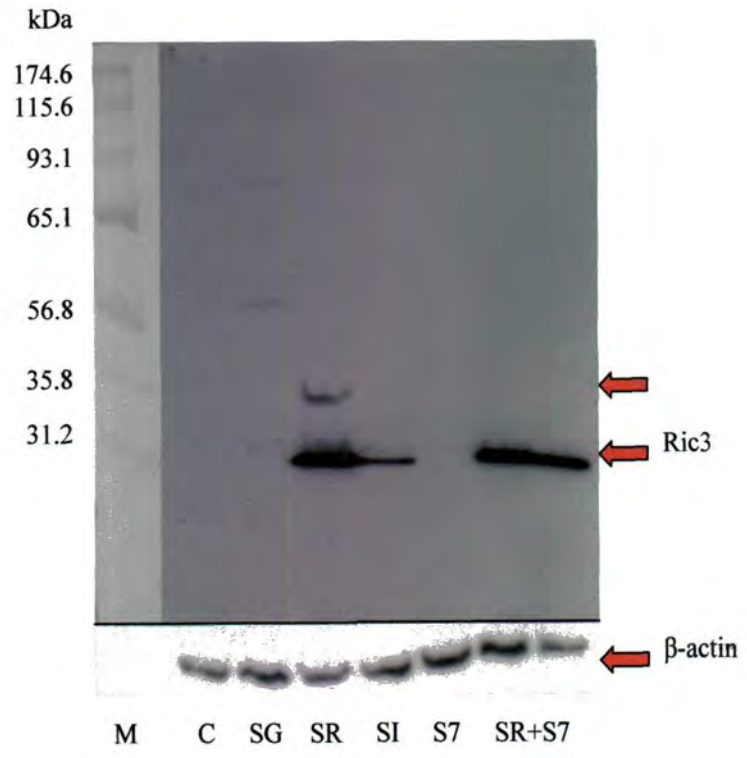
The anti-mRic3 antibody developed in Chapter 3 was used to detect expression of the *ric3* gene in HEK 293 cells infected with either SR-AdV or SI-AdV (Fig. 4.4A). Ric3 protein expression was only detected in samples where infections with either the SR-AdV or the SI-AdV recombinant adenoviruses. The Ric3 protein consists of 228 amino acids and has a theoretical molecular weight of 24.92 kDa. A single band of approximately this size can be found in both the SR-AdV and SI-AdV infected HEK cell samples. The intensity of the SI-AdV generated band is significantly lower than that of the SR-AdV generated band, despite both samples being infected with approximately 10^7 viral particles. Protein quantities loaded unto the gel were also normalized with β-actin (~ 42 kDa) blotting (Fig. 4.4A). This is explained by the fact that the Ric3 protein production from the mRNA of the SI-AdV construct is under the control of the IRES region, which generally cause a ten times lower protein production than the first gene on the same mRNA strand, in this case the α7 nAChR (AAV Helper-Free System Introduction Manual, Stratagene).

The specificity of the anti-mRic3 antibody is very high although three faint non-specific bands can be found in the SG-AdV infected HEK cells at approximate sizes of 80, 56 and 32 kDa. An additional high intensity band of approximately 37 kDa also appears in the SR-AdV infected group. This protein species is, however, not present when Ric3 is co-expressed in the bicystronic IRES-mediated vector (SI-AdV) together with α7 nAChR, or in the two groups where a simultaneous co-infection was performed with SR-AdV and S7-AdV.

Primary antibodies targeted at the α7 nAchR protein are well known for their poor affinity and specificity (Herber *et al.*, 2004; Moser *et al.*, 2007; Jones and Wonnacott, 2005). Despite this, the anti-human α7 nAChR antibody (Santa Cruz α7 # sc-5544) was used in Figure 4.4B for the detection α7 nAChR expression by either the S7-AdV or the SI-AdV recombinant constructs. The reliability of the anti-human α7 nAChR primary antibody in this study is probably due to the fact that the expression of α7 nAChR or any of its alternative splice forms are not native to HEK 293 cells (Williams *et al.*, 2005). The 502 amino acid α7 nAChR peptide has a theoretical molecular weight of 56.63 kDa. A single protein species of this approximate size was detected in all the lanes containing a α7 nAChR-expressing adenovirus. Note, the same nitrocellulose membrane was used as in the Figure 4A, and the Ric3 immunoreactive bands can still clearly be seen at approximately 25 kDa.

These two antibody studies clearly showed that the genes of interest are expressed by the functional recombinant adenoviral constructs, including the double gene expression by the bicystronic adenovirus.

A)



B)

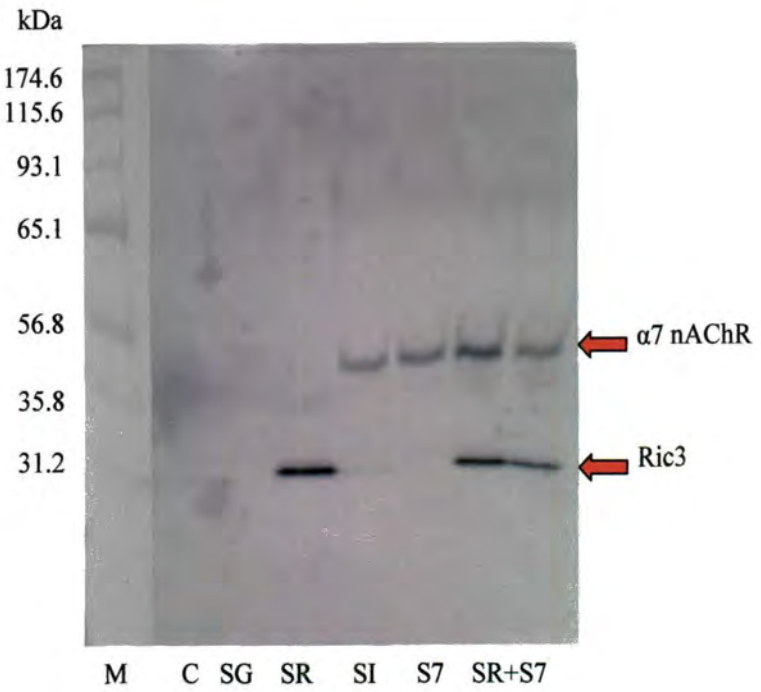


Figure 4.4. HEK 293 cells were infected with recombinant adenoviruses. 50 µl of the 1 ml harvested cell suspension was analysed on a 6 % SDS-PAGE gel. M represents the molecular weight marker used (Precision Plus Protein™ Standards, Bio-Rad), C the control uninfected HEK 293 cells, SG the SG-AdV infected cells, SR the SR-AdV infected cells, SI the bicystronic adenovirus SI-AdV infected cells, S7 the S7-AdV infected HEK cells, and SR+S7 the HEK cells infected with both SR-AdV and S7-AdV

simultaneously. The Ric3-antibody from Chapter 3 was used at a concentration of 2μg/ml in combination with an anti-rabbit horseradish peroxidase linked secondary antibody (1 in 2000 dilution; ECL™) to generate Figure 4.4A. To normalize the protein expression, monoclonal anti-β-actin antibody (Sigma Aldrich) at a 1 in 2000 dilution was used with an anti-mouse horseradish peroxidase linked secondary antibody (ECL™) also at a 1 in 2000 dilution (inserted at the bottom of the Figure 4.4.A). Figure 4.4B was created by using the rabbit polyclonal anti-α7 nAChR antibody (Santa Cruz α7 # sc-5544) (1 in 2000 dilution) in conjugation with a mouse secondary antibody (1 in 2000 dilution).

4.3.7. The Effect of Recombinant Adenoviral Infections on Pure Neuronal Tissue Cultures

Pure neuronal cortical cultures were infected separately with four adenoviruses, SG-AdV, S7-AdV, SR-AdV, SI-AdV, to determine whether increased expression of α7 nAChRs by itself, without pharmacological intervention, is sufficient to increase the brain's tolerance to ischaemia to a level similar to that achieved with potassium chloride preconditioning.

Initially DIV 7 pure neuronal cultures grown in 35 mm dishes or on coverslips in 24 well microplates were infected at a MOI of 1 with the recombinant adenoviruses. The samples from the dishes were harvested on DIV 14 and subjected to Western blot analyses. The samples were first analysed with the newly developed anti-mRic3 antibody (Fig. 4.5). In all the samples, as in chapter 3, a prominent double species was detected at approximately 50 kDa and a lesser expressed species at approximately 25 kDa. In addition, another lesser expressed protein species was detected at approximately 75 kDa. Only in the SR-AdV infected cells was the faint 25 kDa species highly expressed, corresponding to the expected adenoviral expression of the Ric3 protein. No increase in Ric3 protein expression was, however, detected in the SI-AdV infected cells. The SR-AdV infected cells also express an additional faint band of approximately 37 kDa that is not detected in the control or any of the other infected cells.

Western blot analysis of infected pure neuronal cultures with the anti-GFP antibody resulted in the detection of a 27 kDa protein in the SG-AdV infected cells, corresponding to the expected protein production of EGFP (Fig. 4.6). Attempts at detecting α7 nAChR expression with the polyclonal anti-rabbit α7 nAChR from Santa Cruz Biotechnology (sc-

5544) and with the anti-rabbit α7 nAChR from Abcam (ab23832), as in Chapter 2, failed (results not shown). Subsequently, pure neuronal cultures grown on coverslips were infected on DIV 7 at a MOI of 1 and analysed on DIV 14 with α-Bungarotoxin-FITC. Highly variable results were attained due to the use of coverslips and blind counting. An approximate 9.7 % increase in α-Bungarotoxin-FITC binding to functional α7 nAChR was detected in cells infected with the SR-AdV virus, expressing the Ric3 protein, whereas an approximate 13.59 % decrease in binding was detected in SI-AdV infected cells. Pure neuronal cultures infected with S7-AdV had an increase in α-bungarotoxin bound to functional α7 nAChRs of approximately 25.7 %. Despite this increase not being statistically significant, the trend for the upregulation of functional α7 nAChRs in pure neuronal cortical cultures by infection with the S7-AdV recombinant adenovirus was confirmed in a subsequent experiment (results not shown).

Subsequent to confirmation of recombinant adenovirus transgene expression in pure neuronal cortical cultures, DIV 7 cultures were infected at either a MOI of 1 or 100 and left for an additional 7 days. On DIV 14 the cultures received a 10 minute 1 mM glutamate insult, followed by three washes and 24 hour incubation before MTT analyses. As can be seen in Figure 4.8A, the one week incubation of pure neuronal cultures with any of the viruses at a MOI of 100 led to extensive cell death. Both the control recombinant adenovirus SG-AdV and the α7 nAChR expressing recombinant virus, S7-AdV, killed approximately 32 % of the cells. Of interest is the significant reduction in cell death caused by the recombinant adenoviruses expressing Ric3, SR-AdV and SI-AdV. SR-AdV, which expresses approximately ten times more Ric3 than SI-AdV, has an approximate 11 % increase in cell viability when compared to the control SG-AdV infection and the S7-AdV infection, whereas SI-AdV has an approximate 7 % increase in

cell viability. This could possibly indicate a neuroprotective role of Ric3 against adenoviral infection although it is more likely that the cells were infected at a lower MOI than intended, due to titration variations.

The detrimental effect of recombinant viral infections were attenuated at a MOI of one with negligible cell death being observed except for the control SG-AdV infection which reduced neuronal viability by approximately 16 % (Fig. 4.8B). The S7-AdV recombinant showed similar levels of negligible cell death as SR-AdV, contrary to the earlier proposed hypothesis that the Ric3 protein might be neuroprotective against the viral infection. The increased cellular lethality caused by SG-AdV is once again probably due to variations in titration between the adenoviruses.

Infecting pure neuronal cultures with the recombinant adenoviruses expressing the α7 nAChR and/ or its chaperone protein Ric3 had no neuroprotective effects; on the contrary, upregulation of the α7 nAChR seems to increase the susceptibility of the cells to an excitotoxic glutamate insult. The 10 minute 1 mM glutamate insult killed approximately 10 % of the control cells whereas the decrease in viability of MOI of 100 virally infected cells ranged from 31 % for the control SG-AdV infection to approximately 37 % for the S7-AdV recombinant adenovirus infection (Fig. 4.9A). At a MOI of 1 the decrease in cell viability after the excitotoxic insult of the virally infected cells was insignificant when compared to the glutamate-insulted control groups. For all the adenoviral infected cells, only a slight decrease in viability was observed, except for the S7-AdV infected group. In the latter group a highly significant (approximate 22 %) decrease was observed when compared to the glutamate-insulted control group. This decrease is reflected in the MOI of 100 virally infected culture set where cells infected with S7-AdV expressing the α7

nAChR resulted in an approximate 4 % decrease in cellular viability subsequent to the glutamate, when compared to the other virally infected cultures. It should be noted that the cultures in Figures 4.8B and 4.9B were prepared on poly-D-Lysine-coated glass coverslips – an unsuitable surface for cell attachment which resulted in cell loss during the wash steps and a subsequent increase in the standard errors of the experiments.

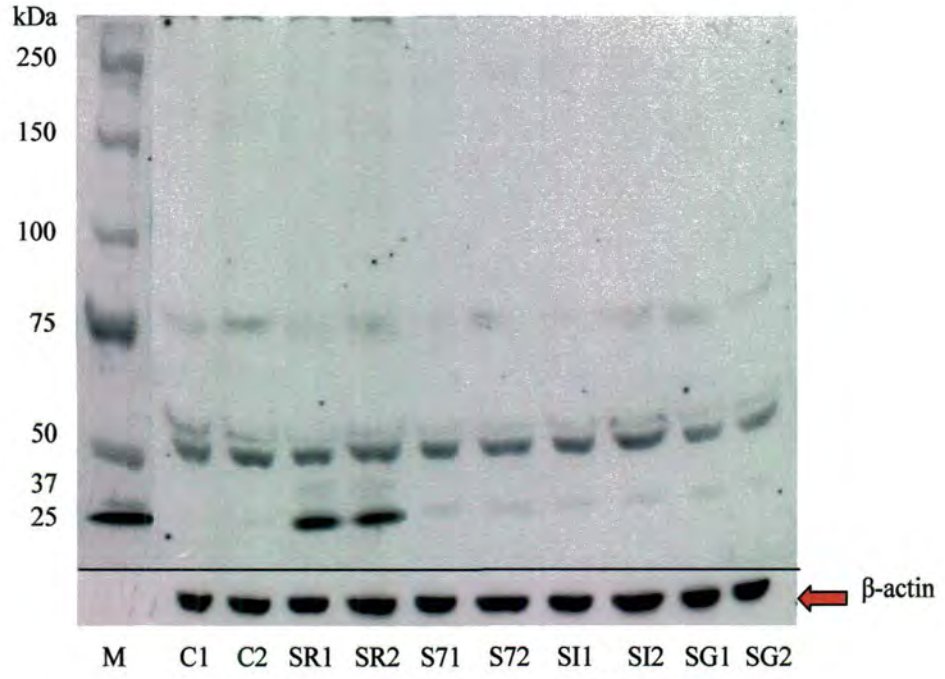


Figure 4.5. Pure neuronal cortical cultures grown in 35 mm dishes at a density of 1500 cells/ mm^2 were infected on DIV 7 with either no virus (C1 and C2), SR-AdV (SR1 and SR2), S7-AdV (S71 and S72), SI-AdV (SI1 and SI2), or SG-AdV (SG1 and SG2). All viral infections occurred at a MOI of 1 and the cultures were harvested on DIV 14 before being analysed on a 6 % SDS polyacrylamide gel and immunostained with the anti-mRic-3 antibody (2 μg/ ml) in conjugation with the anti-rabbit secondary antibody (1 in 2000 dilution). This was followed by immunostaining with a monoclonal anti-β-actin antibody (1 in 2000 dilution) in combination with an anti-mouse secondary antibody to normalize the amounts of protein loaded unto the gel. M represents the molecular weight marker used (Precision Plus Protein™ Standards, Bio-Rad).

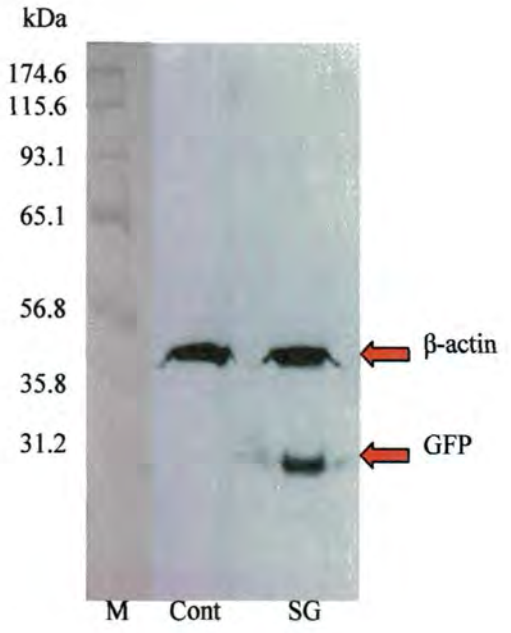


Figure 4.6. Pure neuronal cortical cultures grown in 35 mm dishes at a density of 1500 cells/ mm^2 were infected on DIV 7 with either no virus (C) or SG-AdV (SG). The viral infection occurred at a MOI of 1 and the cultures were harvested on DIV 14 before being analysed on a 6 % SDS polyacrylamide gel and immunostained with the mouse monoclonal anti-GFP antibody (1 in 2000 dilution) (ab1218; Abcam) in conjugation with the anti-mouse secondary antibody (1 in 2000 dilution). This was followed by immunostaining with a monoclonal anti-β-actin antibody (1 in 2000 dilution) in combination with an anti-mouse secondary antibody to normalize the amounts of protein loaded unto the gel. M represents the molecular weight marker used.

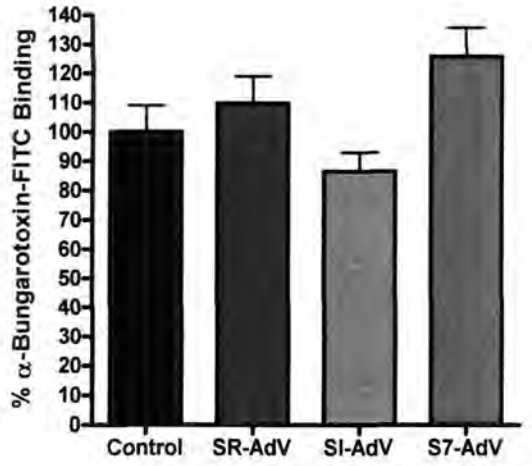
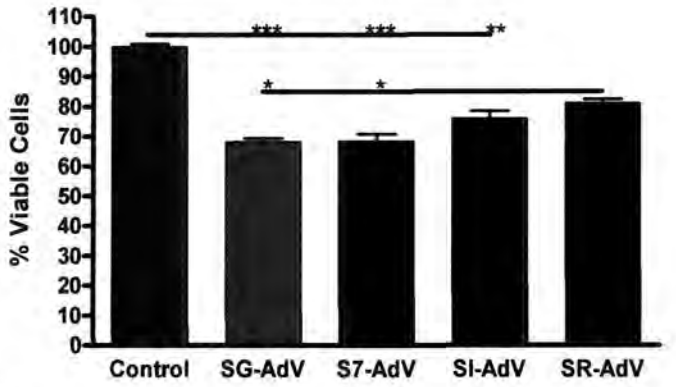


Figure 4.7. DIV 14 pure neuronal cortical cultures grown on glass coverslips were infected on DIV 7 with either no virus for the control, SR-AdV, SI-AdV , or S7-AdV. All viral infections occurred at a MOI of 1 and the cultures were washed on DIV 14 with PBS before being stained with α-Bungarotoxin-FITC. Five digital images were taken of each coverslip and submitted to independent blind counting. The average was calculated for each coverslip, each average representing a single *n*-number. Data are expressed as percentage of controls; the bars represent the mean ±S.E.M. A non-parametric one-way ANOVA statistical analyses was done with $n = 6$.

A)



B)

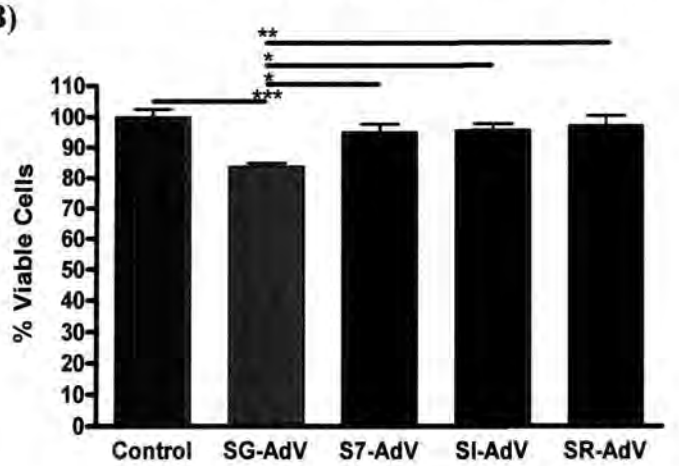


Figure 4.8. A representation of the cell death caused by the various viral infections of pure neuronal cultures infected on DIV 7 for 7 days at either a MOI of 100 (A) or a MOI of 1 (B). Cultures were washed on DIV 14 incubated for 24 hours before MTT readings were taken. The values represent the mean MTT produced/ absorbance. Data are expressed as percentage of controls; the bars represent the mean ±S.E.M from two independent culture experiments. A non-parametric one-way ANOVA statistical analyses was done with significance indicated by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. $n = 12$.

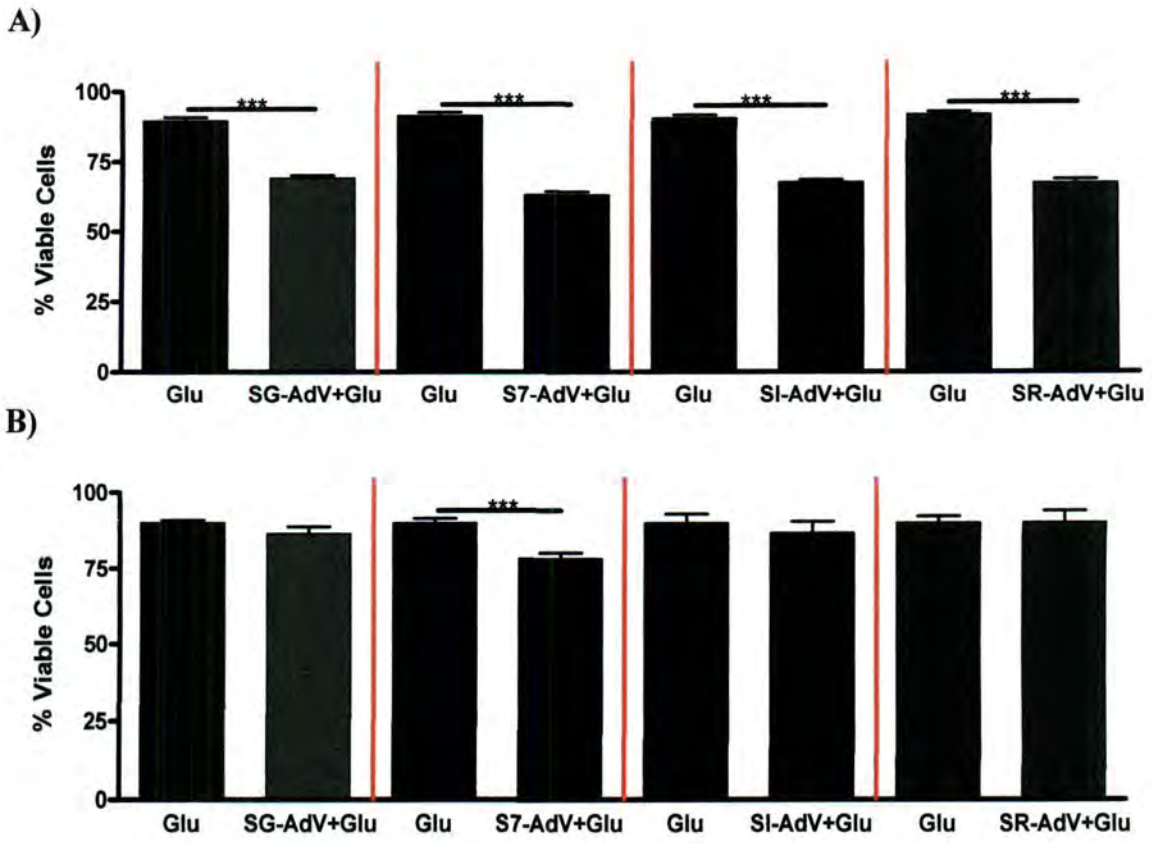


Figure 4.9. Pure neuronal cultures infected on DIV 7 for 7 days at a MOI of 100 (A) or a MOI of 1 (B) were subjected to a 10 minute 1 mM glutamate excitotoxic insult. The cultures received three immediate washes with EBS before being incubated for 24 hour incubation period before the MTT analyses. The glutamate lethal insult group is expressed as a percentage of the control whereas the virally treated group that received a lethal insult is expressed as a percentage of the virally treated cells that did not receive the glutamate insult. A *t*-test was used to compare the two resulting groups with the bars representing the mean ±S.E.M from two independent culture experiments. Significance indicated by *** $P < 0.001$. $n = 12$ except for B) SG-AdV+Glu and S7-AdV+Glu where the data comes from three independent experiments and n = 18.

4.4. Discussion

The aim of this chapter was to create recombinant adenoviral vectors with which the role of alpha 7 nicotinic acetylcholine receptors (α7 nAChRs) in neuroprotection could be studied in isolation, without the interference of additional gene regulation. E1-deleted recombinant adenoviral vectors expressing the mouse α7 nAChR and its function-dependent chaperone protein's gene, *ric3*, were created for the expression of these two genes in neuronal cells. Despite the observation in Chapter 3 that the mouse brain does not express a 25 kDa Ric3 isoform, it was decided to use the truncated mouse sequence identified in that chapter as this was the sequence with the highest homology to the *hric3* gene. In addition, great consideration was given to the Ben-Ami *et al.* (2005) study in which it was shown that the effects of Ric3 on the functional expression and on receptor properties are mediated by the two transmembrane domains and that the variable length random coil domain or coiled-coil domain of the C-terminal is not required.

The probability of infecting a single cell with two different adenoviruses is, however, far lower than infecting a single cell with only one virus. To ensure that all the cells expressing the increased levels of α7 nAChRs have the necessary level of the Ric3 chaperone protein required for a corresponding increase in functional receptor formation, a bicystronic adenoviral construct was also created which expressed both the genes. This construct was made possible by the insertion of an internal ribosome entry site (IRES) between the two proteins, enabling cells to produce the two proteins from a single mRNA transcript. The bicystronic construct is designed to overcome any obstacle of regulation associated with the formation of functional α7 nAChRs. The IRES region has been shown to function in association with the Syn1 promoter (Li *et al.*, 2005) and it has been

noted that the IRES region provides a ten fold decrease in expression of the second gene (*ric3*) than the first (α7 nAChR) of the construct (AAV Helper-Free System Introduction Manual, Stratagene). The recombinant adenoviruses were created from a composite expression cassette containing a neuron-specific 5' human synapsin-1 (Syn1) promoter, as well as a 3' woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) for enhanced expression of the transgenes. A recombinant adenovirus expressing the enhanced green fluorescent protein (EGFP) was used as a control in this study.

Expression of the recombinant adenoviral transgenes were confirmed by exploiting the transient non-specific expression of the Syn1 promoter in HEK 293. Ric3 protein was detected in HEK 293 cells infected with SR-AdV and SI-AdV at 25 kDa. An additional band was detected in cells infected with SR-AdV in the absence of any α7 nAChR protein expression. This protein species of approximately 37 kDa is too small to indicate a Ric3 dimer but might indicate the interaction of Ric3 with another endogenous protein. Co-infection with S7-AdV expressing the α7 nAChR protein leads to the disruption of the putative interaction with the unknown protein, providing evidence for an interaction between the Ric3 and the α7 nAChR protein. In the study by Kato *et al.* (2007), it was found that the γ7 transmembrane AMPA receptor regulatory protein remains associated with various proteins upon electrophoretic analysis. The upregulation of Ric3 upon the SR-AdV infection of pure neuronal cortical cultures once again led to the formation of the 37 kDa protein complex, this time in the presence of the cells' native α7 nAChR protein. The reason for this is possibly due to the high over-expression of the Ric3 protein with the limited native α7 nAChR protein subunits only able to disrupt a part of Ric3's interaction with yet to be identified protein. It would be interesting to identify the

unknown protein by coimmunoprecipitation with the Ric3 protein and proteomic analysis, as this would further the current understanding of the function of Ric3.

Pure neuronal cortical cultures were infected with recombinant adenoviruses expressing either α7 nAChR, Ric3 or both these proteins, in an attempt to study the neuroprotective effect, if any, that their upregulation might have. Transgene expression and gene upregulation in neuronal cells were confirmed using the anti-mRic3 antibody for Ric3 expression and α-Bungarotoxin-FITC binding for the detection of functional α7 nAChR upregulation. A significant increase in Ric3 protein production was detected upon infection with the SR-AdV virus but not for the SI-AdV viral infection. The lack of a detectable Ric3 protein increase for the SI-AdV infection is probably due to the *ric3* gene in this construct being under control of the IRES region, leading to a ten times less expression of this gene than with the SR-AdV construct. Another possibility is that the cultures were infected with a very low number of SI-AdV viral particles due to the inaccuracy of the viral titration technique. This is supported by the lack of an increase in the formation of functional α7 nAChR, as detected by α-Bungarotoxin-FITC binding. As for the S7-AdV infection, a large increase in α-Bungarotoxin-FITC binding was detected, although, due to the blind counting and the necessary cultivation of the neurons on glass coverslips, the increase was insignificant. The trend was, however, repeated in a subsequent repeat of the same experiment (data not shown).

Culture studies were complicated by the choice of the viral transgene promoter, requiring long infection periods to generate sufficient transgene expression, leading to high cell death with increased viral infection. Glover *et al.* (2002) infected primary hippocampal cultures at a maximum MOI of 200 for seven consecutive days while studying the

fluorescence level generated by the inclusion of the WPRE element in the Syn1-EGFP recombinant adenoviral construct. They did not, however, report cellular death associated with such a high adenoviral infection for that period of time, as was noted in this study. Pi *et al.* (2004) infected DIV 12 primary cortical cultures for 24 hours with enough viral particles to infect 95 % of the cells. This concentration of virus led to low levels of cell death. A subsequent 10-fold decrease in viral particles decreased the attained level of transgene expression by 30 % but with no change to virally-induced toxicity being observed. The Cytomegalovirus (CMV) promoter is the popular choice for transgene neuroprotective studies *in vitro* as it requires a short incubation period and low viral infection numbers for the generation of a sufficient quantity of target protein (Tan *et al.*, 2003; Pi *et al.*, 2004; Tsai *et al.*, 2007; Hester *et al.*, 2007). Glover *et al.* (2002) showed that a CMV promoter has approximately 3.5 fold higher expression of the transgene in cultured neurons over the same time period as the Syn1 promoter. The current study utilized the Syn1 promoter due to the use of the recombinant adenoviruses in subsequent *in vivo* experiments (Chapter 5).

For the neuroprotective studies – statistically speaking – the initial effect of adenoviral-induced cell death was nullified by expressing the glutamate lethal insult group as a percentage of the control, and the virally treated group that received a lethal insult as a percentage of the virally treated cells that did not receive the glutamate insult. There seem to be an increase in the susceptibility of virally infected cells to a glutamate insult. It is reasonable to assume that virally infected cells would be more susceptible to a glutamate insult as the cellular survival mechanisms are already strained by the presence of the virus and would be able to cope to a lesser extent with the additional glutamate excitotoxicity than healthy uninfected cells. This is supported by findings that a reduction

in the infective viral particles to cell ratio from 100 to 1 attenuates the level of glutamate-mediated death seen in the adenoviral infected groups. Of interest is tendency for an increase in glutamate excitotoxicity subsequent to the upregulation of functional α7 nAChRs. With a MOI 100 infection approximately 4 % more cells were killed by the glutamate insult in the S7-AdV infected group than in any other virally infected group. This additional vulnerability increases significantly to approximately 22 % at a MOI of 1. The increase in cell death subsequent to α7 nAChR upregulation and glutamate excitotoxicity from a high viral infection of a MOI of 100 to a lower viral infection of a MOI of 1 is due to more cells surviving subsequent to a lower infection, prior to the excitotoxic insult. In Chapter 2 it was observed that contrary to the Chazot *et al.* (2002) study, depolarization with KCl to induce a neuroprotective effect is associated with a decrease in α7 nAChRs. The tendency for cells with more functional α7 nAChRs to have a higher mortality subsequent to a glutamatergic insult complements the *in vitro* depolarization preconditioning model. A future study that might conclude the issue is to use α7 nAChR subunit antisense to down regulate the expression of this receptor followed by excitotoxicity studies to see if any neuroprotection is gained (Brussaard, 1997). Unfortunately this experiment could not be performed due to financial and time constraints.

In conclusion, this chapter discussed the creation and validation of three novel recombinant adenoviruses with which it is possible to upregulate α7 nAChR and/ or Ric3 expression. An investigation was conducted into whether the upregulation of these proteins would lead to neuroprotection against an excitotoxic insult. In contradiction to the prediction made in the Chazot *et al.* (2002) study and complementing results from the

studies performed in Chapter 2, it was found that the upregulation of functional α7 nAChRs leads to an increase in excitotoxic susceptibility of the neuronal cells.

CHAPTER 5: Overall Discussion and Future Direction

The overall aims of this project were to investigate the role of α7 nicotinic acetylcholine receptors (α7 nAChRs) in depolarisation-induced preconditioning *in vitro*, and to develop novel tools and approaches to study α7 receptors *in vivo*. The upregulation of this receptor subunit is putatively reported to play a role in *in vivo* cortical spreading depression-elicited neuroprotection (Chazot *et al.*, 2002). Addressing this concern required the development of a reliable *in vitro* preconditioning model. Initial preconditioning studies with potassium chloride, MK801 and nicotine in mixed neuronal and glial cell cultures were successful yet unreliable due to the heterogeneity of the cultures. Preconditioning in pure neuronal cortical cultures proved to be far more difficult, with the successfully attained effect being less pronounced than that seen with the mixed neuronal and glial cultures. The pure neuronal cortical cultures were, however, far more reliable with the very low variation and high reproducibility leading to relatively low *n*-numbers being required to attain high significance.

Potassium chloride-induced preconditioning in pure neuronal cultures was attained by prolonged exposure of the cells to a low concentration of this agent – an effect that seemed to positively correlate with cellular density and subsequent increased glutamate release. It was confirmed that the induction of neuroprotection in the *in vitro* model follows a similar mechanism to that seen *in vivo*, with tolerance generation being dependent upon cellular glutamate release and NMDA receptor function; blocking of the tolerance generation being possible with the selective NMDA receptor antagonist, MK801. The role of α7 nAChRs in the KCl-induced neuroprotective effect associated with CSD was analysed *in vitro*, first by blocking any possible upregulation of α7 nAChR

mRNA by inhibiting the activation of Ca^{2+}-calmodulin dependent kinase II with KN-62, and second by directly blocking any action of α7 nAChR with the receptor-specific antagonist, α-bungarotoxin. Both these experiments indicated that the activity of this receptor subtype remains essential for the preconditioning mechanism. Quantification of α7 nAChRs *in vitro* with α-Bungarotoxin-FITC showed that, contrary to the findings of Chazot *et al.* (2002) and other various studies in which KCl treatment was shown to increase α7 nAChR protein expression, a decrease in the formation of functional homomeric α7 nAChRs was found subsequent to chronic KCl-induced preconditioning of pure neuronal cortical cultures.

To further investigate changes in functional α7 nAChR formation associated with spreading depression, a study was made of the recently identified obligatory α7 nAChR chaperone protein, Ric3. This required the development of a novel antibody for the identification of the rodent Ric3 protein. Using this novel tool, evidence was discovered for a family of Ric3 related isoforms which are differentially expressed in mouse brain, kidney and lung tissue. A common diffuse species (Mr 50-55 kDa) was found in the mouse and rat brain, together with a prominent Mr 25,000 species in the rat brain, which was expressed at low levels in the adult mouse brain. The larger protein species is likely to be a rodent Ric3 isoform – a potential homolog of the major human isoform. Evidence was also provided for the robust association of Ric3 with an unidentified protein in neuronal and non-neuronal cells. This association was disrupted in the presence of α7 nAChRs, indicting a tripartite regulatory interaction; the identity of this protein is worthy of future study using proteomics or yeast two-hybrid strategies. The Ric3 protein was found to be highly expressed in specific subfields and laminae of the mouse hippocampus and cerebral cortex, respectively. Notable high expression was also found in the thalamic

and hypothalamic paraventricular nuclei, implicating a role in emotional responses to stress. In this thesis, the first evidence was provided for the expression of Ric3 in the choroid plexus non-neuronal epithelial cells, indicating a distinct role for Ric3 independent of α7 nAChRs, possibly for the regulation of 5HT3 receptors. Interestingly, *in vivo* spreading depression increased the expression level of Ric3, although no changes were detected with the *in vitro* KCl-preconditioned pure neuronal cortical cultures. It is postulated that this is due to the simplicity of the culture system, lacking in brain structures (e.g. choroid plexus) and cell types (eg. glia, endothelial cells).

A human Ric3 antibody would be of great value since there has been a recent increased interest in Ric3 expression and receptor regulation during psychiatric disease (Severance and Yolken, 2007). These recent early studies have relied on mRNA analysis, however, it was observed that Ric3 protein expression does not necessarily correspond to mRNA levels. Of importance for future studies is the fact that the amino acid region used for the generation of the rodent Ric3 antibody is conserved within the human homolog of the protein, and, therefore, highly likely to cross-react with the human homolog.

The neuroprotective role, if any, played by the isolated increased expression of either α7 nAChRs, Ric3 or both of these proteins together was studied by the infection of pure neuronal cortical cultures with several newly created recombinant adenoviral vectors. Infection with the adenoviral constructs did not lead to any significant resultant neuroprotection, on the contrary, a tendency for an increase in glutamate excitotoxicity was observed subsequent to the upregulation of functional (calcium-permeable) α7 nAChRs. Taking into account this tendency, the active role played by α7 nAChRs in KCl-induced preconditioning, as well as the decrease in the amount of cell surface α7

nAChRs subsequent to neuroprotective KCl treatment, it would be of interest to study the effect of an artificial decrease in cell surface α7 nAChRs. Such a study would be facilitated by the use of an antisense oligonucleotide sequence targeted to the translational start site of α7 nAChR mRNA (GCA TCA GCG CCC GGA) (Girod *et al.*, 2000). The use of antisense oligonuclotides to induce functional deletion of ligand gated ion channels *in vitro* and *in vivo* has been well documented (Brussaard, 1997). DIV 7 pure neuronal cortical culture medium could be supplemented with antisense oligonuclotide to a final concentration of 20 μM. Controls for the specificity of the antisense oligonucleotides would include the use of sense, missense (scrambled) and mismatch sequences. On DIV 14, a lethal insult can be elicited, with MTT viability assessment the subsequent day. Replenishment of the antisense oligonucleotides may be necessary and might also serve to vary the amount of cell surface α7 nAChRs at any given time, further elucidating the role played by α7 nAChRs in neuroprotection.

One of the main objectives of this study was to develop tools and validated protocols to study the role that non-pharmacological increases in α7 nAChRs and/ or Ric3 play in animal emotional behaviour, subsequent to the anatomical studies reported herein. To date, studies have looked at the behavioral effects of α7 nAChRs knockouts and hyper excitable mutant α7 nAChRs, but no study has looked at the phenotypic repercussions of α7 nAChR upregulation in properly validated animal tests of human anxiety. As stated in the introduction and alluded to in the Ric-3 anatomical study, α7 nAChRs may play a role in anxiety behaviour. In order to perform such studies, a series of suitable novel AdV constructs and behavioural tests were developed. These validated novel adenoviral tools and fully validated behavioural tests (Appendix E) are ideal to assess the effects of α7 and/or Ric3 upregulation upon general activity and anxiety. In fact, the full version of the

behavioural test is ideal to assess, in addition, learning and memory behaviours in the same animal ("all-in-one test" – first of its kind) (Ennaceur *et al.*, 2007; Ennaceur *et al.*, 2008 ; Michalikova *et al.*, 2007).

In conclusion, the antibody and adenoviral vectors created in this project, as well as the presented findings, will form the basis of further novel investigations into the role of α7 nAChRs and its chaperone protein Ric3 in neuroprotection as well as behavioural phenotypes. As is the case with this research, the end of one project paves the way for the beginning of another.

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APPENDICES

Appendix A.1. Complete sequence of pS7-AdV with the α7 nAChR gene highlighted in blue

AATTCATCATCAATAATATACCTTATTTTGGATTGAAGCCAATATGATAATGAGGGGGTGGAGTTTGTGAC
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TGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAA
AAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTT
AAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTT
AAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTAT
CTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGG
AGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCA
GCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTC
TATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTG
CTGCAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGG
CGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAG
TAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCG
TAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGT
TGCTCTTGCCCGGCGTCAACACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGG
AAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTC
GTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAA
AATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTA
TTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAA
TAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTA
ACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTTCAAG

Appendix A.2. CLUSTAL X (1.81) multiple sequence alignment of the α7 nAChR transgene (α7Transg; blue) with its theoretical template.

```
Template    AACATAGTTAAGAATACCAGTCAATCTTTCACAAATTTTGTAATCCAGAGGTTGATTGTC
α7Transg    AACATAGTTAAGAATACCAGTCAATCTTTCACAAATTTTGTAATCCAGAGGTTGATTGTC
            ************************************************************

Template    GACGGATCCTCTAGATGCATGCTCGAGTTAAGCAAAGTCTTTGGACACAGCCTCCACAAA
α7Transg    GACGGATCCTCTAGATGCATGCTCGAGTTAAGCAAAGTCTTTGGACACAGCCTCCACAAA
            ************************************************************

Template    GTTTGGGGCTGACATGAGGATGCCGATGGTGCAGATGATGGTAAAAACCGAAAAGGCCAT
α7Transg    GTTTGGGGCTGACATGAGGATGCCGATGGTGCAGATGATGGTAAAAACCGAAAAGGCCAT
            ************************************************************

Template    GAGGCACAAGCGGTCCACCACACAGGCCGCAAACTTCCACTCACTGCAGATCACCTCACT
α7Transg    GAGGCACAAGCGGTCCACCACACAGGCCGCAAACTTCCACTCACTGCAGATCACCTCACT
            ************************************************************

Template    CTCATCCTGGCAGCGGAAGCGGTTGGCGATGTAGCGGACCTCCTCCAGGATCTTGGCCAG
α7Transg    CTCATCCTGGCAGCGGAAGCGGTTGGCGATGTAGCGGACCTCCTCCAGGATCTTGGCCAG
            ************************************************************

Template    GTCGGGGTCCCCATCAGAGGGGTGTGTACCATGCATGAGGTGCTCATCATGTGTTGGGGA
α7Transg    GTCGGGGTCCCCATCAGAGGGGTGTGTACCATGCATGAGGTGCTCATCATGTGTTGGGGA
            ************************************************************

Template    GCAGGCCAAACGACCACACACGACCCCAGAGTCTGGAGTTGGGGCACAGTGCATGCCCTC
α7Transg    GCAGGCCAAACGACCACACACGACCCCAGAGTCTGGAGTTGGGGCACAGTGCATGCCCTC
            ************************************************************

Template    CAGGCCTCGGAAGCCAATGTAGAGCAGGTTGCCATTGCTGGTGGGTGGCCCCGCACCTGC
α7Transg    CAGGCCTCGGAAGCCAATGTAGAGCAGGTTGCCATTGCTGGTGGGTGGCCCCGCACCTGC
            ************************************************************

Template    GCTCAGCTCCACACTGGCCAGGCTGCAGCGCCGAGGCTTGTGCTGACAAGCCGGCCGCAC
α7Transg    GCTCAGCTCCACACTGGCCAGGCTGCAGCGCCGAGGCTTGTGCTGACAAGCCGGCCGCAC
            ************************************************************

Template    CTTGTCCTCTCCCGGCCTCTTCATGCGCAGAAACCATGCACACCAATTCAGAAGAATGAT
α7Transg    CTTGTCCTCTCCCGGCCTCTTCATGCGCAGAAACCATGCACACCAATTCAGAAGAATGAT
            ************************************************************

Template    CCTGGTCCACTTAGGCATTTTGCCACCATCAGGGTCATGGTGGTGATATCGCAGCACAAT
α7Transg    CCTGGTCCACTTAGGCATTTTGCCACCATCAGGGTCATGGTGGTGATATCGCAGCACAAT
            ************************************************************

Template    CACTGTCACGACCACTGAGAGGCCCACGATGATCATGGTGCTGGCGAAGTACTGTGCTAT
α7Transg    CACTGTCACGACCACTGAGAGGCCCACGATGATCATGGTGCTGGCGAAGTACTGTGCTAT
            ************************************************************

Template    CAAGGGCACGGAATCAGATGTTGCTGGCATGATCTCAGCCACAAGCAGCATGAAGACAGT
α7Transg    CAAGGGCACGGAATCAGATGTTGCTGGCATGATCTCAGCCACAAGCAGCATGAAGACAGT
            ************************************************************

Template    CAGAGAAAGTAAGACAGTTATTCCAAGAGAGATTTTCTCTCCAGAGTCTGCAGGCAGCAA
α7Transg    CAGAGAAAGTAAGACAGTTATTCCAAGAGAGATTTTCTCTCCAGAGTCTGCAGGCAGCAA
            ************************************************************
```

```
Template   GAATACCAGCAAAGCCAGGGCTGAAATGAGCACACAAGGAATGAGCAGGTTGAGGCCATA
α7Transg   GAATACCAGCAAAGCCAGGGCTGAAATGAGCACACAAGGAATGAGCAGGTTGAGGCCATA
           ************************************************************

Template   GTAGAGTGTCCTACGGCGCATGGTTACTGTGTAGGTGACATCTGGGTATGGCTCTTTGCA
α7Transg   GTAGAGTGTCCTACGGCGCATGGTTACTGTGTAGGTGACATCTGGGTATGGCTCTTTGCA
           ************************************************************

Template   GCATTCATAGAACTTCTCATTCCTTTTGCCAGGGATTCCCATGAGATCCCATTCTCCATT
α7Transg   GCATTCATAGAACTTCTCATTCCTTTTGCCAGGGATTCCCATGAGATCCCATTCTCCATT
           ************************************************************

Template   GGGGATATAGCTGCTGATATCTGCCTCTTGCATCTGCAGGTCCAAGGACCACCCTCCATA
α7Transg   GGGGATATAGCTGCTGATATCTGCCTCTTGCATCTGCAGGTCCAAGGACCACCCTCCATA
           ************************************************************

Template   GGACCAGGACCCAAACTTCAGTTTGCACTGCTGCACATCAAAAGGGAACCAGCGTACATC
α7Transg   GGACCAGGACCCAAACTTCAGTTTGCACTGCTGCACATCAAAAGGGAACCAGCGTACATC
           ************************************************************

Template   GATGTAGCAGGAGCTCTTGAATATGCCTGGAGGGAGATACTGGCAATGCCCAGATGCATT
α7Transg   GATGTAGCAGGAGCTCTTGAATATGCCTGGAGGGAGATACTGGCAATGCCCAGATGCATT
           ************************************************************

Template   CACCAAGACGTTGGTGTGGAATGTGGCATCAAAGCGTTCATCTGCACTGTTATAGAGGAG
α7Transg   CACCAAGACGTTGGTGTGGAATGTGGCATCAAAGCGTTCATCTGCACTGTTATAGAGGAG
           ************************************************************

Template   AATGTCTGGTTTCCAAATCTGGCCATCTGGAAAACGAACATTTTTCACTCCGGGGTACTC
α7Transg   AATGTCTGGTTTCCAAATCTGGCCATCTGGAAAACGAACATTTTTCACTCCGGGGTACTC
           ************************************************************

Template   AGACATGTTCCACTGCAAATAGTGATCTGTCCAAGACATTTGTAGCCAAATGTTGGTGGT
α7Transg   AGACATGTTCCACTGCAAATAGTGATCTGTCCAAGACATTTGTAGCCAAATGTTGGTGGT
           ************************************************************

Template   TAAAACTTGGTTCTTCTCATCCACATCCATGATCTGCAGGAGGCTCAGGGAGAAGTACAC
α7Transg   TAAAACTTGGTTCTTCTCATCCACATCCATGATCTGCAGGAGGCTCAGGGAGAAGTACAC
           ************************************************************

Template   GGTGAGCGGCTGCGAGTCGTTGGCCACCGGCCTCTCCAGCGGGTTGTAGTTCTTGACCAG
α7Transg   GGTGAGCGGCTGCGAGTCGTTGGCCACCGGCCTCTCCAGCGGGTTGTAGTTCTTGACCAG
           ************************************************************

Template   CTCCTTGTACAGCCTCCTCTGGAACTCGCCTTGCAGGGACACGTGCAGCAGCGCCGCGGC
α7Transg   CTCCTTGTACAGCCTCCTCTGGAACTCGCCTTGCAGGGACACGTGCAGCAGCGCCGCGGC
           ************************************************************

Template   CAGAGCCAGCCAGATGCCTCCCCGCCGGCCGCACATGGTACCAAGCTCTAGAGTCGACTG
α7Transg   CAGAGCCAGCCAGATGCCTCCCCGCCGGCCGCACATGGTACCAAGCTCTAGAGTCGACTG
           ************************************************************

Template   CGCTCTCAGGCACGACACGACTCCTCCGCTGCCCACCGCAGACTGAGGCAGCGCTGAGTC
α7Transg   CGCTCTCAGGCACGACACGACTCCTCCGCTGCCCACCGCAGACTGAGGCAGCGCTGAGTC
           ************************************************************

Template   GCCGGCGCCGCAGCGCAGATGGTCGCGCCCGTGCCCCCCTATCTCGCGCCTCGCGTGGT
α7Transg   GCCGGCGCCGCAGCGCAGATGGTCGCGCCCGTGCCCCCCTATCTCGCGCCTCGCGTGGT
           ************************************************************
```

Appendix B.1. Complete sequence of pSR-AdV with the *ric3* gene highlighted in red

AATTCATCATCAATAATATACCTTATTTTGGATTGAAGCCAATATGATAATGAGGGGGTGGAGTTTGTGAC
GTGGCGCGGGGCGTGGGAACGGGGCGGGTGACGTAGTAGTGTGGCGGAAGTGTGATGTTGCAAGTGTGGCG
GAACACATGTAAGCGACGGATGTGGCAAAAGTGACGTTTTTGGTGTGCGCCGGTGTACACAGGAAGTGACA
ATTTTCGCGCGGTTTTAGGCGGATGTTGTAGTAAATTTGGGCGTAACCGAGTAAGATTTGGCCATTTTCGC
GGGAAAACTGAATAAGAGGAAGTGAAATCTGAATAATTTTGTGTTACTCATAGCGCGTAATATTTGTCTAG
GGCCGCGGGGACTTTGACCGTTTACGTGGAGACTCGCCCAGGTGTTTTTCTCAGGTGTTTTCCGCGTTCCG
GGTCAAAGTTGGCGTTTTATTATTATAGTCAGTCTAGCTGGTTCCATAGAGCCCACCGCATCCCCAGCATG
CCTGCTATTGTCTTCCCAATCCTCCCCCTTGCTGTCCTGCCCCACCCCACCCCCCAGAATAGAATGACACC
TACTCAGACAATGCGATGCAATTTCCTCATTTTATTAGGAAAGGACAGTGGGAGTGGCACCTTCCAGGGTC
AAGGAAGGCACGGGGGAGGGGCAAACAACAGATGGCTGGCAACTAGAAGGCACAGTCGAGGCTGATCGTAC
CGAGCTCGAATTCCAGGCGGGGAGGCGGCCCAAAGGGAGATCCGACTCGTCTGAGGGCGAAGGCGAAGACG
CGGAAGAGGCCGCAGAGCCGGCAGCAGGCCGCGGGAAGGAAGGTCCGCTGGATTGAGGGCCGAAGGGACGT
AGCAGAAGGACGTCCCGCGCAGAATCCAGGTGGCAACACAGGCGAGCAGCCATGGAAAGGACGTCAGCTTC
CCCGACAACACCACGGAATTGTCAGTGCCCAACAGCCGAGCCCCTGTCCAGCAGCGGGCAAGGCAGGCGGC
GATGAGTTCCGCCGTGGCAATAGGGAGGGGGAAAGCGAAAGTCCCGGAAAGGAGCTGACAGGTGGTGGCAA
TGCCCCAACCAGTGGGGGTTGCGTCAGCAAACACAGTGCACACCACGCCACGTTGCCTGACAACGGGCCAC
AACTCCTCATAAAGAGACAGCAACCAGGATTTATACAAGGAGGAGAAAATGAAAGCCATACGGGAAGCAAT
AGCATGATACAAAGGCATTAAAGCAGCGTATCCACATAGCGTAAAAGGAGCAACATAGTTAAGAATACCAG
TCAATCTTTCACAAATTTTGTAATCCAGAGGTTGATTGTCGACGGATCCTCTAGATGCATGCTCGAGCTAA
CAAGGAAGGGGCATTTTACCTTCCCAGTCCTCCATGTATGGGGCTTCCTCAGCTTCCTTCTCTGGAGATGT
GTCGATGAACTTGCCTTCTTTCATGACCCTGGTGATTTCTCGGAGCTGATGCAGTAATCGTTTCTCTTGGT
CAGAAGTCACAGCCTGTGCTCTGCTCTCACCATTAGGTCCAACTCTGTTGATTAATTTTTCCATGGCTTCT
TCTGTCTCTTTTAGTTTTTCTTGTAGTTGAACAAGCTCAAAGTTGGTAATCTTCCTGTGGGCGTTTCCAGG
TGGGGCAGTGGAGCAGTTCCGATCCTCTGCAGTTTTCCCCTTTGAAAGCTTAAACAAAATGTACAGTATGT
ACAAAAAGATCCCAAAGCCATAGATTGGAATGATCTGGCCCATCAGTCCTCTTCCACTACCCCCTCCTCCA
GCACCTCCACCTGCTCCCTTGGCCTTTGCAAAGGCCTCTGCAAGGTGAGACCTCTGGAAACGAGCCCCTGG
TGTCTGACCATCTGAGGGTGCCGAGTGGTGATGCATCATAGGTGGAAATCGGTCCAATTTTCCTTCCGGGC
CCGGCGGCGGCTCCGGTCGCTTCCCGCGAGACAAGAAGGCCTTGGGCAGCAGCAGCGACACGGCCAGGACG
AGCCCCGAGGCCAGCGCCACTCTCTGCACCGTGGAGTACGCCATACTAGTGGATCCGAGCTCGGTACCAAG
CTCTAGAGTCGACTGCGCTCTCAGGCACGACACGACTCCTCCGCTGCCCACCGCAGACTGAGGCAGCGCTG
AGTCGCCGGCGCCGCAGCGCAGATGGTCGCGCCCGTGCCCCCCTATCTCGCGCCTCGCGTGGTGCGGTCCG
GCTGGGCCGGCGGCGGCGCGGACGCGACCAAGGTGGCCGGGAAGGGGAGTTTGCGGGGGACCGGCGAGTGA
CGTCAGCGCGCCTTCAGTGCTGAGGCGGCGGTGGCGCGCGCCGCCAGGCGGGGGCGAAGGCACTGTCCGCG
GTGCTGAAGCTGGCAGTGCGCACGCGCCTCGCCGCATCCTGTTTCCCCTCCCCCTCTCTGATAGGGGATGC
GCAATTTGGGGAATGGGGGTTGGGTGCTTGTCCAGTGGGTCGGGGTCGGTCGTCAGGTAGGCACCCCCACC
CCGCCTCATCCTGGTCCTAAAACCCACTTGCACTCATACGCAGGGCCCTCTGCAGCTGGCGCCATCGATAC
GCGTATATCTGGCCCGTACATCGCTAGANGATCTGGAAGGTGCTGAGGTACGATGAGACCCGCACCAGGTG
CAGACCCTGCGAGTGTGGCGGTAAACATATTAGGAACCAGCCTGTGATGCTGGATGTGACCGAGGAGCTGA
GGCCCGATCACTTGGTGCTGGCCTGCACCCGCGCTGAGTTTGGCTCTAGCGATGAAGATACAGATTGAGGT
ACTGAAATGTGTGGGCGTGGCTTAAGGGTGGGAAAGAATATATAAGGTGGGGGTCTTATGTAGTTTTGTAT
CTGTTTTGCAGCAGCCGCCGCCGCCATGAGCACCAACTCGTTTGATGGAAGCATTGTGAGCTCATATTTGA
CAACGCGCATGCCCCCATGGGCCGGGGTGCGTCAGAATGTGATGGGCTCCAGCATTGATGGTCGCCCCGTC
CTGCCCGCAAACTCTACTACCTTGACCTACGAGACCGTGTCTGGAACGCCGTTGGAGACTGCAGCCTCCGC
CGCCGCTTCAGCCGCTGCAGCCACCGCCCGCGGGATTGTGACTGACTTTGCTTTCCTGAGCCCGCTTGCAA
GCAGTGCAGCTTCCCGTTCATCCGCCCGCGATGACAAGTTGACGGCTCTTTTGGCACAATTGGATTCTTTG
ACCCGGGAACTTAATGTCGTTTCTCAGCAGCTGTTGGATCTGCGCCAGCAGGTTTCTGCCCTGAAGGCTTC
CTCCCCTCCCAATGCGGTTTAAAACATAAATAAAAAACCAGACTCTGTTTGGATTTGGATCAAGCAAGTGT
CTTGCTGTCTTTATTTAGGGGTTTTGCGCGCGCGGTAGGCCCGGGACCAGCGGTCTCGGTCGTTGAGGGTC
CTGTGTATTTTTTCCAGGACGTGGTAAAGGTGACTCTGGATGTTCAGATACATGGGCATAAGCCCGTCTCT
GGGGTGGAGGTAGCACCACTGCAGAGCTTCATGCTGCGGGGTGGTGTTGTAGATGATCCAGTCGTAGCAGG
AGCGCTGGGCGTGGTGCCTAAAAATGTCTTTCAGTAGCAAGCTGATTGCCAGGGGCAGGCCCTTGGTGTAA
GTGTTTACAAAGCGGTTAAGCTGGGATGGGTGCATACGTGGGGATATGAGATGCATCTTGGACTGTATTTT
TAGGTTGGCTATGTTCCCAGCCATATCCCTCCGGGGATTCATGTTGTGCAGAACCACCAGCACAGTGTATC
CGGTGCACTTGGGAAATTTGTCATGTAGCTTAGAAGGAAATGCGTGGAAGAACTTGGAGACGCCCTTGTGA
CCTCCAAGATTTTCCATGCATTCGTCCATAATGATGGCAATGGGCCCACGGGCGGCGGCCTGGGCGAAGAT
ATTTCTGGGATCACTAACGTCATAGTTGTGTTCCAGGATGAGATCGTCATAGGCCATTTTTACAAAGCGCG

GGCGGAGGGTGCCAGACTGCGGTATAATGGTTCCATCCGGCCCAGGGGCGTAGTTACCCTCACAGATTTGC
ATTTCCCACGCTTTGAGTTCAGATGGGGGGATCATGTCTACCTGCGGGGCGATGAAGAAAACGGTTTCCGG
GGTAGGGGAGATCAGCTGGGAAGAAAGCAGGTTCCTGAGCAGCTGCGACTTACCGCAGCCGGTGGGCCCGT
AAATCACACCTATTACCGGGTGCAACTGGTAGTTAAGAGAGCTGCAGCTGCCGTCATCCCTGAGCAGGGGG
GCCACTTCGTTAAGCATGTCCCTGACTCGCATGTTTTCCCTGACCAAATCCGCCAGAAGGCGCTCGCCGCC
CAGCGATAGCAGTTCTTGCAAGGAAGCAAAGTTTTTCAACGGTTTGAGACCGTCCGCCGTAGGCATGCTTT
TGAGCGTTTGACCAAGCAGTTCCAGGCGGTCCCACAGCTCGGTCACCTGCTCTACGGCATCTCGATCCAGC
ATATCTCCTCGTTTCGCGGGTTGGGGCGGCTTTCGCTGTACGGCAGTAGTCGGTGCTCGTCCAGACGGGCC
AGGGTCATGTCTTTCCACGGGCGCAGGGTCCTCGTCAGCGTAGTCTGGGTCACGGTGAAGGGGTGCGCTCC
GGGCTGCGCGCTGGCCAGGGTGCGCTTGAGGCTGGTCCTGCTGGTGCTGAAGCGCTGCCGGTCTTCGCCCT
GCGCGTCGGCCAGGTAGCATTTGACCATGGTGTCATAGTCCAGCCCCTCCGCGGCGTGGCCCTTGGCGCGC
AGCTTGCCCTTGGAGGAGGCGCCGCACGAGGGGCAGTGCAGACTTTTGAGGGCGTAGAGCTTGGGCGCGAG
AAATACCGATTCCGGGGAGTAGGCATCCGCGCCGCAGGCCCCGCAGACGGTCTCGCATTCCACGAGCCAGG
TGAGCTCTGGCCGTTCGGGGTCAAAAACCAGGTTTCCCCCATGCTTTTTGATGCGTTTCTTACCTCTGGTT
TCCATGAGCCGGTGTCCACGCTCGGTGACGAAAAGGCTGTCCGTGTCCCCGTATACAGACTTGAGAGGCCT
GTCCTCGACCGATGCCCTTGAGAGCCTTCAACCCAGTCAGCTCCTTCCGGTGGGCGCGGGGCATGACTATC
GTCGCCGCACTTATGACTGTCTTCTTTATCATGCAACTCGTAGGACAGGTGCCGGCAGCGCTCTGGGTCAT
TTTCGGCGAGGACCGCTTTCGCTGGAGCGCGACGATGATCGGCCTGTCGCTTGCGGTATTCGGAATCTTGC
ACGCCCTCGCTCAAGCCTTCGTCACTGGTCCCGCCACCAAACGTTTCGGCGAGAAGCAGGCCATTATCGCC
GGCATGGCGGCCGACGCGCTGGGCTACGTCTTGCTGGCGTTCGCGACGCGAGGCTGGATGGCCTTCCCCAT
TATGATTCTTCTCGCTTCCGGCGGCATCGGGATGCCCGCGTTGCAGGCCATGCTGTCCAGGCAGGTAGATG
ACGACCATCAGGGACAGCTTCAAGGATCGCTCGCGGCTCTTACCAGCCTAACTTCGATCACTGGACCGCTG
ATCGTCACGGCGATTTATGCCGCCTCGGCGAGCACATGGAACGGGTTGGCATGGATTGTAGGCGCCGCCCT
ATACCTTGTCTGCCTCCCCGCGTTGCGTCGCGGTGCATGGAGCCGGGCCACCTCGACCTGAATGGAAGCCG
GCGGCACCTCGCTAACGGATTCACCACTCCAAGAATTGGAGCCAATCAATTCTTGCGGAGAACTGTGAATG
CGCAAACCAACCCTTGGCAGAACATATCCATCGCGTCCGCCATCTCCAGCAGCCGCACGCGGCGCATCTCG
GGCAGCGTTGGGTCCTGGCCACGGGTGCGCATGATCGTGCTCCTGTCGTTGAGGACCCGGCTAGGCTGGCG
GGGTTGCCTTACTGGTTAGCAGAATGAATCACCGATACGCGAGCGAACGTGAAGCGACTGCTGCTGCAAAA
CGTCTGCGACCTGAGCAACAACATGAATGGTCTTCGGTTTCCGTGTTTCGTAAAGTCTGGAAACGCGGAAG
TCAGCGCCCTGCACCATTATGTTCCGGATCTGCATCGCAGGATGCTGCTGGCTACCCTGTGGAACACCTAC
ATCTGTATTAACGAAGCGCTGGCATTGACCCTGAGTGATTTTTCTCTGGTCCCGCCGCATCCATACCGCCA
GTTGTTTACCCTCACAACGTTCCAGTAACCGGGCATGTTCATCATCAGTAACCCGTATCGTGAGCATCCTC
TCTCGTTTCATCGGTATCATTACCCCCATGAACAGAAATCCCCCTTACACGGAGGCATCAGTGACCAAACA
GGAAAAAACCGCCCTTAACATGGCCCGCTTTATCAGAAGCCAGACATTAACGCTTCTGGAGAAACTCAACG
AGCTGGACGCGGATGAACAGGCAGACATCTGTGAATCGCTTCACGACCACGCTGATGAGCTTTACCGCAGC
TGCCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTG
TCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCG
CAGCCATGACCCAGTCACGTAGCGATAGCGGAGTGTATACTGGCTTAACTATGCGGCATCAGAGCAGATTG
TACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGC
TCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTC
AAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGC
AAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCAT
CACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCC
TGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTT
CGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAG
CTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTC
CAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATG
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TGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGC
TGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTT
TGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTA
TCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGA
GTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTT
CATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGT
GCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAG
GGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTA
GAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTGCAGGCATCGTGGTGTCACGC
TCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTT
GTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCAC
TCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGT
GAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAACACG

GGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAAC
TCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCA
TCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAG
GGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATT
GTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCC
CGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCAC
GAGGCCCTTTCGTCTTCAAG

Appendix B.2. CLUSTAL X (1.81) multiple sequence alignment of the *ric3* transgene (*ric*Trans; red) with its theoretical template.

```
Template    AACATAGTTAAGAATACCAGTCAATCTTTCACAAATTTTGTAATCCAGAGGTTGATTGTC
ricTrans    AACATAGTTAAGAATACCAGTCAATCTTTCACAAATTTTGTAATCCAGAGGTTGATTGTC
            ************************************************************

Template    GACGGATCCTCTAGATGCATGCTCGAGCTAACAAGGAAGGGGCATTTTACCTTCCCAGTC
ricTrans    GACGGATCCTCTAGATGCATGCTCGAGCTAACAAGGAAGGGGCATTTTACCTTCCCAGTC
            ************************************************************

Template    CTCCATGTATGGGGCTTCCTCAGCTTCCTTCTCTGGAGATGTGTCGATGAACTTGCCTTC
ricTrans    CTCCATGTATGGGGCTTCCTCAGCTTCCTTCTCTGGAGATGTGTCGATGAACTTGCCTTC
            ************************************************************

Template    TTTCATGACCCTGGTGATTTCTCGGAGCTGATGCAGTAATCGTTTCTCTTGGTCAGAAGT
ricTrans    TTTCATGACCCTGGTGATTTCTCGGAGCTGATGCAGTAATCGTTTCTCTTGGTCAGAAGT
            ************************************************************

Template    CACAGCCTGTGCTCTGCTCTCACCATTAGGTCCAACTCTGTTGATTAATTTTTCCATGGC
ricTrans    CACAGCCTGTGCTCTGCTCTCACCATTAGGTCCAACTCTGTTGATTAATTTTTCCATGGC
            ************************************************************

Template    TTCTTCTGTCTCTTTTAGTTTTTCTTGTAGTTGAACAAGCTCAAAGTTGGTAATCTTCCT
ricTrans    TTCTTCTGTCTCTTTTAGTTTTTCTTGTAGTTGAACAAGCTCAAAGTTGGTAATCTTCCT
            ************************************************************

Template    GTGGGCGTTTCCAGGTGGGGCAGTGGAGCAGTTCCGATCCTCTGCAGTTTTCCCCTTTGA
ricTrans    GTGGGCGTTTCCAGGTGGGGCAGTGGAGCAGTTCCGATCCTCTGCAGTTTTCCCCTTTGA
            ************************************************************

Template    AAGCTTAAACAAAATGTACAGTATGTACAAAAAGATCCCAAAGCCATAGATTGGAATGAT
ricTrans    AAGCTTAAACAAAATGTACAGTATGTACAAAAAGATCCCAAAGCCATAGATTGGAATGAT
            ************************************************************

Template    CTGGCCCATCAGTCCTCTTCCACTACCCCCTCCTCCAGCACCTCCACCTGCTCCCTTGGC
ricTrans    CTGGCCCATCAGTCCTCTTCCACTACCCCCTCCTCCAGCACCTCCACCTGCTCCCTTGGC
            ************************************************************

Template    CTTTGCAAAGGCCTCTGCAAGGTGAGACCTCTGGAAACGAGCCCCTGGTGTCTGACCATC
ricTrans    CTTTGCAAAGGCCTCTGCAAGGTGAGACCTCTGGAAACGAGCCCCTGGTGTCTGACCATC
            ************************************************************

Template    TGAGGGTGCCGAGTGGTGATGCATCATAGGTGGAAATCGGTCCAATTTTCCTTCCGGGCC
ricTrans    TGAGGGTGCCGAGTGGTGATGCATCATAGGTGGAAATCGGTCCAATTTTCCTTCCGGGCC
            ************************************************************

Template    CGGCGGCGGCTCCGGTCGCTTCCCGCGAGACAAGAAGGCCTTGGGCAGCAGCAGCGACAC
ricTrans    CGGCGGCGGCTCCGGTCGCTTCCCGCGAGACAAGAAGGCCTTGGGCAGCAGCAGCGACAC
            ************************************************************

Template    GGCCAGGACGAGCCCCGAGGCCAGCGCCACTCTCTGCACCGTGGAGTACGCCATACTAGT
ricTrans    GGCCAGGACGAGCCCCGAGGCCAGCGCCACTCTCTGCACCGTGGAGTACGCCATACTAGT
            ************************************************************

Template    GGATCCGAGCTCGGTACCAAGCTCTAGAGTCGACTGCGCTCTCAGGCACGACACGACTCC
ricTrans    GGATCCGAGCTCGGTACCAAGCTCTAGAGTCGACTGCGCTCTCAGGCACGACACGACTCC
            ************************************************************
```

Appendix C.1. Complete sequence of pSI-AdV with the α7 nAChR gene highlighted in blue, the *ric3* gene in red and the IRES region in green.

AATTCATCATCAATAATATACCTTATTTTGGATTGAAGCCAATATGATAATGAGGGGGTGGAGTTTGTGAC
GTGGCGCGGGGCGTGGGAACGGGGCGGGTGACGTAGTAGTGTGGCGGAAGTGTGATGTTGCAAGTGTGGCG
GAACACATGTAAGCGACGGATGTGGCAAAAGTGACGTTTTTGGTGTGCGCCGGTGTACACAGGAAGTGACA
ATTTTCGCGCGGTTTTAGGCGGATGTTGTAGTAAATTTGGGCGTAACCGAGTAAGATTTGGCCATTTTCGC
GGGAAAACTGAATAAGAGGAAGTGAAATCTGAATAATTTTGTGTTACTCATAGCGCGTAATATTTGTCTAG
GGCCGCGGGGACTTTGACCGTTTACGTGGAGACTCGCCCAGGTGTTTTTCTCAGGTGTTTTCCGCGTTCCG
GGTCAAAGTTGGCGTTTTATTATTATAGTCAGTCTAGCTGGTTCCATAGAGCCCACCGCATCCCCAGCATG
CCTGCTATTGTCTTCCCAATCCTCCCCCTTGCTGTCCTGCCCCACCCCACCCCCCAGAATAGAATGACACC
TACTCAGACAATGCGATGCAATTTCCTCATTTTATTAGGAAAGGACAGTGGGAGTGGCACCTTCCAGGGTC
AAGGAAGGCACGGGGGAGGGGCAAACAACAGATGGCTGGCAACTAGAAGGCACAGTCGAGGCTGATCGTAC
CGAGCTCGAATTCCAGGCGGGGAGGCGGCCCAAAGGGAGATCCGACTCGTCTGAGGGCGAAGGCGAAGACG
CGGAAGAGGCCGCAGAGCCGGCAGCAGGCCGCGGGAAGGAAGGTCCGCTGGATTGAGGGCCGAAGGGACGT
AGCAGAAGGACGTCCCGCGCAGAATCCAGGTGGCAACACAGGCGAGCAGCCATGGAAAGGACGTCAGCTTC
CCCGACAACACCACGGAATTGTCAGTGCCCAACAGCCGAGCCCCTGTCCAGCAGCGGGCAAGGCAGGCGGC
GATGAGTTCCGCCGTGGCAATAGGGAGGGGGAAAGCGAAAGTCCCGGAAAGGAGCTGACAGGTGGTGGCAA
TGCCCCAACCAGTGGGGGTTGCGTCAGCAAACACAGTGCACACCACGCCACGTTGCCTGACAACGGGCCAC
AACTCCTCATAAAGAGACAGCAACCAGGATTTATACAAGGAGGAGAAAATGAAAGCCATACGGGAAGCAAT
AGCATGATACAAAGGCATTAAAGCAGCGTATCCACATAGCGTAAAAGGAGCAACATAGTTAAGAATACCAG
TCAATCTTTCACAAATTTTGTAATCCAGAGGTTGATTGTCGACGGATCCCTAACAAGGAAGGGGCATTTTA
CCTTCCCAGTCCTCCATGTATGGGGCTTCCTCAGCTTCCTTCTCTGGAGATGTGTCGATGAACTTGCCTTC
TTTCATGACCCTGGTGATTTCTCGGAGCTGATGCAGTAATCGTTTCTCTTGGTCAGAAGTCACAGCCTGTG
CTCTGCTCTCACCATTAGGTCCAACTCTGTTGATTAATTTTTCCATGGCTTCTTCTGTCTCTTTTAGTTTT
TCTTGTAGTTGAACAAGCTCAAAGTTGGTAATCTTCCTGTGGGCGTTTCCAGGTGGGGCAGTGGAGCAGTT
CCGATCCTCTGCAGTTTTCCCCTTTGAAAGCTTAAACAAAATGTACAGTATGTACAAAAAGATCCCAAAGC
CATAGATTGGAATGATCTGGCCCATCAGTCCTCTTCCACTACCCCCTCCTCCAGCACCTCCACCTGCTCCC
TTGGCCTTTGCAAAGGCCTCTGCAAGGTGAGACCTCTGGAAACGAGCCCCTGGTGTCTGACCATCTGAGGG
TGCCGAGTGGTGATGCATCATAGGTGGAAATCGGTCCAATTTTCCTTCCGGGCCCGGCGGCGGCTCCGGTC
GCTTCCCGCGAGACAAGAAGGCCTTGGGCAGCAGCAGCGACACGGCCAGGACGAGCCCCGAGGCCAGCGCC
ACTCTCTGCACCGTGGAGTACGCCATGAATTCGGTTGTGGCCATTATCATCGTGTTTTTCAAAGGAAAACC
ACGTCCCCGTGGTTCGGGGGGCCTAGACGTTTTTTAACCTCGACTAAACACATGTAAAGCATGTGCACCGA
GGCCCCAGATCAGATCCCATACAATGGGGTACCTTCTGGGCATCCTTCAGCCCCTTGTTGAATACGCTTGA
GGAGAGCCATTTGACTCTTTCCACAACTATCCAACTCACAACGTGGCACTGGGGTTGTGCCGCCTTTGCAG
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ACCTTGCATTCCTTTGGCGAGAGGGGAAAGACCCCTAGGAATGCTCGTCAAGAAGACAGGGCCAGGTTTCC
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TCCAAGCGGCTTCGGCCAGTAACGTTAGGGGGGGGGGAGGGAGAGGGGCTCGAGTTAAGCAAAGTCTTTGG
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AGGGGTGTGTACCATGCATGAGGTGCTCATCATGTGTTGGGGAGCAGGCCAAACGACCACACACGACCCCA
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GGTGGGTGGCCCCGCACCTGCGCTCAGCTCCACACTGGCCAGGCTGCAGCGCCGAGGCTTGTGCTGACAAG
CCGGCCGCACCTTGTCCTCTCCCGGCCTCTTCATGCGCAGAAACCATGCACACCAATTCAGAAGAATGATC
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TTCTCTCCAGAGTCTGCAGGCAGCAAGAATACCAGCAAAGCCAGGGCTGAAATGAGCACACAAGGAATGAG
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CTCGCCTTGCAGGGACACGTGCAGCAGCGCCGCGGCCAGAGCCAGCCAGATGCCTCCCCGCCGGCCGCACA
TGGTACCAAGCTCTAGAGTCGACTGCGCTCTCAGGCACGACACGACTCCTCCGCTGCCCACCGCAGACTGA
GGCAGCGCTGAGTCGCCGGCGCCGCAGCGCAGATGGTCGCGCCCGTGCCCCCCTATCTCGCGCCTCGCGTG
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TAGGGGATGCGCAATTTGGGGAATGGGGGTTGGGTGCTTGTCCAGTGGGTCGGGGTCGGTCGTCAGGTAGG
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CCATCGATACGCGTATATCTGGCCCGTACATCGCTAGANGATCTGGAAGGTGCTGAGGTACGATGAGACCC
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GGATTCTTTGACCCGGGAACTTAATGTCGTTTCTCAGCAGCTGTTGGATCTGCGCCAGCAGGTTTCTGCCC
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CGGTTTCCGGGGTAGGGGAGATCAGCTGGGAAGAAAGCAGGTTCCTGAGCAGCTGCGACTTACCGCAGCCG
GTGGGCCCGTAAATCACACCTATTACCGGGTGCAACTGGTAGTTAAGAGAGCTGCAGCTGCCGTCATCCCT
GAGCAGGGGGGCCACTTCGTTAAGCATGTCCCTGACTCGCATGTTTTCCCTGACCAAATCCGCCAGAAGGC
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GGCATGCTTTTGAGCGTTTGACCAAGCAGTTCCAGGCGGTCCCACAGCTCGGTCACCTGCTCTACGGCATC
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CAGACGGGCCAGGGTCATGTCTTTCCACGGGCGCAGGGTCCTCGTCAGCGTAGTCTGGGTCACGGTGAAGG
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CATTATCGCCGGCATGGCGGCCGACGCGCTGGGCTACGTCTTGCTGGCGTTCGCGACGCGAGGCTGGATGG
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CAGGTAGATGACGACCATCAGGGACAGCTTCAAGGATCGCTCGCGGCTCTTACCAGCCTAACTTCGATCAC
TGGACCGCTGATCGTCACGGCGATTTATGCCGCCTCGGCGAGCACATGGAACGGGTTGGCATGGATTGTAG
GCGCCGCCCTATACCTTGTCTGCCTCCCCGCGTTGCGTCGCGGTGCATGGAGCCGGGCCACCTCGACCTGA
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GCGCATCTCGGGCAGCGTTGGGTCCTGGCCACGGGTGCGCATGATCGTGCTCCTGTCGTTGAGGACCCGGC
TAGGCTGGCGGGGTTGCCTTACTGGTTAGCAGAATGAATCACCGATACGCGAGCGAACGTGAAGCGACTGC
TGCTGCAAAACGTCTGCGACCTGAGCAACAACATGAATGGTCTTCGGTTTCCGTGTTTCGTAAAGTCTGGA
AACGCGGAAGTCAGCGCCCTGCACCATTATGTTCCGGATCTGCATCGCAGGATGCTGCTGGCTACCCTGTG
GAACACCTACATCTGTATTAACGAAGCGCTGGCATTGACCCTGAGTGATTTTTCTCTGGTCCCGCCGCATC

CATACCGCCAGTTGTTTACCCTCACAACGTTCCAGTAACCGGGCATGTTCATCATCAGTAACCCGTATCGT
GAGCATCCTCTCTCGTTTCATCGGTATCATTACCCCCATGAACAGAAATCCCCCTTACACGGAGGCATCAG
TGACCAAACAGGAAAAAACCGCCCTTAACATGGCCCGCTTTATCAGAAGCCAGACATTAACGCTTCTGGAG
AAACTCAACGAGCTGGACGCGGATGAACAGGCAGACATCTGTGAATCGCTTCACGACCACGCTGATGAGCT
TTACCGCAGCTGCCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGG
TCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGG
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GAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCG
CATCAGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTAT
CAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCA
AAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCC
TGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGG
CGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCC
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TCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATC
GTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGA
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AAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAA
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CATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAA
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CTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATC
TGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGC
CAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGC
CGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTGCAGGCATCGT
GGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGAT
CCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCA
GTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTC
TGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGG
CGTCAACACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCG
GGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTG
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CAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCG
CACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATA
GGCGTATCACGAGGCCCTTTCGTCTTCAAG

Appendix C.2. CLUSTAL X (1.81) multiple sequence alignment of the α7 nAChR gene (blue), IRES region (green) and *ric3* (red) transgenes (7IRTrans) with its theoretical template.

```
Template    AACATAGTTAAGAATACCAGTCAATCTTTCACAAATTTTGTAATCCAGAGGTTGATTGTC
7IRTrans    AACATAGTTAAGAATACCAGTCAATCTTTCACAAATTTTGTAATCCAGAGGTTGATTGTC
            ************************************************************

Template    GACGGATCCCTAACAAGGAAGGGGCATTTTACCTTCCCAGTCCTCCATGTATGGGGCTTC
7IRTrans    GACGGATCCCTAACAAGGAAGGGGCATTTTACCTTCCCAGTCCTCCATGTATGGGGCTTC
            ************************************************************

Template    CTCAGCTTCCTTCTCTGGAGATGTGTCGATGAACTTGCCTTCTTTCATGACCCTGGTGAT
7IRTrans    CTCAGCTTCCTTCTCTGGAGATGTGTCGATGAACTTGCCTTCTTTCATGACCCTGGTGAT
            ************************************************************

Template    TTCTCGGAGCTGATGCAGTAATCGTTTCTCTTGGTCAGAAGTCACAGCCTGTGCTCTGCT
7IRTrans    TTCTCGGAGCTGATGCAGTAATCGTTTCTCTTGGTCAGAAGTCACAGCCTGTGCTCTGCT
            ************************************************************

Template    CTCACCATTAGGTCCAACTCTGTTGATTAATTTTTCCATGGCTTCTTCTGTCTCTTTTAG
7IRTrans    CTCACCATTAGGTCCAACTCTGTTGATTAATTTTTCCATGGCTTCTTCTGTCTCTTTTAG
            ************************************************************

Template    TTTTTCTTGTAGTTGAACAAGCTCAAAGTTGGTAATCTTCCTGTGGGCGTTTCCAGGTGG
7IRTrans    TTTTTCTTGTAGTTGAACAAGCTCAAAGTTGGTAATCTTCCTGTGGGCGTTTCCAGGTGG
            ************************************************************

Template    GGCAGTGGAGCAGTTCCGATCCTCTGCAGTTTTCCCCTTTGAAAGCTTAAACAAAATGTA
7IRTrans    GGCAGTGGAGCAGTTCCGATCCTCTGCAGTTTTCCCCTTTGAAAGCTTAAACAAAATGTA
            ************************************************************

Template    CAGTATGTACAAAAAGATCCCAAAGCCATAGATTGGAATGATCTGGCCCATCAGTCCTCT
7IRTrans    CAGTATGTACAAAAAGATCCCAAAGCCATAGATTGGAATGATCTGGCCCATCAGTCCTCT
            ************************************************************

Template    TCCACTACCCCCTCCTCCAGCACCTCCACCTGCTCCCTTGGCCTTTGCAAAGGCCTCTGC
7IRTrans    TCCACTACCCCCTCCTCCAGCACCTCCACCTGCTCCCTTGGCCTTTGCAAAGGCCTCTGC
            ************************************************************

Template    AAGGTGAGACCTCTGGAAACGAGCCCCTGGTGTCTGACCATCTGAGGGTGCCGAGTGGTG
7IRTrans    AAGGTGAGACCTCTGGAAACGAGCCCCTGGTGTCTGACCATCTGAGGGTGCCGAGTGGTG
            ************************************************************

Template    ATGCATCATAGGTGGAAATCGGTCCAATTTTCCTTCCGGGCCCGGCGGCGGCTCCGGTCG
7IRTrans    ATGCATCATAGGTGGAAATCGGTCCAATTTTCCTTCCGGGCCCGGCGGCGGCTCCGGTCG
            ************************************************************

Template    CTTCCCGCGAGACAAGAAGGCCTTGGGCAGCAGCAGCGACACGGCCAGGACGAGCCCCGA
7IRTrans    CTTCCCGCGAGACAAGAAGGCCTTGGGCAGCAGCAGCGACACGGCCAGGACGAGCCCCGA
            ************************************************************

Template    GGCCAGCGCCACTCTCTGCACCGTGGAGTACGCCATGAATTCGGTTGTGGCCATTATCAT
7IRTrans    GGCCAGCGCCACTCTCTGCACCGTGGAGTACGCCATGAATTCGGTTGTGGCCATTATCAT
            ************************************************************
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Template    CGTGTTTTTCAAAGGAAAACCACGTCCCCGTGGTTCGGGGGGCCTAGACGTTTTTTAACC
7IRTrans    CGTGTTTTTCAAAGGAAAACCACGTCCCCGTGGTTCGGGGGGCCTAGACGTTTTTTAACC
            ************************************************************

Template    TCGACTAAACACATGTAAAGCATGTGCACCGAGGCCCCAGATCAGATCCCATACAATGGG
7IRTrans    TCGACTAAACACATGTAAAGCATGTGCACCGAGGCCCCAGATCAGATCCCATACAATGGG
            ************************************************************

Template    GTACCTTCTGGGCATCCTTCAGCCCCTTGTTGAATACGCTTGAGGAGAGCCATTTGACTC
7IRTrans    GTACCTTCTGGGCATCCTTCAGCCCCTTGTTGAATACGCTTGAGGAGAGCCATTTGACTC
            ************************************************************

Template    TTTCCACAACTATCCAACTCACAACGTGGCACTGGGGTTGTGCCGCCTTTGCAGGTGTAT
7IRTrans    TTTCCACAACTATCCAACTCACAACGTGGCACTGGGGTTGTGCCGCCTTTGCAGGTGTAT
            ************************************************************

Template    CTTATACACGTGGCTTTTGGCCGCAGAGGCACCTGTCGCCAGGTGGGGGGTTCCGCTGCC
7IRTrans    CTTATACACGTGGCTTTTGGCCGCAGAGGCACCTGTCGCCAGGTGGGGGGTTCCGCTGCC
            ************************************************************

Template    TGCAAAGGGTCGCTACAGACGTTGTTTGTCTTCAAGAAGCTTCCAGAGGAACTGCTTCCT
7IRTrans    TGCAAAGGGTCGCTACAGACGTTGTTTGTCTTCAAGAAGCTTCCAGAGGAACTGCTTCCT
            ************************************************************

Template    TCACGACATTCAACAGACCTTGCATTCCTTTGGCGAGAGGGGAAAGACCCCTAGGAATGC
7IRTrans    TCACGACATTCAACAGACCTTGCATTCCTTTGGCGAGAGGGGAAAGACCCCTAGGAATGC
            ************************************************************

Template    TCGTCAAGAAGACAGGGCCAGGTTTCCGGGCCCTCACATTGCCAAAAGACGGCAATATGG
7IRTrans    TCGTCAAGAAGACAGGGCCAGGTTTCCGGGCCCTCACATTGCCAAAAGACGGCAATATGG
            ************************************************************

Template    TGGAAAATAACATATAGACAAACGCACACCGGCCTTATTCCAAGCGGCTTCGGCCAGTAA
7IRTrans    TGGAAAATAACATATAGACAAACGCACACCGGCCTTATTCCAAGCGGCTTCGGCCAGTAA
            ************************************************************

Template    CGTTAGGGGGGGGGGAGGGAGAGGGGCTCGAGTTAAGCAAAGTCTTTGGACACAGCCTCC
7IRTrans    CGTTAGGGGGGGGGGAGGGAGAGGGGCTCGAGTTAAGCAAAGTCTTTGGACACAGCCTCC
            ************************************************************

Template    ACAAAGTTTGGGGCTGACATGAGGATGCCGATGGTGCAGATGATGGTAAAAACCGAAAAG
7IRTrans    ACAAAGTTTGGGGCTGACATGAGGATGCCGATGGTGCAGATGATGGTAAAAACCGAAAAG
            ************************************************************

Template    GCCATGAGGCACAAGCGGTCCACCACACAGGCCGCAAACTTCCACTCACTGCAGATCACC
7IRTrans    GCCATGAGGCACAAGCGGTCCACCACACAGGCCGCAAACTTCCACTCACTGCAGATCACC
            ************************************************************

Template    TCACTCTCATCCTGGCAGCGGAAGCGGTTGGCGATGTAGCGGACCTCCTCCAGGATCTTG
7IRTrans    TCACTCTCATCCTGGCAGCGGAAGCGGTTGGCGATGTAGCGGACCTCCTCCAGGATCTTG
            ************************************************************

Template    GCCAGGTCGGGGTCCCCATCAGAGGGGTGTGTACCATGCATGAGGTGCTCATCATGTGTT
7IRTrans    GCCAGGTCGGGGTCCCCATCAGAGGGGTGTGTACCATGCATGAGGTGCTCATCATGTGTT
            ************************************************************

Template    GGGGAGCAGGCCAAACGACCACACACGACCCCAGAGTCTGGAGTTGGGGCACAGTGCATG
7IRTrans    GGGGAGCAGGCCAAACGACCACACACGACCCCAGAGTCTGGAGTTGGGGCACAGTGCATG
            ************************************************************

Template    CCCTCCAGGCCTCGGAAGCCAATGTAGAGCAGGTTGCCATTGCTGGTGGGTGGCCCCGCA
```

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7IRTrans    CCCTCCAGGCCTCGGAAGCCAATGTAGAGCAGGTTGCCATTGCTGGTGGGTGGCCCCGCA
            ************************************************************

Template    CCTGCGCTCAGCTCCACACTGGCCAGGCTGCAGCGCCGAGGCTTGTGCTGACAAGCCGGC
7IRTrans    CCTGCGCTCAGCTCCACACTGGCCAGGCTGCAGCGCCGAGGCTTGTGCTGACAAGCCGGC
            ************************************************************

Template    CGCACCTTGTCCTCTCCCGGCCTCTTCATGCGCAGAAACCATGCACACCAATTCAGAAGA
7IRTrans    CGCACCTTGTCCTCTCCCGGCCTCTTCATGCGCAGAAACCATGCACACCAATTCAGAAGA
            ************************************************************

Template    ATGATCCTGGTCCACTTAGGCATTTTGCCACCATCAGGGTCATGGTGGTGATATCGCAGC
7IRTrans    ATGATCCTGGTCCACTTAGGCATTTTGCCACCATCAGGGTCATGGTGGTGATATCGCAGC
            ************************************************************

Template    ACAATCACTGTCACGACCACTGAGAGGCCCACGATGATCATGGTGCTGGCGAAGTACTGT
7IRTrans    ACAATCACTGTCACGACCACTGAGAGGCCCACGATGATCATGGTGCTGGCGAAGTACTGT
            ************************************************************

Template    GCTATCAAGGGCACGGAATCAGATGTTGCTGGCATGATCTCAGCCACAAGCAGCATGAAG
7IRTrans    GCTATCAAGGGCACGGAATCAGATGTTGCTGGCATGATCTCAGCCACAAGCAGCATGAAG
            ************************************************************

Template    ACAGTCAGAGAAAGTAAGACAGTTATTCCAAGAGAGATTTTCTCTCCAGAGTCTGCAGGC
7IRTrans    ACAGTCAGAGAAAGTAAGACAGTTATTCCAAGAGAGATTTTCTCTCCAGAGTCTGCAGGC
            ************************************************************

Template    AGCAAGAATACCAGCAAAGCCAGGGCTGAAATGAGCACACAAGGAATGAGCAGGTTGAGG
7IRTrans    AGCAAGAATACCAGCAAAGCCAGGGCTGAAATGAGCACACAAGGAATGAGCAGGTTGAGG
            ************************************************************

Template    CCATAGTAGAGTGTCCTACGGCGCATGGTTACTGTGTAGGTGACATCTGGGTATGGCTCT
7IRTrans    CCATAGTAGAGTGTCCTACGGCGCATGGTTACTGTGTAGGTGACATCTGGGTATGGCTCT
            ************************************************************

Template    TTGCAGCATTCATAGAACTTCTCATTCCTTTTGCCAGGGATTCCCATGAGATCCCATTCT
7IRTrans    TTGCAGCATTCATAGAACTTCTCATTCCTTTTGCCAGGGATTCCCATGAGATCCCATTCT
            ************************************************************

Template    CCATTGGGGATATAGCTGCTGATATCTGCCTCTTGCATCTGCAGGTCCAAGGACCACCCT
7IRTrans    CCATTGGGGATATAGCTGCTGATATCTGCCTCTTGCATCTGCAGGTCCAAGGACCACCCT
            ************************************************************

Template    CCATAGGACCAGGACCCAAACTTCAGTTTGCACTGCTGCACATCAAAAGGGAACCAGCGT
7IRTrans    CCATAGGACCAGGACCCAAACTTCAGTTTGCACTGCTGCACATCAAAAGGGAACCAGCGT
            ************************************************************

Template    ACATCGATGTAGCAGGAGCTCTTGAATATGCCTGGAGGGAGATACTGGCAATGCCCAGAT
7IRTrans    ACATCGATGTAGCAGGAGCTCTTGAATATGCCTGGAGGGAGATACTGGCAATGCCCAGAT
            ************************************************************

Template    GCATTCACCAAGACGTTGGTGTGGAATGTGGCATCAAAGCGTTCATCTGCACTGTTATAG
7IRTrans    GCATTCACCAAGACGTTGGTGTGGAATGTGGCATCAAAGCGTTCATCTGCACTGTTATAG
            ************************************************************

Template    AGGAGAATGTCTGGTTTCCAAATCTGGCCATCTGGAAAACGAACATTTTTCACTCCGGGG
7IRTrans    AGGAGAATGTCTGGTTTCCAAATCTGGCCATCTGGAAAACGAACATTTTTCACTCCGGGG
            ************************************************************

Template    TACTCAGACATGTTCCACTGCAAATAGTGATCTGTCCAAGACATTTGTAGCCAAATGTTG
7IRTrans    TACTCAGACATGTTCCACTGCAAATAGTGATCTGTCCAAGACATTTGTAGCCAAATGTTG
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                ************************************************************

Template        GTGGTTAAAACTTGGTTCTTCTCATCCACATCCATGATCTGCAGGAGGCTCAGGGAGAAG
7IRTrans        GTGGTTAAAACTTGGTTCTTCTCATCCACATCCATGATCTGCAGGAGGCTCAGGGAGAAG
                ************************************************************

Template        TACACGGTGAGCGGCTGCGAGTCGTTGGCCACCGGCCTCTCCAGCGGGTTGTAGTTCTTG
7IRTrans        TACACGGTGAGCGGCTGCGAGTCGTTGGCCACCGGCCTCTCCAGCGGGTTGTAGTTCTTG
                ************************************************************

Template        ACCAGCTCCTTGTACAGCCTCCTCTGGAACTCGCCTTGCAGGGACACGTGCAGCAGCGCC
7IRTrans        ACCAGCTCCTTGTACAGCCTCCTCTGGAACTCGCCTTGCAGGGACACGTGCAGCAGCGCC
                ************************************************************

Template        GCGGCCAGAGCCAGCCAGATGCCTCCCCGCCGGCCGCACATGGTACCAAGCTCTAGAGTC
7IRTrans        GCGGCCAGAGCCAGCCAGATGCCTCCCCGCCGGCCGCACATGGTACCAAGCTCTAGAGTC
                ************************************************************

Template        GACTGCGCTCTCAGGCACGACACGACTCCTCCGCTGCCCACCGCAGACTGAGGCAGCGCT
7IRTrans        GACTGCGCTCTCAGGCACGACACGACTCCTCCGCTGCCCACCGCAGACTGAGGCAGCGCT
                ************************************************************
```

Appendix D. Complete sequence of pSG-AdV with the EGFP gene highlighted green.

AATTCATCATCAATAATATACCTTATTTTGGATTGAAGCCAATATGATAATGAGGGGGTGGAGTTTGTGAC
GTGGCGCGGGGCGTGGGAACGGGGCGGGTGACGTAGTAGTGTGGCGGAAGTGTGATGTTGCAAGTGTGGCG
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GGGAAAACTGAATAAGAGGAAGTGAAATCTGAATAATTTTGTGTTACTCATAGCGCGTAATATTTGTCTAG
GGCCGCGGGGACTTTGACCGTTTACGTGGAGACTCGCCCAGGTGTTTTTCTCAGGTGTTTTCCGCGTTCCG
GGTCAAAGTTGGCGTTTTATTATTATAGTCAGTCTAGCTGGTTCCATAGAGCCCACCGCATCCCCAGCATG
CCTGCTATTGTCTTCCCAATCCTCCCCCTTGCTGTCCTGCCCCACCCCACCCCCCAGAATAGAATGACACC
TACTCAGACAATGCGATGCAATTTCCTCATTTTATTAGGAAAGGACAGTGGGAGTGGCACCTTCCAGGGTC
AAGGAAGGCACGGGGGAGGGGCAAACAACAGATGGCTGGCAACTAGAAGGCACAGTCGAGGCTGATCGTAC
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CGGAAGAGGCCGCAGAGCCGGCAGCAGGCCGCGGGAAGGAAGGTCCGCTGGATTGAGGGCCGAAGGGACGT
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CCCGACAACACCACGGAATTGTCAGTGCCCAACAGCCGAGCCCCTGTCCAGCAGCGGGCAAGGCAGGCGGC
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AACTCCTCATAAAGAGACAGCAACCAGGATTTATACAAGGAGGAGAAAATGAAAGCCATACGGGAAGCAAT
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Research report

Models of anxiety: Responses of mice to novelty and open spaces in a 3D maze

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Abstract

The present report describes the emotional responses of different strains of mice to exposure to a novel open space model of anxiety using a 3D spatial navigation task. The 3D maze is modification of the radial maze with flexible arms that can be raised above or lowered below the horizontal level of a central platform. To access the arms animals need to cross a bridge linking the arms to the central platform. In this model, mice are exposed to novelty in an unfamiliar open space setting with no safe alternative. Fear from novelty is compounded with the need to explore. The drive to escape and the drive to approach are intermingled making this open space model radically different from the current models of anxiety which provide animals with the choice between safe and anxiogenic spaces.

In a series of experiments, we examined the behaviour of different groups of mice from C57, C3H, CD1 and Balb/c strains. In the first experiment, different groups of C57 mice were tested in one of the three arms configurations. In the second experiment, C57 mice were compared to C3H mice. In the third experiment, C57 mice were compared to CD1 and Balb/c mice in the raised arm configuration over three successive sessions. In the fourth experiment, we examined the behaviour of C57 mice in the lowered arm configuration with an open and an enclosed central. In the final experiment, we examined the difference between C57 and C3H mice of both genders.

Using several spatio-temporal parameters of the transition responses between central platform, bridges and arms, we have been able to show consistent results demonstrating significant differences between C57 and C3H mice, and between Balb/c and both C57 and CD1 mice. C3H appear more anxious than C57 mice, and Balb/c mice seem more anxious than C57 and CD1 mice. We also observed significant differences between sexes in C3H mice but not in C57 mice. C3H male mice appear more anxious than C3H female mice and than both C57 male and female mice. In the lowered arm configuration with an enclosed central platform, C57 mice took longer time to make a first entry to an arm, made more visits to bridges before first entry to an arm and required longer time between re-entries to arms, spent longer time on the central platform and shorter time on arms compared to mice in the other arm configurations. They also made frequent entries to the centre and bridges compared to mice in the lowered arm with an open central platform.

These results demonstrate not only the sensitivity of the parameters of the test but also the consistencies and concordances of the results which make this 3D maze a valuable new tool in the study of the underlying neural mechanisms of anxiety responses in addition to learning and memory, and in assessing the effects of potential anxiolytic drugs. In this report we examine methodological issues related to the design of animal behavioural paradigms and question the value and the construct validity of the current models of human anxiety.

Keywords: Neophobia; Habituation; Tolerance; Benzodiazepines; GABAA; Amygdala; Hippocampus; Sex; Rats

1. Introduction

In everyday life, humans are faced with challenges that are often anxiogenic such as sitting for exams, interviews, sport competitions and public oral communications. Some spatial settings also have been proved to be anxiogenic, such as unfamiliar enclosed or large spaces and elevated surfaces. While most subjects would feel the need to face challenges and overcome their anxiety and fear, some may be more fearful and attempt to avoid the challenge or escape. Fear is an emotional response which can be induced by exposure to novelty and can be expressed through escape, avoidance or anxiety responses. Escape and avoidance

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responses should not be confused with anxiety responses. In the former, the subject is not and/or does not feel compelled to confront the threatening stimuli and there is a safe alternative to choose from while in the latter, the subject is and/or feels obliged to confront real or a potential threat either immediately or in the near future and there is no obvious safe alternative option to escape to Gray and McNaughton wrote that there is "a distinction between pure fear and anxiety. In pure fear, defence is simple and requires only escape or active avoidance controlled by the fight/flight/freeze system. In anxiety, defence is more complicated and fear is *compounded* with a tendency to approach the source of potential threat—producing an approach-avoidance conflict" [106].

Fear is a common feature and an indissociable component of escape, avoidance and anxiety. What makes escape and avoidance distinct from anxiety is that in the latter, fear is compounded with the need to approach the source of threat. This means that the drive to escape or avoid and the drive to explore are intermingled. They are expressed simultaneously, in combination; they are not separate.

A wide range of behavioural tests have been developed to assess anxiety [27,40,59,57,74,104,115,116,152,167,258,265], they are all based on two alternative choices and are claimed to induce an approach-avoidance conflict in animals. This conflict is seen to be induced by the presence of open spaces alongside enclosed or protected spaces in the elevated T-maze, plus-maze and zero-maze and, in the light–dark box and the open-field [28,51,73,137,180,170,265]. It seems to us that this assumption is based on the juxtaposition of the physical static state of contrasting elements present in the outside world (open versus enclosed, light versus dark and white versus black) to a dynamic state of mind of animals which would mirror this contrast through approach and avoidance responses. It is not clear how animals that are mostly escaping to protected spaces or mostly avoiding open spaces can be described as expressing the drive or need to explore anxiogenic spaces. Furthermore, it is not clear whether animals experience anxiety when engaging in an open space, when retracting in an enclosed space or when moving anywhere in the apparatus. The use of these two-choice models does not discriminate between fear inducing escape or avoidance responses from fear resulting in anxiety responses. In fact, reports in the literature seem to suggest that escape and avoidance responses reflect some forms of anxiety and are used indiscriminately [17,33,36,35,46,49,51,56,76, 103,129,160,187,203,212,215,219,221,229,166]. In our view, this association of escape and avoidance responses of open spaces to an anxious state is possible when a subject feels that it will or may be forced to confront the source of threat in the near future. This prospective emotional response has yet to be explored in animals.

This confusion between escape, avoidance and anxiety responses is further exacerbated by another confusion currently rife amongst many scientists between animal models of human behaviour and animal models of human psychopathologies or neurological disorders [6,25,33,37,38,69,70,93,130,152, 180,195,264]. This confusion arises from the perceived need for animal behaviourists to mimic largely questionable clinical assessments of psychological and neurological pathologies [18,138,158,197,217,218,274,262]. The current models of anxiety are expected to produce symptoms and determine the aetiology of these symptoms in normal animals that are challenged with anxiolytic as well as anxiogenic compounds [6,38,45,52, 62,78,97,116,117,128,130,132,133,136,152,157,162,184,189, 195,214,216,220,254]. The pharmacological validity of the current anxiety tests imply that the purpose of a behavioural test is to provide symptoms for benzodiazepines and SSRI to act upon. Apparently, without benzodiazepines and SSRIs there would be no valid test of anxiety.

This confusion between escape, avoidance and anxiety responses and between animal models of human behaviour and animal models of human psychopathologies or neurological disorders account for the inconsistent and contradictory results observed with the current models of anxiety. Because of this confusion, it is not surprising that various physiological and pharmacological data have been accumulated supporting a wide range of theories which are unfortunately based on divergent results. It is not clear up to now what the elevated T-maze, plus-maze, the open-field, the light–dark box are really measuring. Which behavioural measurement is supposed to reflect or measure anxiety without being confused with other forms of behaviour, affective state and cognitive processes? [84].

The entries in open arms, in a lit box or in the centre of the field may induce anxiety but they do not represent the natural behaviour expressed by animals when exposed to these experimental settings. The spontaneous responses of animals in these settings are avoidance or escape. Most of these tests rely mainly on the use of anxiolytic drugs obtained from clinical studies to increase the number of entries and duration of entries in open spaces [41,47,49,51,58,69,103,107,116, 172,190,195,212,244,247,257]. Amphetamine also increases the number of entries in the open spaces of these experimental settings [67,264]. However, because of its known stimulant effects it sparked a debate over the value of open-arm entries and it was, thus, denied any potential anxiolytic properties [67,69]. Is the definition of benzodiazepines any less problematical than amphetamine in terms of their well known cognitive effects? Moreover, there have been no objections raised to the introduction of SSRI as if anxiety is just another variant of depression [9,80,94,111,169,175,177,178,185,226,224,228,275].

Studies based on the current models of anxiety are constrained by the limitation of clinical methods that is imposed on them. Clinical studies are biased by their reliance on verbal communication. The tests used for the assessment of anxiety and affective states are based on questionnaires and the scoring of patients' answers [101,231,232,243,272]. It is unclear whether results obtained from clinical studies concerning mental states and functions are always accurate and reliable [13,43,71, 158,217,218,252,262]. The positive effect of drugs can be the result of expectancy as patients are told for ethical reasons about the possible outcomes of a treatment [99,230,239,241]. Furthermore, when dealing with human feelings and perception, it is inevitable that words lead to some misinterpretation and confusion; they are and remain subjective measures [3,114]. Following administration of benzodiazepines, a subject may seem

relieved or report being relieved from anxiety but numerous evidence shows that perception and cognitive processes are altered by these drugs [21,44,64,95,151,188,198,230,236,238,268]. Patients under the influence of these drugs are not encoding and processing normally the information from the outside world and might feel detached from it. Therefore, these compounds may be not anxiolytic after all. Clinical evidence is not compelling [12,14,19,178,235,236,158,169,268,267,275]. Furthermore "anxiolytics" and "antidepressants" seem to be interchangeable in both clinical and laboratory settings [6,8,39,41,80,86,92,117,176,207,228,233,236,94,169,175,226, 263]. It is our view that the lack of verbal communication between human and animals in scientific research is a blessing. Behaviour, cognition and emotion are now subjects of laboratory studies in diverse fields of science and have been largely removed from the psychologists' monopoly. Scientists from different disciplines ranging from physics, mathematics, computer modelling, chemistry, molecular biology and genetics are currently involved in the study of behaviour, cognition and emotion. With these numerous inputs from most of the fields of science, animal studies should lead the way to the understanding of human behaviour, the underlying mechanisms and drug discoveries. They do not need to trail behind clinical research to support and confirm its findings as is currently the case. Animal behavioural studies have yet to reveal any drug with potential therapeutic action on depression and anxiety disorders [119,200,201,227]. We do not wish to provide further ammunition for those who devalue the importance of animal studies [194,201] but we feel it is time for an unbiased, objective critical evaluation of the methods and techniques used in behavioural neuroscience. The current anxiolytic compounds did not arise from drug discovery through laboratory experiments but are the result of fortuitous clinical observations and assessments of the effects of drugs that were not specifically designed for anxiety [77,119,200,227]. Chlorpromazine was the result of Laborit fortuitous clinical observations. It was considered as a pre-anaesthetic sedative in 1930 until Laborit in 1950 observed its mood-altering side effects on patients. He reported that it has a calming effect which led to the drug being prescribed as a major antipsychotic drug [72,147,148]. Chlorpromazine which was preceded earlier by anti-histaminergic drugs, widely used in the clinic as CNS depressants at that time, led chemists and clinicians to the discovery of the tricyclic antidepressants (imipramine) and benzodiazepines (Chlordiazepoxide and Diazepam) [146,161,237,245,246,120,200]. Animal studies did not play a major role and animal neuroscientists were since being tunnelled by clinicians to follow their lead. When a drug is attributed anxiolytic properties by clinicians, behavioural neuroscientists are all forcefully tuned to these properties. Drug discovery is initiated and conducted by clinicians. Laboratory neuroscientists are left to a secondary role to account and justify the purpose of a "pharmaceutical" compound. They seem constrained within a "clinically" restricted view of pre-defined action properties of a drug and resigned to accommodate any findings within this view. They try to confirm the existence of these properties and when these properties are not revealed, they barely think to question the clinical methods that led to these drugs. It would be more appropriate and advantageous that animal neuroscientists pursue their investigation of a compound independent from clinical influences. However, this mission cannot be achieved without a thorough methodological validation of animal models of human behaviour.

In order to study anxiety responses as distinct from escape and avoidance responses it is necessary to expose or subject animals to a situation where they would be tempted to explore but cannot escape or avoid. We should view anxiety as one of many forms of emotional responses [16,179,182,222] that might be experienced by animals during a test and which can evolve to maladaptive responses. We propose the use of a 3D maze as an open space model of anxiety. It is an open space as there are no walls around the central platform and the arms, and animals cannot feel protected from potential threat. Animals are introduced to the central platform and are left to explore the spatial settings. They would naturally attempt to explore and adventure in different areas of the maze in search for an escape route.

The 3D maze is a modification of the classic eight-arm radial with arms divided in two segments. The first segment attached to the central platform constitutes a bridge to a flat, a raised or a lowered arm which provides the possibility to run the maze in three spatial configurations (Fig. 1). Animals have to cross a

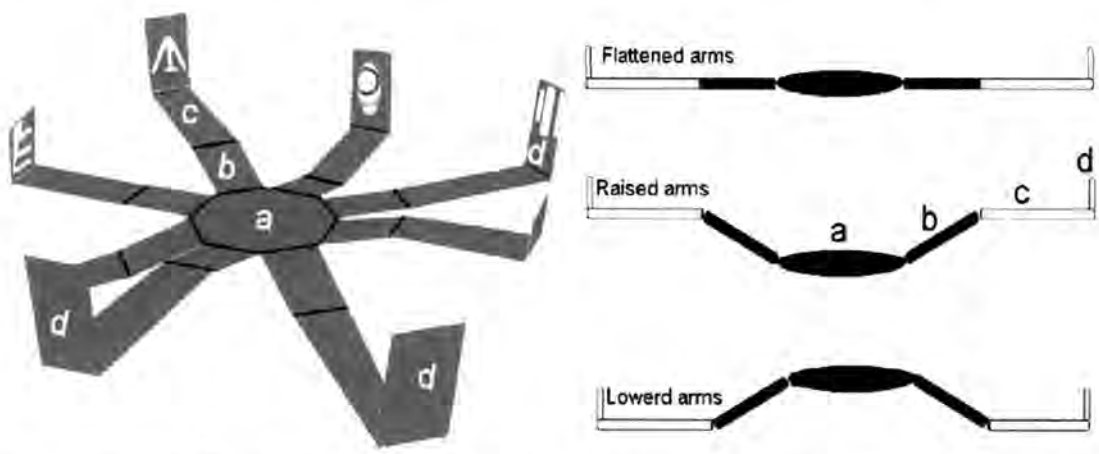

Fig. 1. Schematic representation of the 3D maze. (a) Central platform, (b) ramp for access to arms of the maze, (c) horizontal arm, and (d) panel for displaying visual stimuli.

bridge to reach an arm. A panel is attached to the end of each arm with a display of a distinctive stimulus that can be used as spatial navigation cue. The maze is surrounded with a curtain preventing animals to use external cues. This maze can offer an open space model of anxiety as animals exposed for the first time to a novel and unfamiliar spatial setting are compelled to explore while at the same time may experience fear without an alternative to avoid or escape. Animals that are placed in the central platform for a session of 10–12 min would spend some time exploring the central platform and would attempt to explore the bridges and arms.

Anxiety can be measured by the number of entries to bridges, arms and re-entries to central platform as well as by latencies to and duration in these areas of the maze. In anxious animals, the number of entries to bridges or re-entries to central platform is always higher than the number of entries to arms on the first sessions of exposure to the test. In non-anxious animals and/or with repeated exposures to the test the number of entries to bridges would reduce to equal the number of entries to arms. When less anxious, an animal would engage in a bridge and continue up to the arm linked to that bridge whereas animal that is anxious would make many hesitations before adventuring in an arm. These hesitations are reflected by number of entries to bridges.

Our test is based on the spontaneous exploratory activity of animals and relies on the measure of crossing responses from/to defined areas of the maze—the central platform, bridges and arms. Our analysis of behaviour is therefore based on spatio-temporal parameters which seem unwelcome by some scientists and which are progressively overshadowed by ethological measurements [10,45,47,53,62,112,125,127,135,136, 206,209,213,225,264,223]. These ethological parameters have been introduced recently after repeated failure and frustration reported with the current models of anxiety where results were often inconsistent and sometimes contradictory [24,33,41,69,91,122,172,204,208,260]. They are in fact a global term attributed to independent measures of a variety of responses from animals. They cannot be compared to spatio-temporal parameters which are a set of measures of the same behavioural response. The current use of ethological parameters involves the recording of several variables that are mostly based on subjective appreciations and are subject to non unequivocal interpretations [47,48,109,122,152,205,209,260,271,84]. In addition, the results of these ethological parameters seem insufficient and need to be cross-examined in a battery of behavioural tests [7,42,60,256].

The motive for such unnecessary complexity seems to be a lack of confidence in the primary purpose of the behavioural paradigms that were originally promoted as models of anxiety. The philosophy behind the recording of several variables – each variable may have several associated parameters – is that any of these variables may produce something significant that may cross-link with results from other studies. This strategy is justified by File [90] who wrote that in a free exploratory paradigm "the experimenter has little control over the animal's responses in a test of exploration, it is therefore important that as many different behaviours as possible should be recorded". This strategy is unscientific as the experimenter should define the precise conditions under which a behavioural test takes place and delimits beforehand the variables to assess and/or manipulate. In most current studies of anxiety, a behavioural test in a laboratory experiment involves too many independent variables which remain undefined and there is almost unlimited number of dependent variables that could be measured.

Partisans of ethological validity claims that "unknown behavioural characteristics occurring in genetically modified animals" can be detected with ethological parameters [47,180]. It is puzzling how any behavioural test can be designed to assess undefined behavioural responses that genetic manipulation may 'create'. This claim justifies the eclectic strategy adopted by current scientists to resolve methodological issues that arose from the poor construct validity of their animal models of human behaviour. When dealing with "unknown behavioural characteristics" following physiological, pharmacological or genetic manipulations, the use of a battery of tests becomes justified and standardization becomes the main issue that would promote the debates over contradictions and inconsistencies of results [255,270] and distracts attention from the core problem which is the construct validity of the behavioural tests.

Construct validity is defined as the extent to which a test measures a theoretical construct such as learning, memory, emotion, attention in animal models of human behaviour or such as amnesia, anxiety, depression in animal models of human psycho or neuropathology. It is concerned with the relationship of the measures of the underlying attributes it is attempting to assess. Construct validity includes a wider range of types of validity among which content validity, concurrent validity, convergent and discriminant validity [4,61,96,118,149,192].

In the current behavioural neuroscience studies, a novel behavioural test is often challenged with pharmacological, physiological or genetic manipulations in order to establish its 'construct' validity while very often these manipulations themselves remains still to be validated. This led to inconsistencies and controversies in anxiety studies and some confusion between methodological issues which concern animal models of human behaviour from those concerning animal models of human psycho or neuropathologies (see Section 4).

In the present report, we describe a series of experiments which covers several aspects of the construct validity of our novel animal model of human anxiety. We examined the behaviour of a range of commonly used experimental mice strains including C57, C3H, CD1 and Balb/c strains in the 3D maze. We are opposed to pharmacological validation of a novel animal behavioural test of anxiety as we consider that a behavioural test should be able to stand alone and reveal the functional properties of any test compound (perceived anxiolytic or unknown). A difference between strains of mice is more appropriate for the examination of the sensitivity and the reliability of the measures of a behavioural test. In the first experiment, different groups of C57 mice were tested each in one of the three spatial arm configurations of the maze. The purpose of this experiment is to examine whether these maze configurations would produce comparable emotional responses in animals. In the second experiment, C57 mice were compared to C3H mice.

This experiment would try to confirm the pattern of responses observed in C57 mice in experiment 1. In addition, in lowered arm configuration the bridges were lined with wire mesh to examine whether motor activity would be improved in mice when walking up or down a bridge and if it contributes to anxiety responses. In the third experiment, C57 mice were compared to CD1 and Balb/c mice in the raised arm configuration over three successive sessions. In our laboratory, the 3D maze is used to test memory in mice and rats. We introduce animals directly to a working memory paradigm without the usual habituation procedure. The first sessions of exposure to the 3D maze provide an opportunity to examine animal emotional responses at an early stage of learning and memory processes. In the fourth experiment, a group of C57 were tested in lowered arm configuration with an enclosed central platform and compared to other groups tested in other arm configurations with an open central platform. The purpose of this experiment is to demonstrate that the presence of an enclosed space encourages escape/avoidance responses and that the open space models do not involve such responses. In the final experiment, we examined the difference between C57 and C3H mice of both sexes. This experiment was run on a mixed spatial configuration of the arms which is another option that is offered by the test, but unfortunately there were only eight arms. It would have been more appropriate with an equal number of raised, lowered and flat arms.

The video and data analysis of all experiments were performed at later stage after all experiments were finished. Therefore the design of each experiment was based on some assumptions made from gross observations which were revealed sometimes incorrect such as the selection in experiment 4 of C57 mice which were the least anxious strain.

2. General methods

2.1. Animals

The animal weight was 20–25 g at the time of their arrival. The colony room was held under a 12 h light/12 h dark cycle (light 07:00–19:00 h at 180 lx) and at 23 ± 1 °C. In order to avoid unequal light exposure, the upper shelf was occupied with empty plastic cages filled with sawdust. Mice were housed in a group of five to six mice per cage. Individual mice could be identified by their cage number and their ear tags. Mice were allowed to acclimatize for a month before the start of the experiment. All mice had *ad libitum* access to food and water. During their stay in respective housing conditions, they were removed twice a week from their cages for cleaning the cages and renewing their food and water supply. Animal treatment and husbandry were in accordance with approved use of animals in scientific procedures regulated by the Animals (Scientific Procedures) Act 1986, UK.

2.2. Apparatus

It consists of eight arms radiating from a central platform (Fig. 1). Each arm (51 cm × 11.2 cm) is made from two segments, extended from an octagonal shaped central hub (30 cm in diameter) and can be manipulated independently. The first segment of an arm (15.2 cm × 11.2 cm) directly attached to the central platform can be tilted (max. 90°) and constitutes a bridge that allow access to the second segment (35 cm × 11.2 cm) of the arm which is presented horizontally either at the same, below or above the level of a central platform. Sidewalls, about 1 cm high, extended the length of each arm that is raised 60 cm from the ground. In order to change the configurations of the maze, the central platform is raised or lowered by 15 cm. The bridge forms a slope which is inclined by about 60°. The maze is placed in a sound attenuated room with a masking noise of 70 db above the human thresholds. The end of each arm is extended with panels of identical size (20.2 cm × 11.2 cm). These panels are used for holding intra-maze cues made of distinctive pattern drawings designed on plastic adhesive material and attached to a PVC board (18 cm × 11.2 cm). A small cup (2.5 cm wide) was placed at the end of each arm. The maze is made from grey PVC (5 mm thick).

The maze is totally surrounded with a heavy beige-light coloured curtain. A camera is centred 200 cm above the central platform. The ambient light at the surface of the central platform was 180 lx.

2.3. Tools and recording measures

During the test, mice were observed on a screen monitor connected to a video camera suspended above the test arena. The video record of the experiment is run on a computer screen monitor through a capture card. The live scene is shown alongside a console window (EventLog) that simulates the arena of the box apparatus. The experimenter needs only to press either pre-defined keyboard keys whenever a mouse approaches an object or moves the mouse key on predefined areas of the screen. Since the experiment is videotaped, the experimenter can review the tape and check the accuracy of the recording at any time. In the present experiment, the recording was performed from a video tape played on a VCR. The EventLog program is written in C++ and can be used for many purposes as it requires only visual definition of the layouts of the test apparatus and, the experimenter has only to press the appropriate related key whenever a mouse approaches a predefined area. EventLog provides a record in sequential order of the start and end of each approach, the duration of each approach, and the list of key codes of the predefined area of the test that have been activated. The data record of each mouse can be saved on a text document and, can be exported to any spreadsheet for data analysis. From these records, it is possible to generate a variety of measures such as frequency, latencies between approaches and the sequence order of responses (see section below).

2.3.1. Measurements and statistical analysis

Several measurements were considered:

(1) *Number of bridge and arm entries.* An entry to an arm or a bridge is recorded whenever a mouse crosses with all four paws the line that delimits these areas. The same bridge and the same arm can be visited several times within a session.
(2) *Number of bridge entries before first arm visit.* An entry to a bridge is recorded whenever a mouse crosses with all four paws the line that delimits this area. The same bridge can be visited several times. A mouse that did not enter an arm receives the highest score registered from any mice +1.
(3) *Latency of first entry to a bridge.* The time spent by a mouse in the central platform before it enters for the first time in one of the bridges with all four paws. A mouse that did not enter a bridge receives the highest score which is 10–12 min.
(4) *Latency of first entry to an arm.* The time spent by a mouse in the central platform and bridges before it enters for the first time in one of the arms with all four paws. A mouse that did not enter an arm receives the highest score which is 10–12 min.
(5) *Latency of first entry in the eighth arm.* The time spent by a mouse before entering the last remaining non-visited arm. This should not be confused with eighth choices. Some mice may have visited only a few arms and up to seven arms in a session and may have made many repeated entries to these same arms in a session that lasted 10–12 min, their latency score is 10–12 min.
(6) *Total duration and average duration of bridge entries.* The time is recorded from the entry of a mouse with all four paws in a bridge until it exits to the central platform or to an arm. The average duration is based on the total duration of entries to bridge divided by number of entries to bridges.
(7) *Total duration and average duration of arm entries.* The time is recorded from the entry of a mouse with all four paws in an arm until it exits to the bridge. The average duration is based on the total duration of arm entries divided by number of entries to arms.
(8) *Total duration and average duration of centre re-entries.* The time is recorded from the entry of a mouse with all four paws in the central plat-

form until it exits to a bridge. The average duration is based on the total duration of entries to the centre divided by number of entries to the centre.

(9) *Average number of entries to centre between arm visits.* A mouse can return many times to the centre by visiting the bridges without engaging in an arm. This measure will examine the average number of entries of the centre between consecutive visits to arms.

(10) *Total latencies and average latency between exits from and entries to arms.* The time is recorded when a mouse exits from an arm with all four paws until its next re-entry to an arm (the same or another arm). The average latency is based on the total latencies between exit and entries to arms divided by the number of entries to the centre. A mouse that did not enter an arm receives the highest score which is 10 or 12 min.

(11) *Number of unique arm visits.* In an eight arms radial maze, there can be only eight unique visits maximum in a session. Any arm can be visited more than once within a session that lasted 10 or 12 min and the total number of visits can be higher than eight but some arms may receive no visit. This measure will indicate how many arms were left unvisited and avoided.

(12) *Session duration.* When animals are left in the maze until eight choices were made or 10–12 min elapsed the latency of first entry in the eighth arm do not apply and is replaced here by the measure of the time it took for animals to make eight choices in a session. A choice can be any visit or repeated visit to an arm.

Differences among group means for each of the above measurements were tested for significance with one-way ANOVA in the first two experiments. Two-way ANOVA was performed in the third experiment with strains as variables and sessions as repeated measures. This was followed up with Newman–Keuls post hoc comparisons. We also performed correlation analyses to examine the relations between the different measurements in the three arm maze configurations. Statistics were calculated using the statistical package Statistica for Windows (Version 5.5). Results were considered significant when $p \leq 0.05$. When $p \leq 0.10$, it was reported and rounded up to the nearest value.

2.4. Experiment 1: reaction to novelty and open spaces in C57 mice

Different groups of C57 mice were examined in the 3D maze. Each group was tested in a unique session in one arm configuration only. These mice were exposed for the first time to the apparatus and were not previously food deprived. The purpose of this experiment is to examine the behavioural responses of animals to open spaces and novelty in the different configurations of the maze. It is possible that emotional responses would be best observed in one configuration than another and would justify its selection in future experiments.

2.4.1. Subjects

Thirty C57 male mice supplied by Charles River Laboratories (Kent, UK) were used in the present study.

2.4.2. Testing procedures

Mice were tested in a unique session in one of the three arm maze configurations. The bridges were not covered with wire mesh grid. The order of testing was randomly alternated between batches of five mice from the same cage; each batch of mice being randomly selected for testing in one of the three arm maze configurations. We used three groups of mice ($n = 10$ each). Each group was exposed to one configuration of the maze only, either raised, flat or lowered arms. A mouse is removed from its cage, put in a small bucket in which it is weighted and then poured gently in the centre platform of the maze. It is left in the maze until 12 min elapsed.

2.4.3. Results

2.4.3.1. Number of bridge entries (Fig. 2 (left)). One-way ANOVA revealed significant differences between groups ($F_{2,27} = 13.05, p < 0.0001$). Post hoc comparisons show that the number of entries to bridges is significantly low in lowered arm configurations compared to flattened ($p < 0.0003$) and raised arm ($p < 0.0005$) configurations.

2.4.3.2. Number of arm entries. One-way ANOVA revealed no significant differences between groups ($F_{2,27} = 1.30$, $p > 0.10$) for the number of entries to arms in each arm configurations.

2.4.3.3. Number of bridge entries before first arm visit (Fig. 2 (right)). One-way ANOVA revealed significant differences between groups ($F_{2,27} = 4.73, p < 0.02$). Post hoc comparisons show that the number of entries to bridges is significantly high in flat arm configuration compared to lowered ($p < 0.02$) and raised arm ($p < 0.02$) configurations.

2.4.3.4. Latency of first entry to a bridge (Fig. 3 (left)). One-way ANOVA revealed significant differences between groups ($F_{2,27} = 3.91, p < 0.03$). Post hoc comparisons show that the latency of the first entry to a bridge is significantly low in flat arm configuration compared to lowered ($p < 0.03$) and raised arm ($p < 0.05$) configurations.

2.4.3.5. Latency of first entry to an arm (Fig. 3 (right)). One-way ANOVA revealed no significant differences between groups ($F_{2,27} = 2.90$, $p \leq 0.07$). The latency of first entry to an arm in raised arms appears lower than in flat and lowered arms.

2.4.3.6. Latency of first entry to the eighth arm. One-way ANOVA revealed no significant differences between groups ($F_{2,27} = 1.81$, $p > 0.10$) for the latency to visit the eighth arm of the maze.

2.4.3.7. Average number of entries to centre between arm visits. One-way ANOVA revealed no significant differences between groups ($F_{2,27} = 0.89$, $p > 0.10$) for the number of entries to the centre between arm visits.

2.4.3.8. Total duration and average duration of bridge entries. One-way ANOVA revealed no significant differences between groups ($F_{2,27} = 1.74$,

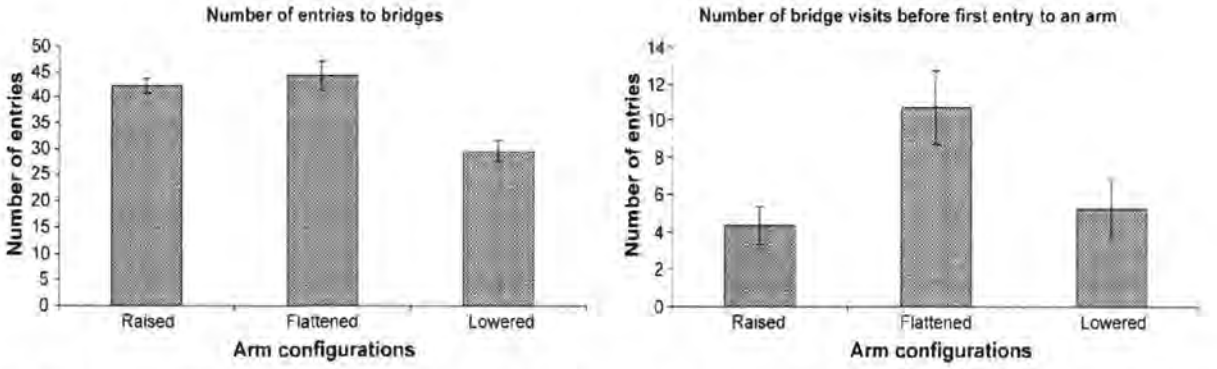

Fig. 2. Number of entries to bridges (left) and number of entries to bridges before first visit to an arm (right) in each maze configuration. See text above for statistic results.

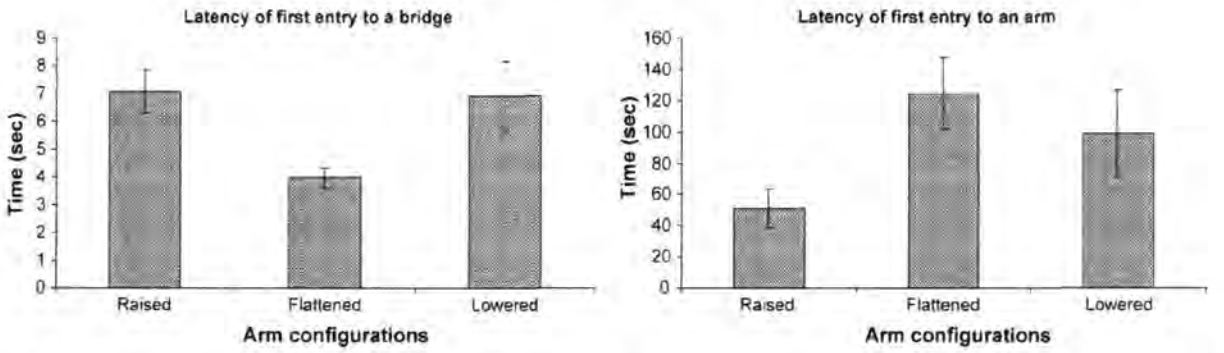

Fig. 3. Latency of first entry to a bridge (left) and latency of first entry to an arm (right) in each maze configuration. See text above for statistic results.

$p > 0.10$) for the total duration of entries to bridges. However, there were significant differences between groups on the average duration of entries ($F_{2,27} = 3.50$, $p < 0.04$). Fig. 4 (left) shows that mice in lowered arms spent significantly more time on bridges than mice in raised ($p < 0.05$) and flattened arms ($p < 0.04$).

2.4.3.9. Total duration and average duration of arm entries. One-way ANOVA revealed no significant differences between groups for the total duration ($F_{2,27} = 0.13$, $p > 0.10$) and average duration ($F_{2,27} = 2.06$, $p > 0.10$) of entries to arms.

2.4.3.10. Total duration and average duration of centre re-entries. One-way ANOVA revealed no significant differences between groups ($F_{2,27} = 1.08$, $p > 0.10$) for the total duration of entries to the centre in each arm configuration. However, there were significant differences between groups on the average duration of entries ($F_{2,27} = 15.18$, $p < 0.0001$). Fig. 4 (right) shows that mice in lowered arms spent significantly more time on the central platform than mice in raised ($p < 0.0002$) and flattened arms ($p < 0.0002$).

2.4.3.11. Total latencies and average latency between exits from and entries to arms. One-way ANOVA revealed no significant differences between groups for the total latencies ($F_{2,27} = 1.89$, $p > 0.10$) and average latency ($F_{2,27} = 0.68$, $p > 0.10$) between exits and entries to arms.

2.4.3.12. Number of unique arm visits. One-way ANOVA revealed no significant differences between groups for the number of unique visits to the arms of the maze ($F_{2,27} = 0.99$, $p > 0.10$).

2.4.4. Summary

C57 mice in the maze configuration with raised arms spent less time in the central platform compared to mice in lowered arm configuration. They took longer time to enter a bridge for the first time compared to flat arm configuration and less time to enter an arm for the first time compared to mice in flat and lowered arm configurations. They also made large number of visits to bridges compared to mice in lowered arm configuration and required fewer bridge entries before first arm visit compared to mice in flat arm configuration.

C57 in the maze configuration with flat arms spent less time in the central platform compared to mice in lowered arm configuration. They took less time to enter a bridge for the first time compared to raised and lowered arm configurations but required more time to enter an arm for the first time compared to raised arm configuration. They also made large number of visits to bridges compared to mice in lowered arm configuration and required high number of bridge entries to first arm visit compared to mice in raised and lowered arm configuration.

C57 in the maze configuration with lowered arms spent more time in the central platform compared to mice in raised and flat arm configurations. They took less time to enter a bridge for the first time compared to flat arm configuration and more time to enter an arm for the first time compared to mice in raised arm configuration. They also made a few number of visits to bridges compared to mice in raised and flat arm configurations and required fewer bridge entries before first arm visit compared to mice in flat arm configuration.

There were no differences between groups in the number of entries to arms and the time spent in the arms, in the latency to enter the eighth arm, in the average number of entries to the centre between arm visits, in the latencies between re-entries to arms as well as in the number of unique visits to arms.

The examination of the different measurements suggests that raised arm configuration provides the most consistent results and this is supported by the correlation analysis (Table 1).

2.5. Experiment 2: reaction to novelty and open spaces in C57 and C3H mice

In the previous experiment, C57 mice made several visits to bridges and arms but these seem less important in the lowered arm configurations than in the raised one but some measures were not significant which might be due to a ceiling performance level in this strain of mice. There are indeed results in the literature suggesting that C57 mice are less emotional than other strains of mice in the T-maze, open-field and light–dark box. These results, however, are not supported by all studies. In this second experiment, we used C57 mice to confirm our results from experiment 1 and to compare and contrast their behaviour to C3H strain in the three configurations of the maze. In addition, we thought that a wire mesh grid on the floor of the bridges may help mice to climb up and

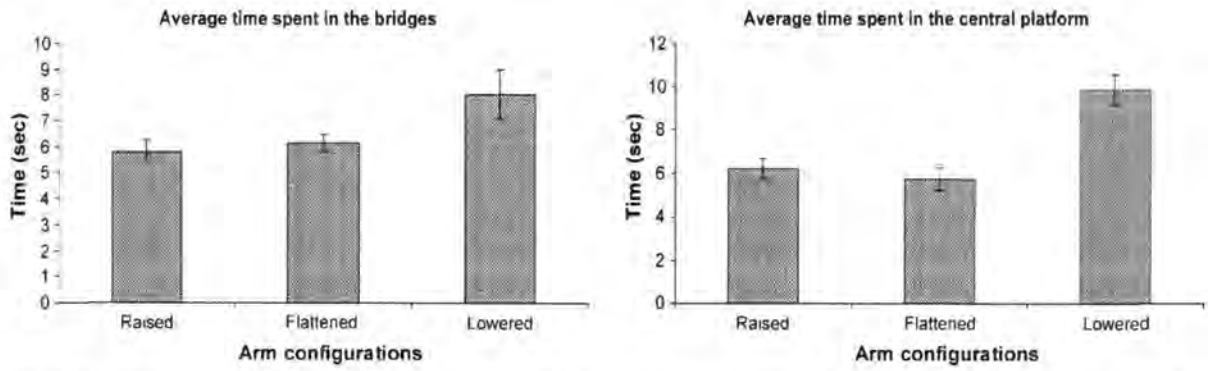

Fig. 4. Average time spent in the bridges (left) and in the central platform (right) in each maze configuration. See text above for statistic results.

return back to the central platform more easily. Animals in several experiments seemed not having any problems walking up and down the bridges except that we observed consistently that in lowered arm configuration mice made less visits to arms. Both in raised and lowered maze configurations the bridges between the central platform and the arms have comparable slopes. In this experiment we would examine whether motor activity would be improved when walking up or down a bridge and if it contributes to our measures of anxiety responses. Therefore we examined a separate group of animals from each strain of mice on lowered arm configuration with the bridges covered with wire mesh in addition to other groups tested separately in the three configurations of the maze without a wire mesh on the bridges.

2.5.1. Subjects

Twenty C57 and 24 C3H male mice supplied by Charles River Laboratories (Kent, UK) were used in the present study.

2.5.2. Testing procedures

Different groups of C57 and C3H mice were examined in the 3D maze. Each group was tested in a unique session in one of the three arm configurations of the maze without wire mesh grid on the floors of the bridges. In addition to these groups, one group of C57 mice and one group of C3H mice were included in the experiment and were tested in lowered maze configuration with a wire mesh grid covering the floor of the bridges. All mice were exposed for the first time to the apparatus and were tested in a unique session in one of these four arm configurations of the maze. They were not previously food deprived. There were five C57 and six C3H mice in each test configuration. They were released by hand on the central platform and were left in the maze until 12 min elapsed.

2.5.3. Results

2.5.3.1. Number of bridge entries (Fig. 5 (left)). One-way ANOVA revealed significant differences between groups ($F_{7,36} = 8.10$, $p < 0.0001$). In C57, the number of bridge entries is comparable between the different arm configurations. In C3H mice, the number of entries in the bridges is significantly high in flattened arms compared to raised arms ($p < 0.04$), to lowered arms ($p < 0.008$) and to lowered arms with wire mesh ($p < 0.04$).

There are also significant differences between C57 and C3H mice on all arm configurations except flattened arms. C57 mice, compared to C3H, made more entries in the bridges of raised arms ($p < 0.002$), lowered arms ($p < 0.001$) and lowered arms with wire mesh ($p < 0.04$).

2.5.3.2. Number of arm entries (Fig. 5 (right)). One-way ANOVA revealed significant differences between groups ($F_{7,36} = 13.92$, $p < 0.0001$). In C57, the number of arms entries is comparable between the different arm configurations. In C3H mice, the number of entries in the arms is significantly high in flattened

Table 1
Correlations between the different measurements of the behaviour of mice in lowered (A), flat (B), raised (C) arms configurations, and of the behaviour of all animals (pooled data) independent of arm configurations (D). *Significant correlations at $p \leq 0.05$ ($n = 16$, $r \geq 0.50$ for **A**, **B**, **C** and $n = 48$, $r \geq 0.29$ for **D**). †Significant correlations at $p \leq 0.10$ ($n = 16$, $r \geq 0.42$ and $r \leq 0.50$ for **A**, **B**, **C** and $n = 48$, $r \geq 0.24$ and $r \leq 0.29$ for **D**). Greyed areas highlight the common significant correlations observed in the three arm configurations of the maze

(A)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2	0.15															
3	0.49	**0.59***														
4	**0.70***	**0.54***	**0.73***													
5	0.06	0.39	**0.65***	0.07												
6	-0.29	***-0.46†***	**-0.74***	-0.36	**-0.87***											
7	***-0.43†***	-0.09	**-0.60***	-0.32	**-0.72***	**0.90***										
8	0.30	0.38	**0.85***	***0.49†***	**0.59***	**-0.54***	***-0.44†***									
9	0.36	***0.45†***	**0.92***	**0.53***	**0.71***	**-0.70***	**-0.59***	**0.98***								
10	-0.11	-0.05	0.30	-0.34	**0.82***	**-0.60***	**-0.57***	***0.47†***	**0.52***							
11	0.39	***0.45†***	**0.96***	**0.69***	**0.61***	**-0.66***	**-0.57***	**0.85***	**0.89***	0.26						
12	0.39	***0.44†***	**0.80***	**0.60***	**0.60***	**-0.70***	**-0.64***	***0.45†***	**0.58***	0.14	**0.83***					
13	**0.59***	**0.58***	0.34	**0.62***	-0.16	-0.02	0.12	0.22	0.21	-0.37	0.14	0.04				
14	0.05	-0.06	**-0.56***	-0.11	**-0.56***	0.29	0.26	**-0.78***	**-0.73***	**-0.56***	**-0.67***	-0.34	0.27			
15	**-0.52***	**-0.57***	**-1.00***	**-0.73***	**-0.65***	**0.72***	**0.60***	**-0.85***	**-0.92***	-0.29	**-0.96***	**-0.81***	-0.35	**0.57***		
16	-0.22	-0.23	**-0.75***	-0.28	**-0.64***	***0.42†***	0.35	**-0.89***	**-0.86***	**-0.57***	**-0.78***	***-0.48†***	-0.05	**0.90***	**0.77***	
17	0.06	0.27	0.22	0.37	-0.17	-0.08	0.00	0.03	0.04	-0.35	0.20	0.27	0.13	0.18	-0.16	0.16

(B)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2	-0.23															
3	-0.29	***0.48†***														
4	-0.16	0.41	**0.56***													
5	-0.14	**0.51***	0.29	-0.22												
6	-0.09	-0.35	-0.28	0.36	**-0.88***											
7	-0.09	-0.04	-0.41	0.02	**-0.68***	**0.76***										
8	-0.17	**0.59***	**0.74***	**0.50***	**0.62***	**-0.51***	**-0.63***									
9	-0.15	**0.61***	**0.72***	***0.45†***	**0.69***	**-0.58***	**-0.68***	**0.99***								
10	0.20	0.05	0.07	***-0.43†***	**0.77***	**-0.84***	**-0.69***	0.38	***0.44†***							
11	***-0.45†***	**0.60***	0.29	0.34	0.23	-0.09	-0.04	0.30	0.31	-0.24						
12	-0.05	-0.20	***-0.44†***	-0.21	***-0.48†***	0.41	***0.49†***	**-0.70***	**-0.70***	**-0.52***	0.41					
13	-0.03	-0.28	-0.01	0.22	**-0.63***	**0.62***	0.36	-0.26	-0.32	**-0.58***	0.19	**0.55***				
14	0.20	**-0.52***	-0.41	-0.17	**-0.75***	**0.61***	**0.53***	**-0.74***	**-0.77***	**-0.55***	-0.04	**0.81***	**0.81***			
15	0.19	**-0.60***	**-0.57***	-0.39	**-0.65***	**0.54***	**0.54***	**-0.85***	**-0.87***	-0.39	-0.13	**0.81***	**0.62***	**0.94***		
16	0.21	***-0.49†***	**-0.51***	-0.31	**-0.67***	**0.52***	**0.54***	**-0.83***	**-0.84***	***-0.49†***	-0.03	**0.87***	**0.62***	**0.95***	**0.97***	
17	***0.49†***	0.23	0.13	0.27	-0.19	0.20	0.16	0.03	0.01	-0.35	0.23	0.20	0.37	0.27	0.14	0.22

Table 1 (*Continued*)

(C)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2	0.16															
3	-0.18	0.38														
4	-0.30	**0.58***	**0.64***													
5	0.07	0.41	**0.54***	-0.01												
6	-0.10	***-0.45†***	**-0.51***	-0.05	**-0.97***											
7	-0.01	-0.35	**-0.54***	-0.11	**-0.92***	**0.97***										
8	0.09	***0.47†***	***0.43†***	0.00	**0.96***	**-0.96***	**-0.93***									
9	0.10	***0.47†***	***0.45†***	0.02	**0.96***	**-0.97***	**-0.94***	**1.00***								
10	0.17	0.00	0.19	***-0.48†***	**0.81***	**-0.75***	**-0.71***	**0.75***	**0.75***							
11	-0.25	**0.53***	**0.84***	**0.74***	***0.46†***	***-0.45†***	***-0.44†***	0.39	0.41	-0.05						
12	-0.34	0.02	0.38	**0.64***	***-0.43†***	***0.44†***	0.41	**-0.55***	**-0.53***	**-0.67***	**0.53***					
13	-0.02	***0.42†***	***0.44†***	**0.70***	-0.10	0.05	-0.04	-0.01	0.00	-0.37	0.38	0.24				
14	-0.07	-0.23	-0.24	0.29	**-0.91***	**0.88***	**0.81***	**-0.89***	**-0.88***	**-0.85***	-0.22	**0.54***	***0.45†***			
15	0.17	**-0.60***	**-0.90***	**-0.76***	**-0.53***	**0.54***	**0.57***	**-0.50***	**-0.53***	-0.07	**-0.91***	-0.32	**-0.63***	0.21		
16	-0.01	***-0.43†***	**-0.73***	-0.20	**-0.90***	**0.88***	**0.88***	**-0.88***	**-0.89***	**-0.71***	**-0.58***	0.28	-0.24	**0.70***	**0.74***	
17	-0.05	0.04	0.26	**0.54***	-0.34	0.34	0.27	-0.31	-0.32	**-0.62***	0.27	***0.46†***	**0.53***	**0.54***	-0.26	0.21

(D)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2	-0.05															
3	0.10	**0.52***														
4	0.03	**0.43***	**0.55***													
5	0.01	**0.40***	**0.48***	-0.10												
6	-0.16	**-0.35***	**-0.44***	0.14	**-0.88***											
7	-0.21	-0.08	**-0.42***	-0.04	**-0.74***	**0.88***										
8	0.04	**0.41***	**0.64***	**0.37***	**0.71***	**-0.60***	**-0.58***									
9	0.08	**0.45***	**0.67***	**0.35***	**0.78***	**-0.69***	**-0.66***	**0.99***								
10	0.09	-0.03	0.18	**-0.35***	**0.79***	**-0.71***	**-0.62***	**0.55***	**0.59***							
11	0.08	**0.45***	**0.74***	**0.38***	**0.45***	**-0.38***	**-0.35***	**0.46***	**0.50***	0.05						
12	0.05	0.13	0.21	0.07	-0.09	0.03	0.02	**-0.39***	**-0.32***	**-0.36***	**0.58***					
13	-0.07	0.11	0.17	**0.31***	**-0.27†**	**0.35***	**0.26†**	0.03	-0.03	**-0.35***	0.16	0.22				
14	-0.06	-0.21	**-0.39***	-0.09	**-0.62***	**0.49***	**0.42***	**-0.65***	**-0.66***	**-0.54***	**-0.29***	**0.38***	**0.64***			
15	-0.17	**-0.43***	**-0.64***	**-0.40***	**-0.54***	**0.52***	**0.50***	**-0.64***	**-0.68***	**-0.27†**	**-0.47***	0.16	**0.42***	**0.82***		
16	-0.12	**-0.27†**	**-0.57***	**-0.24†**	**-0.61***	**0.47***	**0.45***	**-0.72***	**-0.73***	**-0.50***	**-0.40***	**0.31***	**0.41***	**0.93***	**0.89***	
17	0.00	0.22	0.16	**0.27†**	**-0.25†**	0.15	0.14	-0.17	-0.16	**-0.49***	0.18	**0.33***	**0.24†**	**0.29†**	0.01	0.22

1: latency of entry to first bridge; 2: latency of entry to first arm; 3: total latencies between exit from and return to arms; 4: average latency between exit from and return to arms; 5: average number of entries to centre between visits to arms; 6: number of bridge entries before visit to first arm; 7: number of entries to centre; 8: number of entries to bridges; 9: number of entries to arms; 10: number of repeated visited to arms; 11: duration in centre; 12: average duration in centre; 13: duration in bridges; 14: average duration in bridges; 15: duration in arms; 16: average duration in arms; 17: latency of first entry to the eighth arm.

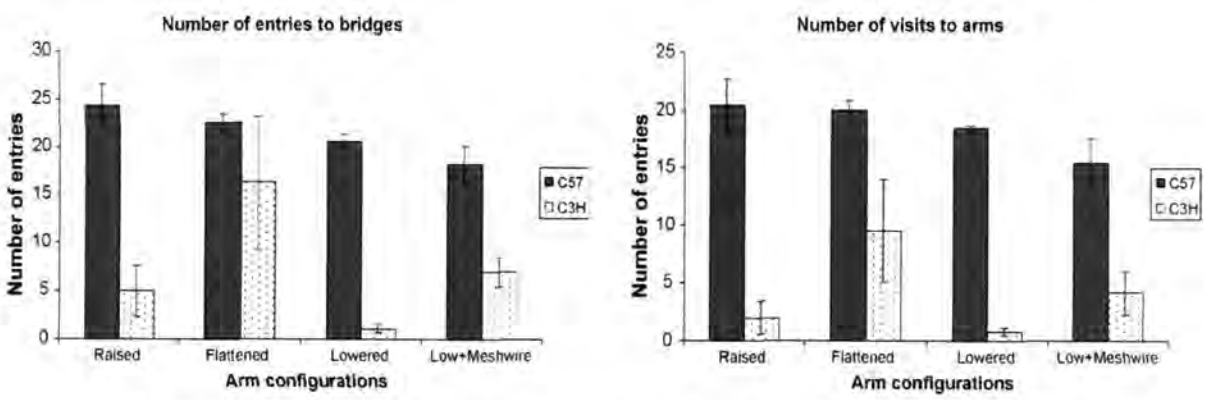

Fig. 5. Number of entries to bridges (left) and to arms (right) in each maze configuration. See text above for statistic results.

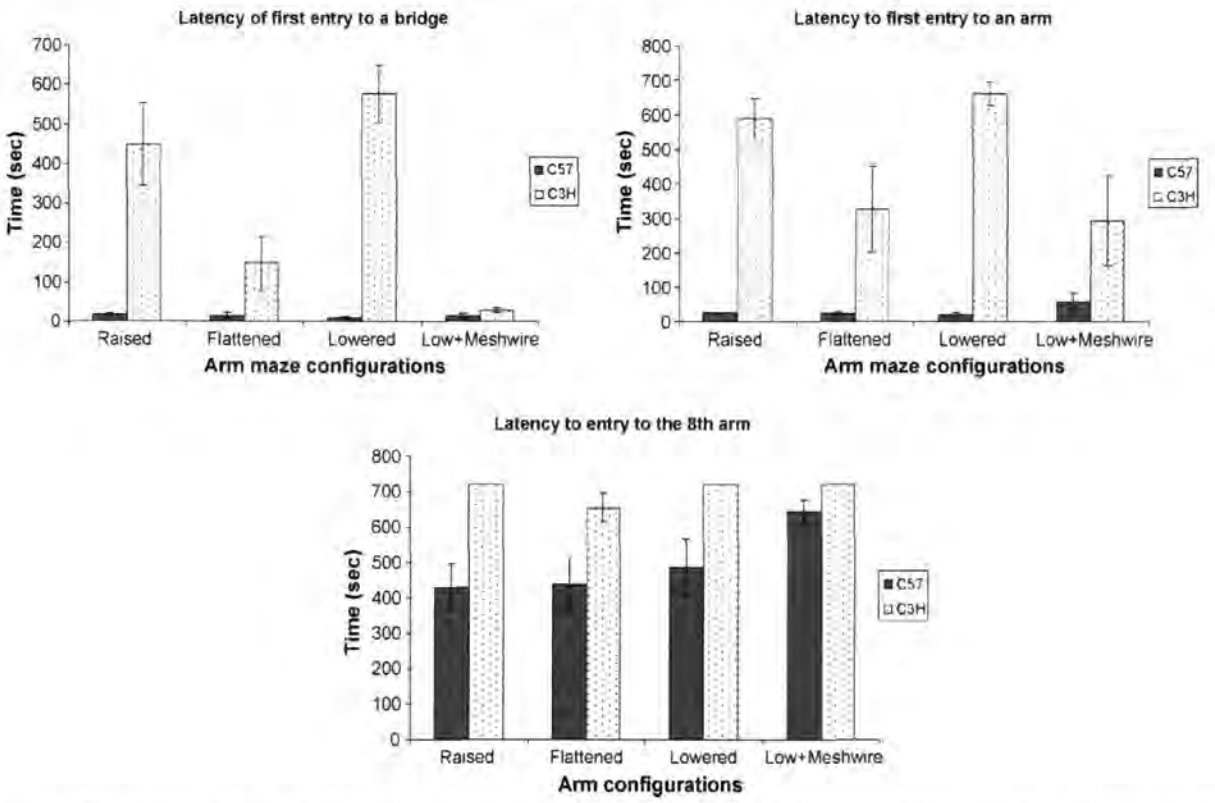

Fig. 6. Latency of first entry to a bridge (top-left) and an arm (top-right) and latency of first entry to the last non-visited arm in each maze configuration. See text above for statistic results.

arms compared to raised arms ($p<0.05$), to lowered arms ($p<0.004$) but not to lowered arms with wire mesh ($p<0.10$).

There are also significant differences between C57 and C3H mice on all arm configurations. C57 mice, compared to C3H, made more entries in raised arms ($p<0.0001$), flattened arms ($p<0.01$), lowered arms ($p<0.0002$) and lowered arms with wire mesh ($p<0.003$).

2.5.3.3. Number of bridge entries before first arm visit. One-way ANOVA revealed no significant differences between groups ($F_{7,36}=0.90$, $p>0.10$) for the number of entries to bridges before first visit to an arm.

2.5.3.4. Latency of first entry to a bridge (Fig. 6 (top-left)). One-way ANOVA revealed significant differences between groups ($F_{7,36}=16.95$, $p<0.0001$). In C57, the latency of first entry to a bridge is comparable between the different arm configurations. In C3H mice, the latency of first entry to a bridge is significantly low in flattened arms compared to raised arms ($p<0.0007$) and to lowered arms ($p<0.0001$). It is also significantly low in lowered arms with wire mesh compared to raised arms ($p<0.001$) and lowered arms ($p<0.0002$).

There are also significant differences between C57 and C3H mice on raised and lowered arm configurations. C57 mice, compared to C3H, took less time to enter for the first time a bridge in the raised ($p<0.0002$), and lowered ($p<0.0001$) arm configurations.

2.5.3.5. Latency of first entry to an arm (Fig. 6 (top-right)). One-way ANOVA revealed significant differences between groups ($F_{7,36}=12.47$, $p<0.0001$). In C57, the latency of first entry to a bridge is comparable between the different arm configurations. In C3H mice, the latency of first entry to an arm is significantly low in flattened arms compared to raised arms ($p<0.02$) and to lowered arms ($p<0.01$). It is also significantly low in lowered arms with wire mesh compared to raised arms ($p<0.02$) and lowered arms ($p<0.008$).

There are also significant differences between C57 and C3H mice on the different arm configurations. C57 mice, compared to C3H, took less time to enter an arm for the first time in raised arms ($p<0.0002$), flattened arms ($p<0.05$), lowered arms ($p<0.0001$) and lowered arms with wire mesh ($p<0.03$).

2.5.3.6. Latency of first entry to the eighth arm (Fig. 6 (bottom)). One-way ANOVA revealed significant differences between groups ($F_{7,36}=8.04$, $p<0.0001$). In C57, the number of bridge entries before entry to the eighth arm is significantly high in lowered arm with wire mesh compared to raised ($p<0.01$), to flattened ($p<0.008$) and lowered ($p<0.02$) arm configurations. In C3H, the latency is comparable between the different arm configurations.

There are also significant differences between C57 and C3H mice on all arm configurations except for lowered arms with wire mesh. C57 mice, compared to C3H, took less time to visit the eighth arm in the raised ($p<0.001$), flattened ($p<0.009$), lowered ($p<0.004$) arm configurations.

2.5.3.7. Average number of entries to centre between arm visits. One-way ANOVA revealed no significant differences between groups ($F_{7,36}=0.64$, $p>0.10$) for the number of entries to the centre between arm visits.

2.5.3.8. Total duration and average duration of bridge entries. One-way ANOVA revealed significant differences between groups for the total duration ($F_{7,36}=4.61$, $p<0.0009$) and the average duration ($F_{7,36}=3.45$, $p<0.006$) of entries to bridges.

In C57, the total duration (Fig. 7, top-left) and average duration of entries to the bridges (Fig. 7, top-right) is comparable between the different arm configurations. In C3H mice, the total duration and average duration of entries to the bridges is significantly low in raised and lowered arms compared to lowered arms with wire mesh ($p<0.02$).

There are also significant differences between C57 and C3H mice on the lowered arm configurations. The total duration of visits to bridges of lowered arms was significantly high in C57 mice compared to C3H ($p<0.02$) and the average duration of visits to bridges of lowered arm with wire mesh was significantly low in C57 compared to C3H.

2.5.3.9. Total duration and average duration of arm entries. One-way ANOVA revealed significant differences between groups for the total duration ($F_{7,36}=7.91$, $p<0.0001$) but not for the average duration ($F_{7,36}=1.18$, $p>0.10$) of entries to arms.

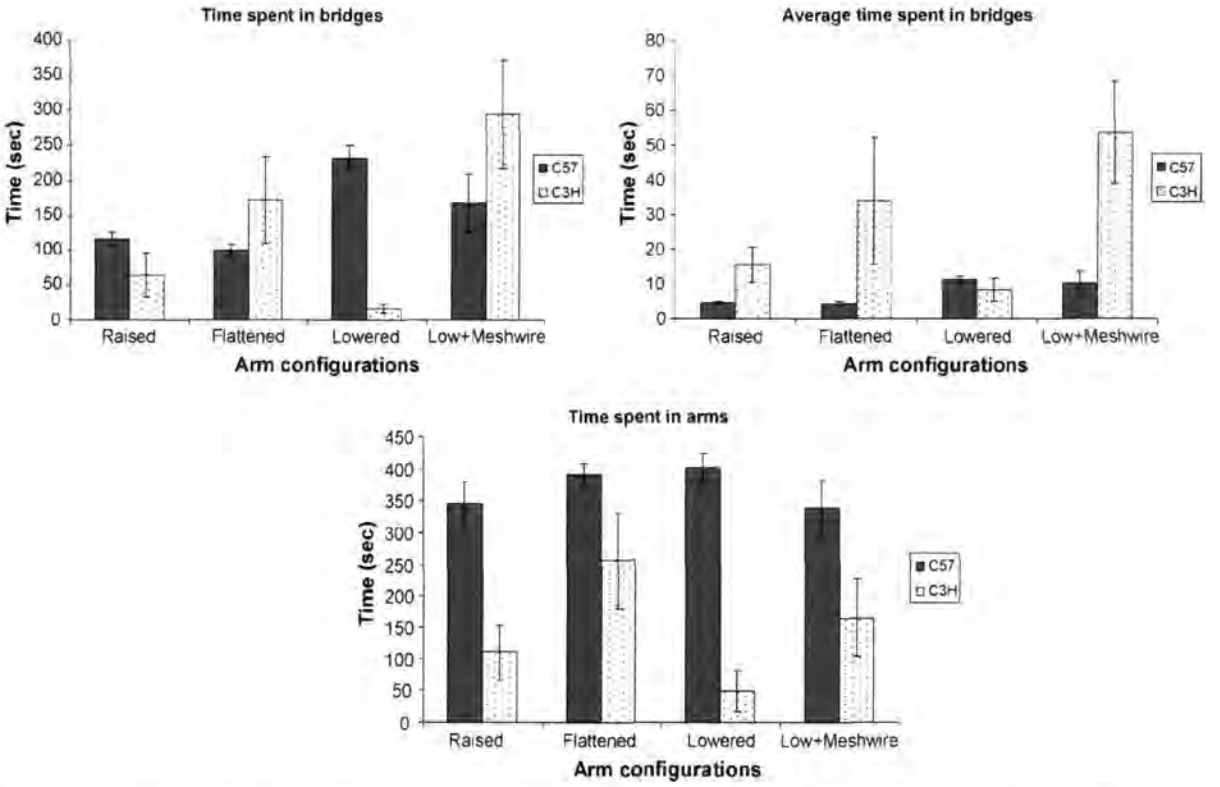

Fig. 7. Total duration (top-left) and average duration (top-right) of entries to bridges and total duration of entries to arms (bottom) in each maze configuration. See text above for statistic results.

In C57, the total duration of entries to arms is comparable between the different arm configurations. In C3H mice, the total duration of entries to arms is significantly low in lowered arms compared to flattened arms ($p < 0.02$). It was not significant between raised and lowered arms ($p < 0.09$).

There are also significant differences between C57 and C3H mice on all arm configurations except for the flattened arms (Fig. 7, bottom). C57 mice, compared to C3H mice, spent more time on raised arms ($p < 0.01$), on lowered arms ($p < 0.003$) and lowered arms with wire mesh ($p < 0.04$).

2.5.3.10. Total duration and average duration of centre re-entries. One-way ANOVA revealed significant differences between groups for the total duration ($F_{7,36} = 18.37$, $p < 0.0001$) and the average duration ($F_{7,36} = 8.60$, $p < 0.0001$) of entries to the central platform.

In C57, the total duration of entries to the central platform is comparable between the different arm configurations. In C3H mice, the total duration and average duration of entries to the central platform is significantly low in flattened and lowered arms with wire mesh compared to raised and lowered arms ($p < 0.0004$).

There are also significant differences between C57 and C3H mice on raised and lowered arm configurations. The total time (Fig. 8, left) and average time (Fig. 8, right) spent on the platform is significantly low in C57 mice compared to C3H on raised arms ($p < 0.003$) and on lowered arms ($p < 0.0004$).

2.5.3.11. Total latencies and average latency between exits from and entries to arms. One-way ANOVA revealed significant differences between groups for the total latencies ($F_{7,36} = 3.07$, $p < 0.01$) and the average latency ($F_{7,36} = 5.47$, $p < 0.0002$) between entries to arms.

In both C57 and C3H mice, the total latencies (Fig. 9, left) and the average latency (Fig. 9, right) between entries to arms is comparable between the different arm configurations.

The total latencies was significantly high C3H on raised arms compared to C57 on flattened ($p < 0.04$) and lowered arms ($p < 0.05$). The average latency

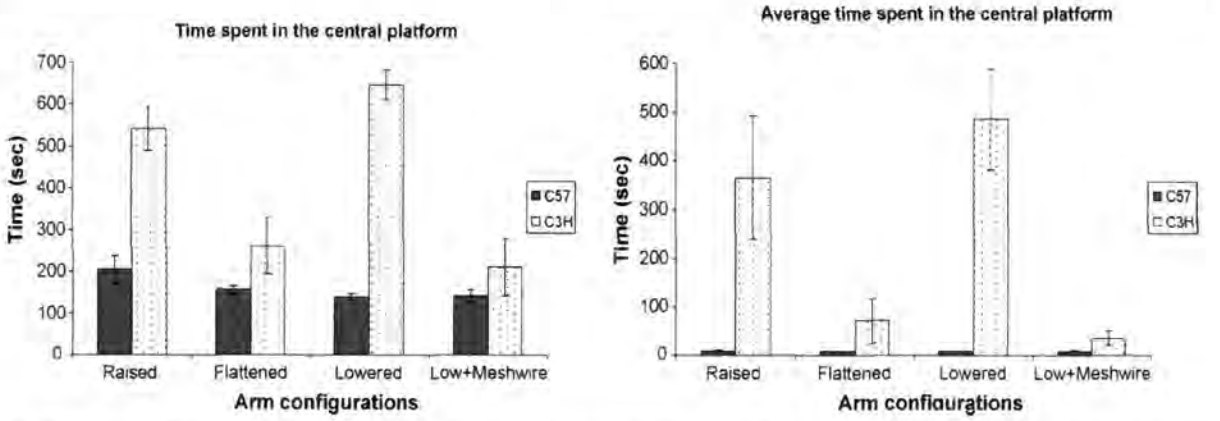

Fig. 8. Total duration (left) and average duration (right) of entries to the central platform in each maze configuration. See text above for statistic results.

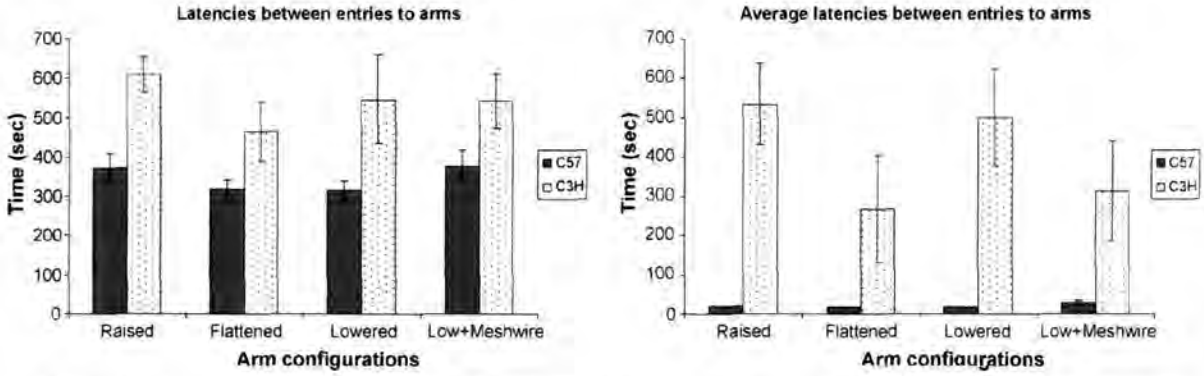

Fig. 9. Total latencies (left) and average latency (right) between entries to arms in each maze configuration. See text above for statistic results.

between entries to arms, however, revealed that C3H mice took significantly much longer time to return to arms than C57 mice on raised ($p < 0.007$) and lowered arms ($p < 0.01$).

2.5.3.12. Number of unique arm visits. One-way ANOVA revealed significant differences between groups for the number of unique visits to arms ($F_{7,36} = 15.49$, $p < 0.0001$).

In C57, the number of unique visits to arms is comparable between the different arm configurations. In C3H mice, the number of unique visits to arms is significantly high in flattened compared to raised ($p < 0.05$) and lowered arms ($p < 0.02$).

There are also significant differences between C57 and C3H mice on all arm configurations (Fig. 10). The number of unique visits to arms is significantly high in C57 mice compared to C3H on raised arms ($p < 0.0001$), flattened arms ($p < 0.007$), lowered arms ($p < 0.0001$) and on lowered arms with wire mesh ($p < 0.002$).

2.5.4. Summary

2.5.4.1. Difference between arm configurations within C57 and C3H mice. In C57 mice, there were no differences between the measurements on the different configurations of the test except for the latency of the first unique visit to the eighth arm. C57 took longer time to enter the eighth arm.

C3H mice, in the flat arm configuration compared the raised and lowered arm configurations spent less time in the central platform, took less time to enter the first bridge and the first arm; they made more entries to bridge and arms and more unique visits to arms.

C3H mice in lowered arm + wire mesh configuration compared to raised an lowered arm configuration spent less time in the central platform and more time on the bridges; they took less time to the enter the first bridge and the first arm.

2.5.4.2. Difference between C57 and C3H on each arm configuration. C3H mice compared to C57 mice, in the raised lowered arm configurations, spent more time in the central platform and less time in the arms, made fewer visits to bridges and arms, took longer time to enter the first bridge and the first arm. They also required longer latencies between arm entries and visited a few number of unique arms.

C3H mice compared to C57 mice, in the flat arm configuration, made a few number of arm visits, took longer time to visit a bridge and an arm for the first time, and made a few number of unique arm visits.

C3H mice compared to C57 mice, in the lowered + wire mesh arm configuration, made a few number of visits to arms and bridges, took longer time to enter an arm of the first time, spent more time on bridges and less time on arms, and made a few number of unique arm visits.

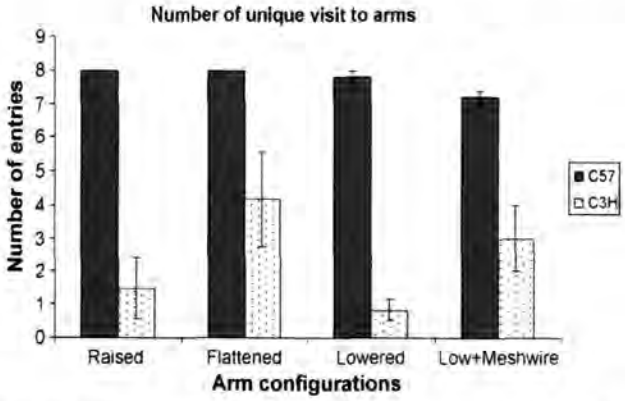

Fig. 10. Number of unique visits to arms (right) in each maze configuration. See text above for statistic results.

2.5.4.3. Effects of the wire mesh grid. The introduction of a wire mesh grid on the floor of the bridge had no significant effects on any parameter of the test in C57 mice except on the latency of entry to eighth arm ($p < 0.02$). However, in C3H, it reduced significantly the total time ($p < 0.0002$) and average time ($p < 0.0003$) spent in the central platform. It also reduced significantly the latency of first entry to a bridge ($p < 0.0002$) and to an arm ($p < 0.008$) of the maze and increased significantly the total time ($p < 0.002$) and average time ($p < 0.02$) spent in the bridges.

The number of entries to arms, the latency to first entry to bridge and arms and both total duration and average duration of entries to arms in the lowered arm configuration with wire mesh are comparable to those observed in the flat arm configuration.

2.6. Experiment 3: reaction to novelty and open spaces in food deprived C57, CD1 and Balb/c mice over three successive sessions

In a previous study, we demonstrated how to take advantage of the habituation phase of an object recognition test to assess anxiety [83]. We suggest here that it is possible to assess anxiety responses in animals during the first phase of a learning and memory tests without any changes to the testing conditions. Indeed in our laboratory, we are able to introduce rats and mice directly to a spatial maze and train rats for a maximum of 12–16 sessions without the introduction of pre-training or shaping. Animals reach a high performance level in much shorter period of time than if they had undergone habituation or pre-training. In this study, we present only results from the first three sessions. A group of C57, CD1 and Balb/c mice were examined in the raised arm configuration of the 3D maze. Each group was tested in three successive sessions, one session a day. In the present study, animals were food deprived.

2.6.1. Subjects

Thirty male mice (Balb/c, C57 and CD1, $n = 10$ each) supplied by Charles River Laboratories (Kent, UK) were used in the present study.

2.6.2. Testing procedures

Mice were tested in a unique session in the elevated arm maze configuration. The floors of the bridges were covered with wire mesh grid. The cups at the end of each arm contained one reward pellet (20 mg P.J. Noyes Company Inc., UK). One hour after the completion of the session animals had a free access to 5 g of biscuits.

The order of testing was randomly alternated between batches of five mice from the same cage and between cages from different strains of mice. A mouse is removed from its cage, put in a small bucket in which it is weighted and then poured gently in the centre platform of the maze. Unlike in the previous experiments, mice were left in the maze until eight choices were made or 10 min elapsed.

2.6.3. Results

2.6.3.1. Number of bridge entries (Fig. 11, top-left). There are a significant effect between groups ($F_{2,27} = 5.28$, $p < 0.01$), between sessions ($F_{2,54} = 57.64$, $p < 0.0001$) and significant interactions between groups and sessions ($F_{4,54} = 13.81$, $p < 0.0001$).

CD1 made fewer visits to bridges than Balb/c ($p < 0.05$) and C57 ($p < 0.009$). The number of entries to bridges decreased significantly from session to session ($p < 0.003$).

Post hoc comparisons revealed that CD1 visited more bridges than Balb/c ($p < 0.04$) and less than C57 ($p < 0.03$) and in the first session, and they visited a few number of bridges in session 2 compared to C57 ($p < 0.0008$) and Balb/c ($p < 0.001$) and in session 3 compared to Balb/c only ($p < 0.004$). C57 mice visited more bridges than Balb/c in session 1 ($p < 0.0006$) and fewer bridges in session 3 ($p < 0.008$).

2.6.3.2. Number of arm entries (Fig. 11, top-right). There are a significant differences between groups ($F_{2,27} = 30.77$, $p < 0.0001$), between sessions ($F_{2,54} = 20.57$, $p < 0.0001$) but no significant interactions between groups and sessions ($F_{4,54} = 1.67$, $p > 0.10$).

C57 and CD1 made more visits to arms than Balb/c ($p < 0.0001$). The number of arm entries increased significantly from session to session ($p < 0.005$).

Post hoc comparisons revealed that C57 and CD1 visited more arms than Balb/c in each of the three sessions of the test ($p < 0.001$). C57 mice visited also more arms than CD1 in the second session ($p < 0.04$).

2.6.3.3. Number of bridge entries before first arm visit (Fig. 11, bottom). There are significant differences between groups ($F_{2,27} = 23.96$, $p < 0.0001$), between sessions ($F_{2,54} = 31.24$, $p < 0.0001$) and significant interactions between groups and sessions ($F_{4,54} = 4.24$, $p < 0.005$).

C57 and CD1 made a few number of visits to bridges before first entry to an arm than Balb/c ($p < 0.0002$). The number of entries to bridges before first visit to an arm decreased significantly from session to session ($p < 0.002$).

Post hoc comparisons revealed that CD1 and C57 visited a few number of bridges than Balb/c in sessions 2 and 3 ($p < 0.0004$). The difference between CD1 and C57 in session 1 did not reach significance ($p < 0.09$).

2.6.3.4. Latency of first entry to a bridge. There are a no significant differences between groups ($F_{2,27} = 1.91$, $p > 0.10$), but significant differences between sessions ($F_{2,54} = 7.63$, $p < 0.001$) and no significant interactions between groups and sessions ($F_{4,54} = 0.77$, $p > 0.10$). The latency of first entry to a bridge decreased significantly from session to session ($p < 0.01$).

2.6.3.5. Latency of first entry to an arm (Fig. 12, left). There are significant differences between groups ($F_{2,27} = 33.51$, $p < 0.0001$), between sessions ($F_{2,54} = 19.17$, $p < 0.0001$) but no significant interactions between groups and sessions ($F_{4,54} = 1.06$, $p > 0.10$).

C57 and CD1 took less time to visit an arm for the first time than Balb/c ($p < 0.0001$). The difference between C57 and CD1 did not reach significance ($p < 0.08$). The latency of first entry to an arm decreased significantly from session to session ($p < 0.01$).

Post hoc comparisons revealed that C57 and CD1 mice took significantly less time to visit an arm for the first time than Balb/c ($p < 0.02$) in the second ($p < 0.002$) and third ($p < 0.0001$) sessions. C57 but not CD1 took less time to visit an arm for the first time than Balb/c ($p < 0.02$) in the first session.

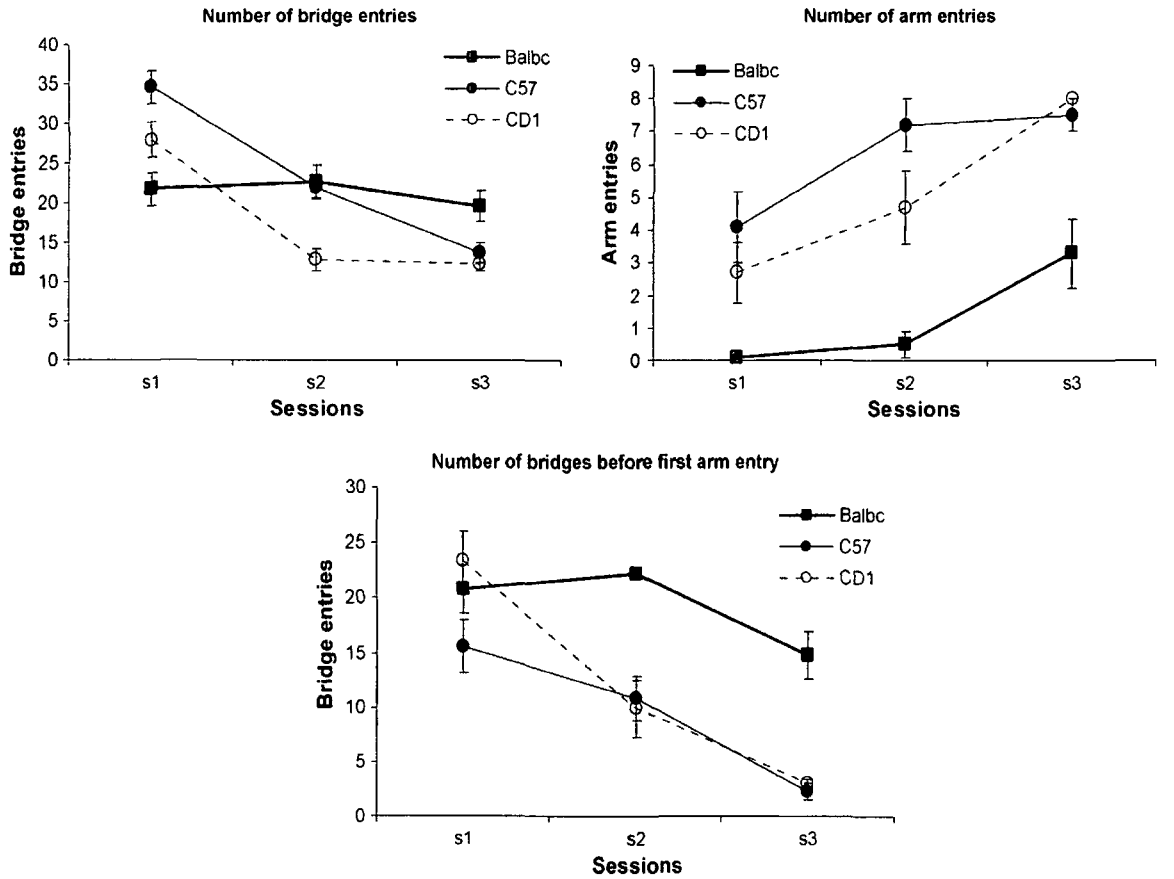

Fig. 11. Number of bridge entries (left), arms entries (right) and number of bridge entries before first visit to an arm (bottom) in each maze configuration. See text above for statistic results.

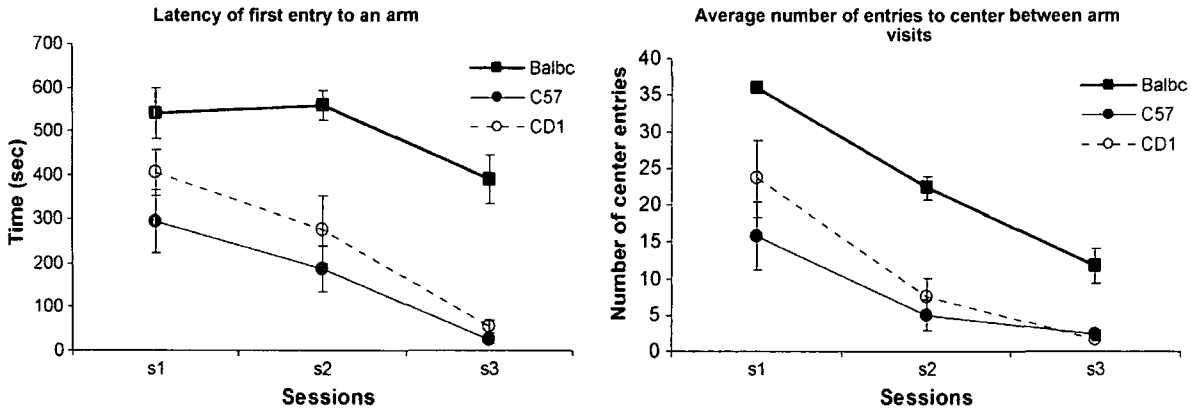

Fig. 12. Latency of first entry to an (left) and average number of entries to the centre between arm visits (right) in each maze configuration. See text above for statistic results.

2.6.3.6. Average number of entries to centre between arm visits (Fig. 12, right). There are significant differences between groups ($F_{2,27} = 29.22$, $p < 0.0001$), between sessions ($F_{2,54} = 39.67$, $p < 0.0001$) but no significant interactions between groups and sessions ($F_{4,54} = 1.16$, $p > 0.10$).

C57 and CD1 returned to visit an arm after fewer visits to the centre than Balb/c ($p < 0.0001$). The number of entries to the central platform between arm visits decreased significantly from session to session ($p < 0.007$).

Post hoc comparisons revealed that C57 and CD1 mice returned to visit an arm after fewer visits to the centre than Balb/c in each of the three sessions ($p < 0.0002$).

2.6.3.7. Total duration and average duration of bridge entries (Fig. 13, left). There are significant differences between groups (respectively, $F_{2,27} = 19.34$, $p < 0.0001$ and $F_{2,27} = 12.04$, $p < 0.0001$) and between sessions (respectively, $F_{2,54} = 43.92$, $p < 0.0001$ and $F_{2,54} = 9.19$, $p < 0.0004$). There are, however, significant interactions between groups and sessions on the average duration of bridge entries ($F_{4,54} = 3.35$, $p < 0.02$) but not for the total duration ($F_{4,54} = 2.80$, $p < 0.07$).

The total time and average time spent on the bridges was lower in C57 and CD1 than in Balb/c ($p < 0.0002$). The total time on the bridges decreased significantly from session to session ($p < 0.0001$).

Post hoc comparisons revealed that the total duration time spent on the bridges was significantly low in CD1 compared to Balb/c in session 1 ($p < 0.05$) and in both C57 and CD1 compared to Balb/c in sessions 2 ($p < 0.02$) and 3 ($p < 0.0001$). The average duration, however, was significantly low in both C57 and CD1 in sessions 1 ($p < 0.001$) and 3 ($p < 0.0006$) but not in session 2 ($p > 0.10$).

2.6.3.8. Total duration and average duration of arm entries (Fig. 14). The total duration revealed significant differences between groups ($F_{2,27} = 21.38$, $p < 0.0001$) and between sessions ($F_{2,54} = 13.57$, $p < 0.0001$) but no significant interactions ($F_{4,54} = 2.23$, $p < 0.08$). The average duration, however, was not significant between groups ($F_{2,27} = 3.01$, $p < 0.07$), between sessions ($F_{2,54} = 7.33$, $p < 0.002$) and no significant interactions ($F_{4,54} = 2.37$, $p < 0.06$).

The total time spent on arms was higher in C57 and CD1 than in Balb/c ($p < 0.0001$) and it increased significantly from session to session ($p < 0.01$).

Post hoc comparisons revealed that the total and average time duration spent on arms was significantly high in C57 and CD1 compared to Balb/c in sessions 1 ($p < 0.04$) and 2 ($p < 0.04$). The total time alone was significantly high in CD1 compared to Balb/c ($p < 0.01$) in session 3.

2.6.3.9. Total duration and average duration of centre re-entries (Fig. 15). The total and average duration revealed significant differences between groups (respectively, $F_{2,27} = 17.52$, $p < 0.0001$ and $F_{2,27} = 5.31$, $p < 0.01$) and between sessions (respectively, $F_{2,54} = 16.24$, $p < 0.0001$ and $F_{2,54} = 5.28$, $p < 0.008$). The interactions between groups and sessions was significant for the total duration ($F_{4,54} = 3.24$, $p < 0.02$) but not for the average duration ($F_{4,54} = 1.94$, $p > 0.10$) of centre entries.

The total time spent on the central platform was lower in C57 and CD1 than in Balb/c ($p < 0.002$) and it is also lower in C57 compared to CD1 ($p < 0.03$). The average time spent on the central platform, however, is low in C57 compared to Balb/c ($p < 0.02$) and CD1 ($p < 0.01$). The total time decreased significantly in session 3 compared to sessions 1 and 2 ($p < 0.0001$).

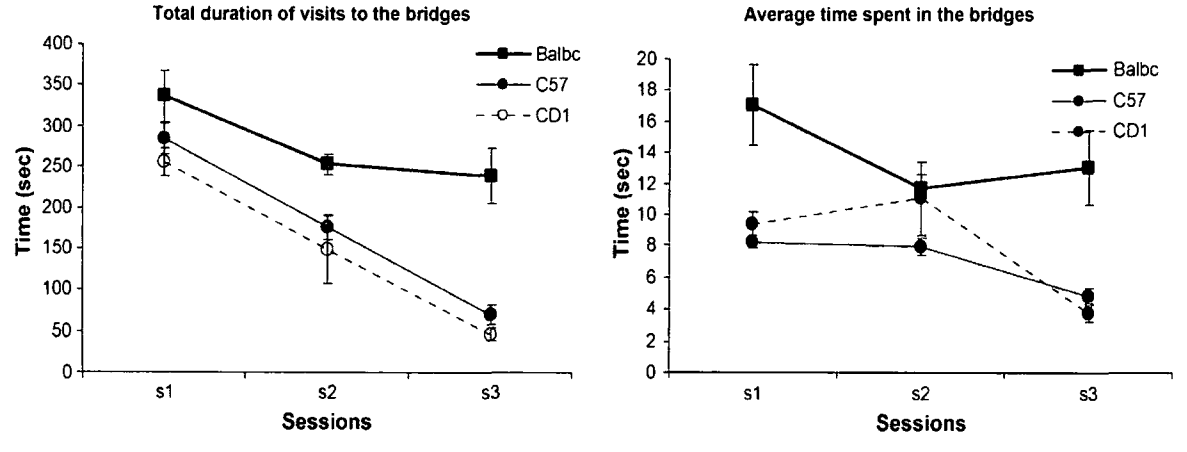

Fig. 13. Total duration of entries (left) and average duration of entries to bridges (right) in each maze configuration. See text above for statistic results.

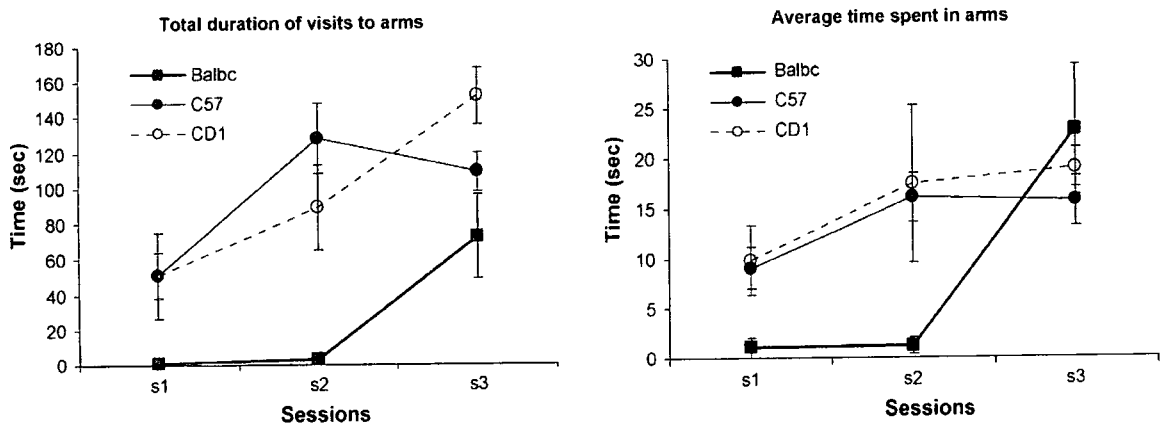

Fig. 14. Total duration of entries (left) and average duration of entries to bridges (right) in each maze configuration. See text above for statistic results.

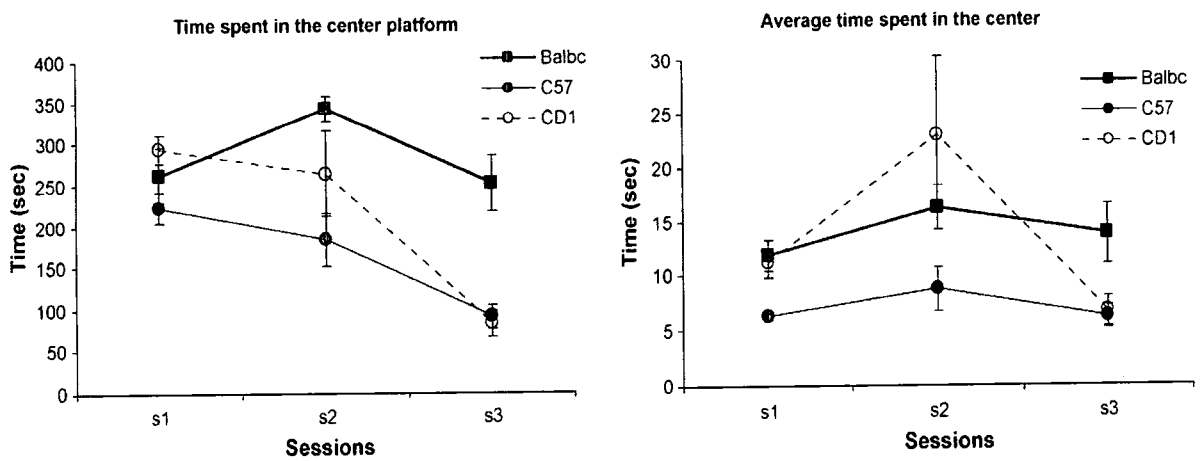

Fig. 15. Total duration (left) and average duration of entries to central platform (right) in each maze configuration. See text above for statistic results.

Post hoc comparisons revealed that the total time spent on the central platform was significantly low in C57 compared to Balb/c in session 2 ($p<0.01$), and in C57 and CD1 compared to Balb/c in session 3 ($p<0.0002$).

The average duration spent on arms was significantly low in C57 compared to Balb/c in session 1 ($p<0.006$) and in C57 and CD1 compared to Balb/c in session 3 ($p<0.02$).

2.6.3.10. Total latencies and average latency between exits from and entries to arms (Fig. 16). The total and average latencies revealed significant differences between groups (respectively, $F_{2,27}=33.72$, $p<0.0001$ and $F_{2,27}=29.10$, $p<0.0001$) and between sessions (respectively, $F_{2,54}=21.97$, $p<0.0001$ and $F_{2,54}=17.91$, $p<0.0001$). The interactions between groups and sessions was not significant for the total latencies ($F_{4,54}=1.54$, $p>0.10$) and the average latencies ($F_{4,54}=2.03$, $p=0.10$).

The total and average latencies in C57 and CD1 were lower than in Balb/c ($p<0.0001$). They also decreased significantly from session to session ($p<0.0001$).

Post hoc comparisons revealed that the total and average latencies between exits and entries to arms were significantly low in C57 and CD1 compared to Balb/c in sessions 1 ($p<0.004$) and 2 ($p<0.01$).

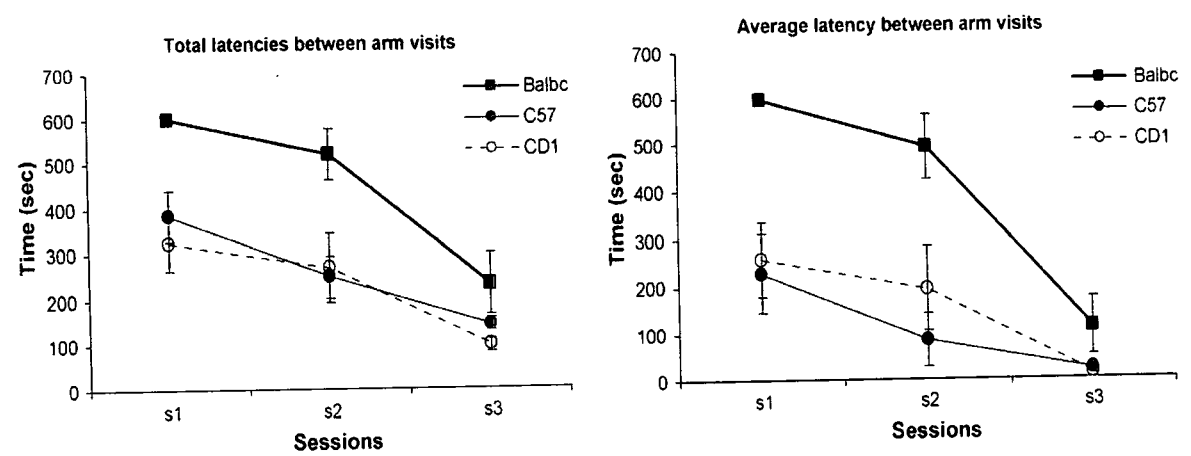

Fig. 16. Total latencies (left) and average latencies between arm visits (right) in each maze configuration. See text above for statistic results.

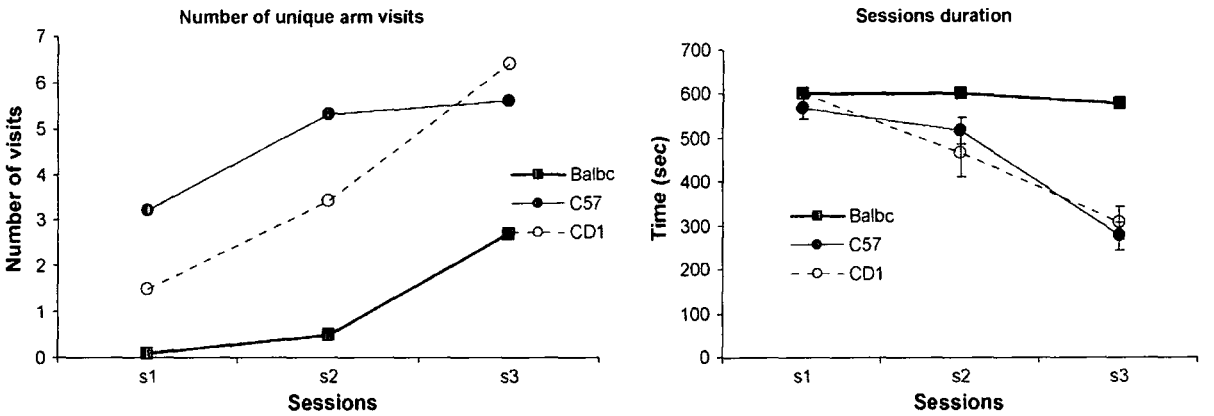

Fig. 17. Total number of unique arm vists (top-left) and session duration (top-right) in each maze configuration. See text above for statistic results.

2.6.3.11. Number of unique visits (Fig. 17, top-right). There are significant differences between groups ($F_{2,27} = 30.17$, $p < 0.0001$), between sessions ($F_{2,54} = 21.79$, $p < 0.0001$) but no significant interactions between groups and sessions ($F_{4,54} = 2.09$, $p = 0.09$).

C57 and CD1 made more unique visits to arms than Balb/c ($p < 0.0001$). The number of unique visits to arms increased significantly from session to session ($p < 0.005$).

Post hoc comparisons revealed that C57 and CD1 made more unique visits to arms than Balb/c in sessions 2 ($p < 0.004$) and 3 ($p < 0.001$). In addition, C57 made more unique visits to arms than Balb/c in session 1 ($p < 0.003$) and more than CD1 in session 2 ($p < 0.05$).

2.6.3.12. Session duration (Fig. 17, bottom). There are significant differences between groups ($F_{2,27} = 22.57$, $p < 0.0001$), between sessions ($F_{2,54} = 44.06$, $p < 0.0001$) and significant interactions between groups and sessions ($F_{4,54} = 9.37$, $p < 0.0001$).

C57 and CD1 too less time to perform eight choices in the maze than Balb/c ($p < 0.0001$) and the duration of the test decreased significantly from session to session ($p < 0.008$).

Post hoc comparisons revealed no significant differences between groups in session 1 ($p > 0.10$). In session 2 CD1 spent less time in the maze than Balb/c ($p < 0.04$) and in session 3 both CD1 and C57 spent less time than Balb/c ($p < 0.0001$).

2.6.4. Summary

In comparisons to Balb/c, CD1 and C57 visited more arms, spent more time on the arms, less time on the bridges and less time on the central platform. They were fast to visit the first arm and required less number of entries to bridges before visit to first arm. They also made fewer re-entries to the central platform between consecutive entries to arms and shorter latencies between re-entries to arms. They visited most of the eight arms of the maze at least once and were quicker to make eight arm choices. The only differences between CD1 and C57 are that CD1 made fewer entries to bridges and spent longer time in the central platform than C57.

After repeated exposure to the maze, the number of entries to bridges and to the central platform decreased significantly while the number of arms entries as well as the number of unique visits to arms increased. In addition, the latency of first entry to a bridge and to an arm, the total latencies between entries to arms, the number of entries to bridges before first visit to an arm, the total time on the bridges and the total time to perform eight choices decreased significantly while the total time on arms increased from session to session.

2.7. Experiment 4: reaction to novelty in presence and absence of a protected space in C57 and Balb/c mice

Most current behavioural models of anxiety provide a choice between an anxiogenic and a safe alternative. It has been suggested that this situation involves a conflict between the drive to escape or avoid and the drive to explore. In our view these models involve escape or avoidance and the assumption that animals "need" or "want" to explore is very speculative. Results from a series of experiments [84] comparing the behavioural reactions of rats to novelty in an open space and an enclosed space suggest that the two testing conditions do not involve the same construct. In an enclosed space rats avoid entry to the central area of a box and spend most of their time against walls and in corners while in a comparable space without walls, animals have to avoid every part of the platform and therefore need to explore in order to find an escape route. The need to explore and the need to escape are more evident in the open space than in the enclosed space. Fear inducing escape or avoidance need to be discriminated from fear inducing anxiety.

In the present experiment, we examined the behaviour of C57 mice in the lowered arm configuration with an enclosed central platform. We expected mice to prefer the enclosed space and avoid entry to bridges and arms of the maze. Mice behaviour in this configuration was compared to that of other groups of mice exposed to different arm configurations of the maze with an open central platform.

2.7.1. Subjects

Thirty C57 mice supplied by Charles River Laboratories (Kent, UK) were used in the present study.

2.7.2. Testing procedures

A cylinder (20 cm high) with small openings (5 cm × 5 cm) leading to each bridge of the maze was used to provide an enclosed central platform. The diameter of the cylinder was exactly the same as the diameter of the central platform (30 cm). Mice were tested in a unique session in different arm configurations of the maze. One group ($n = 6$) was tested in an enclosed central platform with lowered arms. All other groups ($n = 6$ each) were tested with open central platform either in lowered, raised or flat arm configuration. A fourth group ($n = 6$) was tested in a mixed arm configuration with three raised arms, three lowered arms and two flat arms. Mice were released by hand in the centre platform of the maze. They were left in the maze until 12 min elapsed. The floors of the bridges were not covered with wire mesh grid.

2.7.3. Results

2.7.3.1. Number of bridges, arms and central platform entries. There are significant differences between groups for the number of bridges entries ($F_{4,25} = 2.92$, $p < 0.04$, Fig. 18 left) and central platform entries in ($F_{4,25} = 2.69$, $p < 0.05$, Fig. 18 right) but not for the number of arm entries ($F_{4,25} = 2.08$, $p > 0.10$). Mice in the lowered arms with an enclosed central platform made more entries in the bridges and in the central platform than mice in the enclosed arm with an open central platform ($p < 0.04$). However, they were not significantly different from the other groups ($p > 0.10$). Mice in the lowered arms with an open central platform made less bridge and central platform entries compared to mice in raised arms ($p < 0.03$) but they were not significantly different from those in flat arms for the number of bridge entries ($p < 0.07$) and they were not significantly different from those in mixed arms for the number of central platform entries ($p < 0.10$).

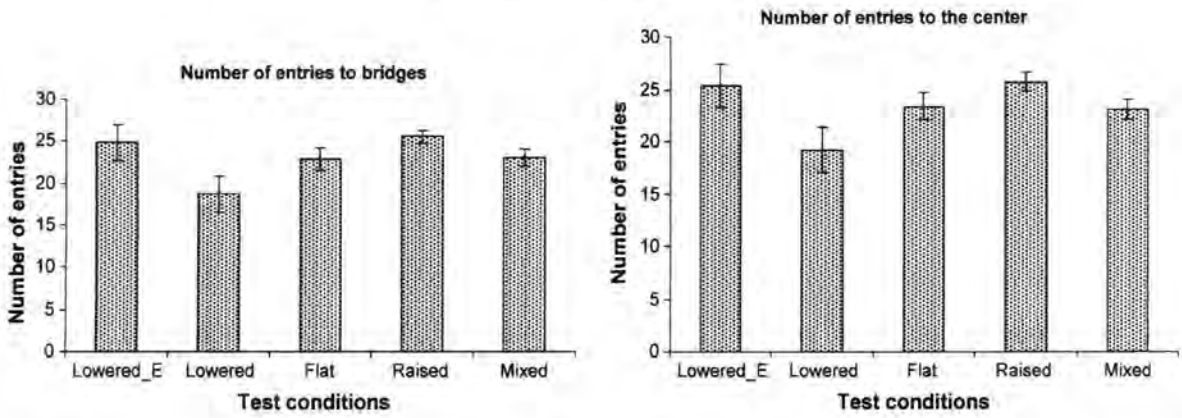

Fig. 18. Number of entries to bridges (left) and to the centre (right) in each maze configuration. See text above for statistic result. Lowered_E: lowered arms with enclosed central platform.

2.7.3.2. Number of bridge entries before first arm visit (Fig. 19, top-left). There are significant differences between groups for the number of bridges entries before the first visit to an arm of the maze ($F_{4,25} = 4.41$, $p < 0.008$). Mice tested in lowered arms with an enclosed central platform required more bridge entries before first visit to an arm of the maze compared to all other groups ($p < 0.02$).

2.7.3.3. Latency of first entry to a bridge. One-way ANOVA revealed no significant differences between groups ($F_{4,25} = 1.09$, $p > 0.10$).

2.7.3.4. Latency of first entry to an arm (Fig. 19, top-right). One-way ANOVA revealed significant differences between groups ($F_{4,25} = 2.94$, $p < 0.04$). Mice in the lowered arm with an enclosed central platform took significantly longer time to visit for the first time an arm of the maze compared to all other groups of mice ($p < 0.05$).

2.7.3.5. Latency of first entry in the eighth arm. One-way ANOVA revealed no significant differences between groups ($F_{4,25} = 0.33$, $p > 0.10$).

2.7.3.6. Total latencies and average latency between exits from and entries to arms (Fig. 19, bottom). One-way ANOVA revealed significant differences between groups on the total latencies ($F_{4,25} = 7.28$, $p < 0.0005$) but not on average between entries in the arms ($F_{4,25} = 1.48$, $p > 0.10$). Mice in the lowered arm with an enclosed central platform took significantly longer time to return to an arm of the maze compared to all other groups of mice ($p < 0.02$). Mice in the lowered

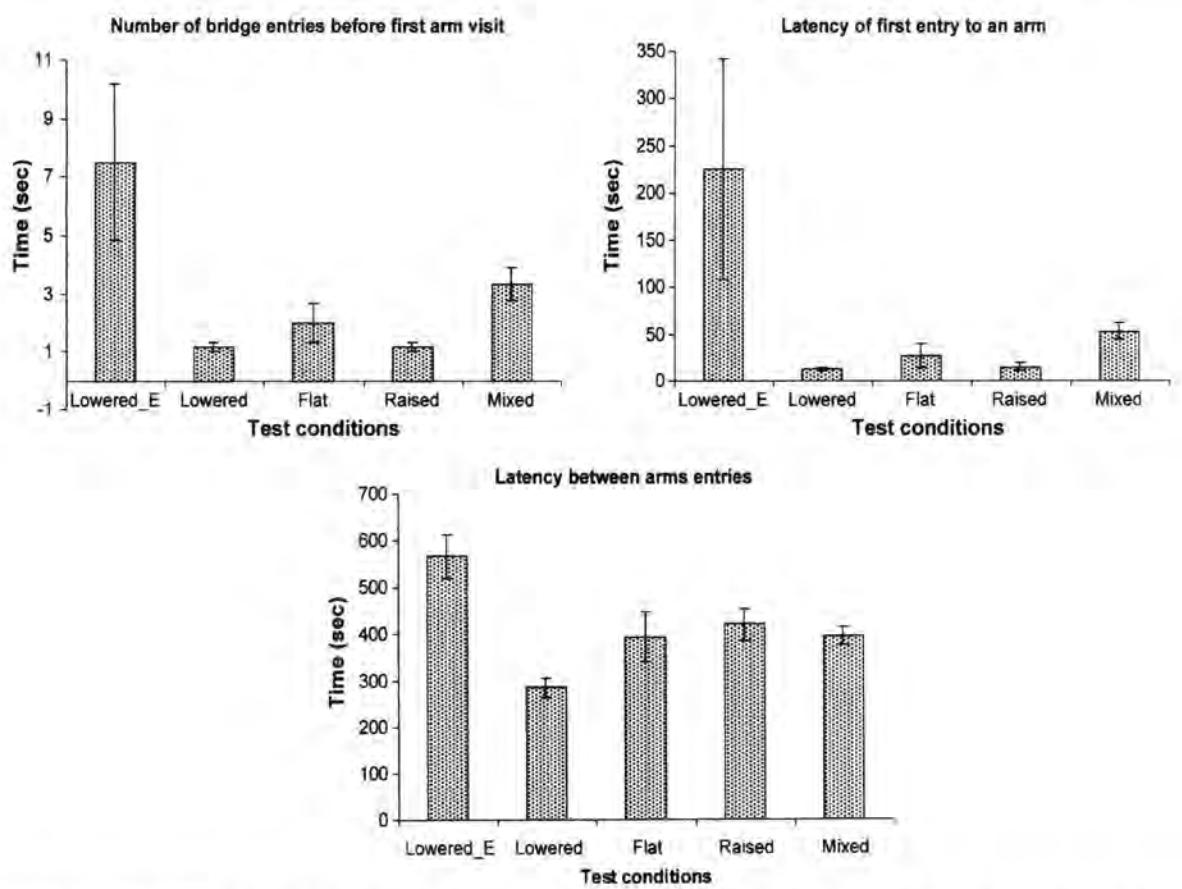

Fig. 19. Number of entries to bridges before first visit to an arm (top-left), latency of first entry to an arm (top-right) and latency between entries to arms (bottom) in each maze configuration. See text above for statistic results.

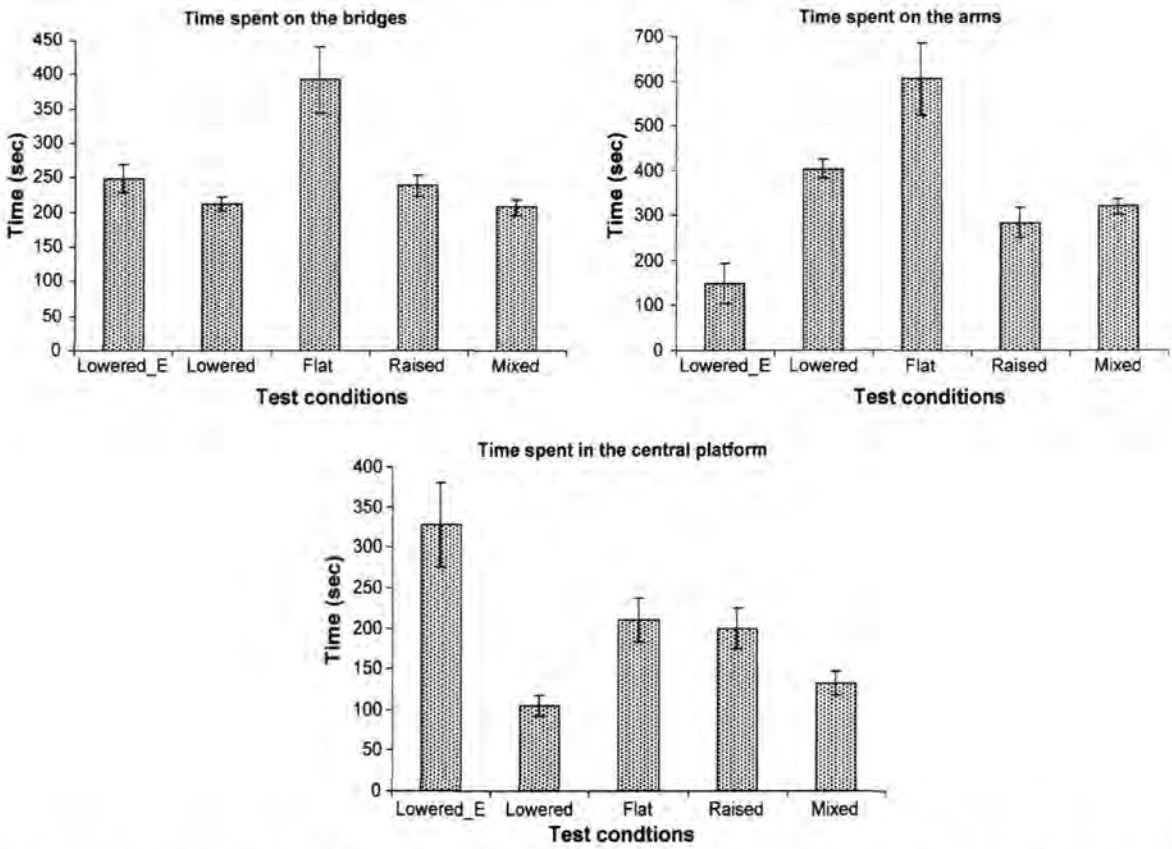

Fig. 20. Total duration of entries in bridges (top-left), in arms (top-right) and in the central platform (bottom) in each maze configuration. See text above for statistic results.

arm with an open central platform took less time to return to an arm of the maze compared to mice on flat arms ($p < 0.05$) and raised arm ($p = 0.07$) configurations.

2.7.3.7. Total duration and average duration of bridge entries (Fig. 20, top-left). One-way ANOVA revealed significant differences between groups on the duration ($F_{4,25} = 8.82$, $p < 0.0001$) and average duration of entries in the bridges ($F_{4,25} = 5.07$, $p < 0.0004$).

The total duration and average duration of visits to the bridges shows that mice in the flat arms spent significantly longer time than mice in the bridges of lowered arms with ($p < 0.0007$) and without ($p < 0.0004$) enclosed central platform and compared to mice in raised ($p < 0.0009$) and mixed arm configurations ($p < 0.0004$).

2.7.3.8. Total duration and average duration of arm entries (Fig. 20, top-right). One-way ANOVA revealed significant differences between groups on the total duration ($F_{4,25} = 13.63$, $p < 0.0001$) but not on average duration of entries in the arms ($F_{4,25} = 0.82$, $p > 0.10$). Mice in the enclosed central platform spent significantly less time on the arms of the maze than the other groups of mice ($p < 0.04$). Mice in the flat arm configurations spent significantly more time in the arms of the maze compared to all other groups ($p < 0.005$).

2.7.3.9. Total duration and average duration of centre re-entries (Fig. 20, bottom). One-way ANOVA revealed significant differences between groups on the total duration ($F_{4,25} = 8.82$, $p < 0.0001$) and average duration of entries in the central platform ($F_{4,25} = 5.07$, $p < 0.0004$). Mice in the lowered arms with an enclosed central platform spent significantly more time in the centre than any other group of mice ($p < 0.01$). Mice in the lowered arms with an open central platform spent less time than mice in flat and raised arms but the difference was not significant ($p < 0.09$).

2.7.3.10. Average number of entries to centre between arm visits. One-way ANOVA revealed no significant differences between groups on the average number of entries to the central platform between visits to arms ($F_{4,25} = 1.66$, $p > 0.10$).

2.7.3.11. Number of unique visits. One-way ANOVA revealed no significant differences between groups on the number of unique visits to arms ($F_{4,25} = 1.48$, $p > 0.10$).

2.7.4. Summary

In the lowered arm configuration with an enclosed central platform, C57 mice took longer time to make a first entry to an arm, made more visits to bridges before first entry to an arm and required longer time between re-entries to arms, spent longer time on the central platform and shorter time on arms compared to mice in the lowered arm with an open central platform and compared to mice in the other arm configurations. They also made frequent entries to the centre and bridges compared to mice in the lowered arm with an open central platform. This experiment demonstrates the significant difference between the behaviour of mice in an open space as compared to an enclosed space which is supported by our previous results in different experimental designs [84]. Each experiment described in this report was designed without any a priori knowledge of the outcome of each one. The video analysis took place at the end of all set of experiments described in this report. C57 revealed less emotionally reactive than any other strains of mice and therefore their selection for the purpose of this experiment appears inappropriate. However, the experiment provides evidence that the presence of an enclosed space reduces the exploratory activity of mice and delays the exploration of the arms of the maze. In our view, an enclosed space involves avoidance and escape responses. We expect that an enclosed space will discourage animals to explore open spaces as they provide safety; this effect would have been more obvious with Balb/c and C3H mice.

2.8. Experiment 5: strain and gender differences in mixed arm configurations of the maze

In this experiment mice were tested in a single session in mixed arm configurations. Three arms were raised, three arms were lowered and, unfortunately

there are only eight arms, two arms were left flat. A group of C57 was compared to C3H mice. This experiment would confirm the differences between these two strains of mice and examine gender differences within strains.

2.8.1. Subjects

Eleven C57 mice (six females and five males) and 12 C3H mice (six males and six females) supplied by Charles River Laboratories (Kent, UK) were used in the present study.

2.8.2. Testing procedures

Mice were tested in a unique session in a mixed arm maze configuration with three raised arms, three lowered arms and two flat arms. The floors of the bridges were not covered with wire mesh grid. Mice were released by hand in the centre platform of the maze. They were left in the maze until 10 min elapsed. We recorded only entries to arms. At that time we did not have the means to record entries to bridges. Therefore, it is not possible to provide the frequency of entries to and the time spent on the bridges and central platform. An entry to an arm is recorded when a mouse leaves a bridge with all four paws and an exit is recorded when all four paws leave an arm.

2.8.3. Results

2.8.3.1. Latency of first visit to arms (Fig. 21, left). There are significant differences between groups ($F_{3,19} = 6.17$, $p < 0.004$). C3H male mice were significantly different from C3H female mice ($p < 0.007$) and from both C57 male ($p < 0.006$) and female mice (0.01).

2.8.3.2. Frequency of visits to arms (Fig. 21, right). There are significant differences between groups ($F_{3,19} = 19.80$, $p < 0.0001$). C3H male and females mice visited less arms than C57 male and females mice ($p < 0.0004$).

2.8.3.3. Duration of visits to arms (Fig. 22, top-left). There are significant differences between groups ($F_{3,19} = 13.26$, $p < 0.0001$). C3H males spent less time in the arms than C3H female ($p < 0.003$) and both C57 male and female mice ($p < 0.0003$). C3H female mice spent less time than C57 female mice, but this is not significant ($p = 0.09$).

2.8.3.4. Average duration of visits to arms (Fig. 22, top-right). There are significant differences between groups ($F_{3,19} = 4.09$, $p < 0.02$). C3H females spent longer time in the arms than both C57 male ($p < 0.03$) and female ($p < 0,05$) mice while C3H male mice spent longer time than C57 male mice ($p < 0.05$). The difference between these results based on average duration and the results above based on total duration is that the number of arms visited by C3H is significantly very low compared to that of C57 mice (mean = 4.7 ± 1.30 for females and 2.7 ± 0.8 for males).

2.8.3.5. Latencies between visits to arms (Fig. 22, bottom-left). There are significant differences between groups ($F_{3,19} = 3.40$, $p < 0.04$). C3H male mice took longer time to re-enter in the arms of the maze compared to C3H and C57 female mice ($p < 0.05$).

2.8.3.6. Average latency between visits to arms (Fig. 22, bottom-right). There are significant differences between groups ($F_{3,19} = 5.34$, $p < 0.008$). C3H male mice took longer time to re-enter in the arms of the maze compared to C3H female mice ($p < 0.03$) and to both C57 male and female mice ($p < 0.01$).

2.8.4. Summary

The main significant results of this study are from C3H males which seem more anxious than C3H female and C57 mice. C3H males took longer time to visit an arm for the first time and to return to arms. They made a very few visits to arms. Though their total duration of visits to arms was shorter than that of the other groups (C3H females and C57 male and female mice), their average duration per visit was longer than that of C57 male and female mice which is accounted for by their low number of entries to arms.

2.9. Correlations analysis

In the above experiments, we described a wide range of measurements that we selected for assessing the reactions of animals to novelty in different arm maze configurations. Most of these measurements revealed sensitivity to differences between test conditions, strains and genders. They also showed consistency between experiments. Correlation analyses were performed to examine whether the different measurements of the tests measure the same construct.

Experiments 1 and 4 contain comparable groups of mice tested in three arm maze configurations. The groups from the same test conditions were comparable ($p > 0.10$) and they were therefore combined to provide a large sample ($n = 16$) for correlation analysis.

As shown in Table 1A–C (separate arm configurations) and Table 1D (combined arm configurations), most parameters of the tests were correlated significantly to each other (bold, $p \leq 0.05$) while a few remained below significance (italics, $p \leq 0.10$) except for the measure of latency of first entry to a bridge which did not correlate with other measurements ($p > 0.10$) in any of the three arm configurations of the maze. The average duration of visits to the central platform and the latency of first entry to the eighth arm did not correlate with any latency measurements and they did not correlate with the average number of entries to centre between visits to arms and number of bridge entries before first visit an arm.

3. Discussion

A wide range of behavioural tests have been introduced for the study of anxiety in rats and mice. Most of these tests offer the choice between open spaces and enclosed spaces. Because of the presence of these contrasting physical entities, it is claimed that these tests induce a conflict between the drive to explore

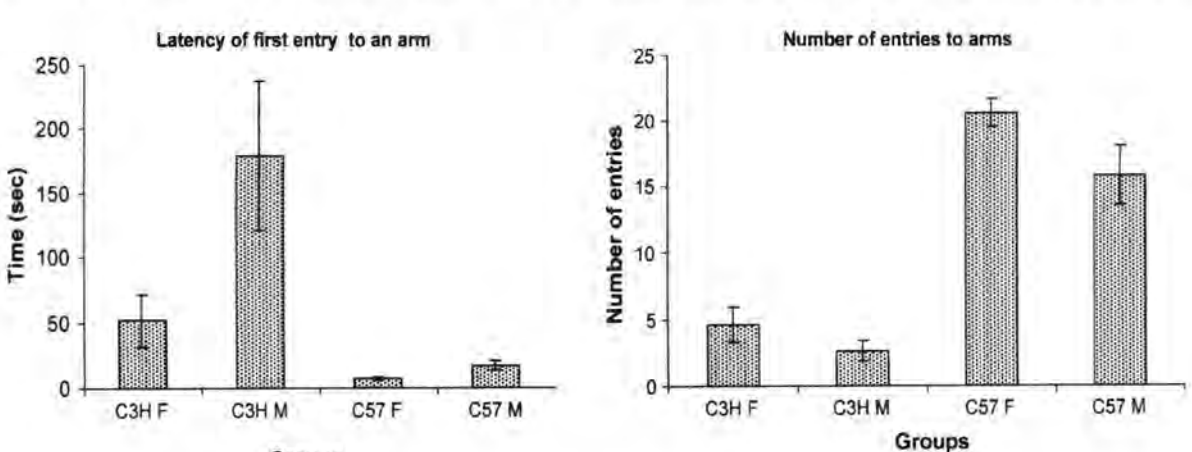

Fig. 21. Latency of first visit to an arm (left) and frequency of visits to arms. F: females; M: males.

there are only eight arms, two arms were left flat. A group of C57 was compared to C3H mice. This experiment would confirm the differences between these two strains of mice and examine gender differences within strains.

2.8.1. Subjects

Eleven C57 mice (six females and five males) and 12 C3H mice (six males and six females) supplied by Charles River Laboratories (Kent, UK) were used in the present study.

2.8.2. Testing procedures

Mice were tested in a unique session in a mixed arm maze configuration with three raised arms, three lowered arms and two flat arms. The floors of the bridges were not covered with wire mesh grid. Mice were released by hand in the centre platform of the maze. They were left in the maze until 10 min elapsed. We recorded only entries to arms. At that time we did not have the means to record entries to bridges. Therefore, it is not possible to provide the frequency of entries to and the time spent on the bridges and central platform. An entry to an arm is recorded when a mouse leaves a bridge with all four paws and an exit is recorded when all four paws leave an arm.

2.8.3. Results

2.8.3.1. Latency of first visit to arms (Fig. 21, left). There are significant differences between groups ($F_{3,19} = 6.17$, $p < 0.004$). C3H male mice were significantly different from C3H female mice ($p < 0.007$) and from both C57 male ($p < 0.006$) and female mice (0.01).

2.8.3.2. Frequency of visits to arms (Fig. 21, right). There are significant differences between groups ($F_{3,19} = 19.80$, $p < 0.0001$). C3H male and females mice visited less arms than C57 male and females mice ($p < 0.0004$).

2.8.3.3. Duration of visits to arms (Fig. 22, top-left). There are significant differences between groups ($F_{3,19} = 13.26$, $p < 0.0001$). C3H males spent less time in the arms than C3H female ($p < 0.003$) and both C57 male and female mice ($p < 0.0003$). C3H female mice spent less time than C57 female mice, but this is not significant ($p = 0.09$).

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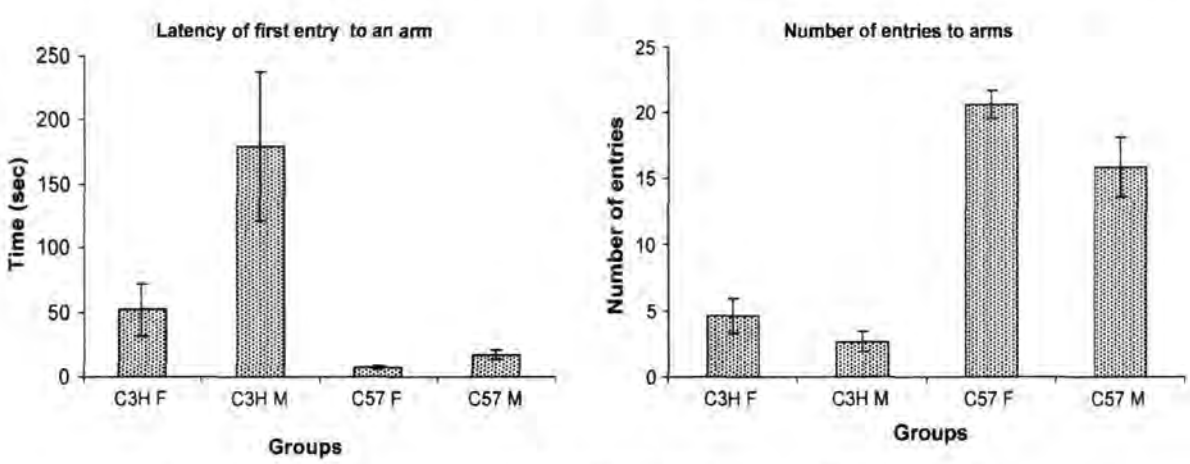

Fig. 21. Latency of first visit to an arm (left) and frequency of visits to arms. F: females; M: males.

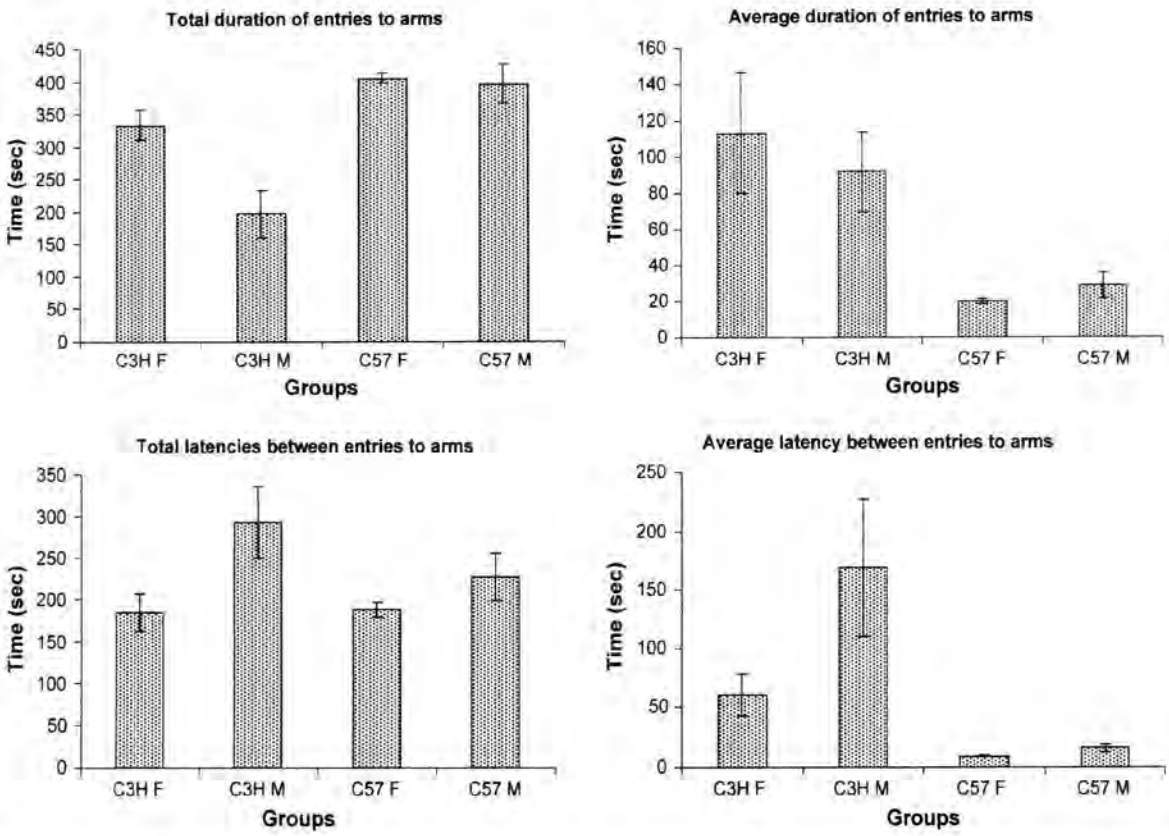

Fig. 22. Total duration (top-left) and average duration of visits to arms (top-right) and total latency (bottom-left) and average latency (bottom-right) between visits to arms. F: females; M: males.

and the drive to escape or avoid. This claim makes an assumption about animals' intentions and needs to explore the source of threat. The spontaneous and natural response of animals is to escape to enclosed spaces and avoid novel unfamiliar open spaces and objects. In order to induce and measure anxiety, animals need to be challenged with stimuli that they have to explore and cannot escape or avoid. This seems to us an obvious way to bring about a conflict in animals. There are numerous situations where we may feel anxious such as in exams, job interviews, public oral communications, and sport competitions. We can also avoid or escape these challenging events by staying at home or with friends. It is possible, however, for anxiety to be expressed before exposure to an anxiogenic environment or after escape or avoidance. In this case, escape or avoidance did not terminate the manifestation of fear but it becomes rather associated with the need to approach the source of threat—not facing a challenge and worrying about consequences. This form of anxiety is not currently accessible to testing in animals as it involves episodic memory through recollection of stressful events.

The present studies provide an open space model of anxiety based on exploratory activity of mice in a 3D maze which is a spatial navigation apparatus that was recently introduced by our lab for testing working memory from different view perspectives [17]. Animals can be exposed to the apparatus for a unique session or directly introduced to a memory test without the usual habituation phase. The unique session as well as the first three sessions of the memory test could reflect emotional responses of animal to novelty in an open space. These emotional responses would subside over time with animals' progression in learning about the conditions and requirements of the test.

In this novel model, animals are introduced to the central platform of the test apparatus and are left free to explore. Because it is an unfamiliar open space animals may feel unsafe in the central platform and would need to explore the maze in order to find an escape route. Some mice may be more anxious than others and would avoid entering the bridges, while others may adventure no further than the bridges whereas other mice may visit a few arms. These apparent differences in maze exploration would reflect different degrees of emotional responses in animals. In addition, the test provides a wide range of measurements of the transition responses between the different parts of the maze, such as latencies, duration and number of entries to bridges and arms which can be useful in the analysis and interpretations of the results.

In the first experiment, different groups of C57 mice were each exposed to one of the three arm configurations of the maze. Contrary to our expectations, this strain of mice explored the different areas of the maze and did not hesitate to visit the arms in each maze configuration. Significant differences between the three maze configurations were observed on a limited number of measurements which seem to indicate a preference for raised arms. Results from the second experiment demonstrate significant differences between maze configurations in C3H mice but not in C57 mice. C3H mice displayed a very low level of exploratory activity in the maze and showed a preference for

of the one-trial phenomenon are mostly based on speculative interpretations; they are not based on experimental evidence in which the internal validity of the test was challenged. It is very likely that open-arm exploration would be increased by an introduction of novelty in these arms. Behavioural experiments assessing the internal validity of a behavioural test are very scarce; a few examples can be found in Ennaceur and Aggleton [81], Miller and Murphy [164] and in Klebaur and Bardo [141] reports. The one-trial phenomenon provides further evidence in support of our view that the current two-choice models involve fear inducing escape or avoidance responses and that the drive to explore the source of potential threat which is assumed by many scientists to be the source of conflict inducing anxiety becomes less evident on the second exposure to the test. In the 3D maze, most arms remain unexplored after the first session of the test in Balb/c and C3H mice, and even the least emotional mice such as C57 and CD1 mice would have visited only a few arms (unique visits). Therefore repeated exposures do not prevent mice from attempting to adventure in unexplored and unprotected areas of the maze. In the present experiment, mice were food deprived but we have now data (not shown here) confirming the present results with non food-deprived C57 and Balb/c mice.

The final experiment confirms, furthermore, the difference between C3H and C57 mice. In addition, it demonstrates that male C3H mice are more anxious than C3H female mice. C3H males took longer time to visit an arm for the first time and to return to arms. They made a very few number of visits to arms and spent a very short amount of time in the arms of the maze. There are no differences between genders in C57 mice. These differences between strains and between genders reveal the sensitivity and consistencies of the different measures that we have selected for assessing anxiety. The reduced anxiety in female C3H mice in this study and in different strains of mice and rats in other studies [2,83,84,105,193,196,259,273] contrasts with the prevalence of anxiety and depression in human females [186]. While the ecological context of both species may account for this difference, sex differences in human studies refers mainly to clinical subjects [186] and it is possible that clinical diagnostics do not discriminate clearly between anxiety and other emotional disorders. However, sex differences in animal studies are mostly contradictory [34,42,55,89,131,98,154,269,240,259]. In order to compare to sex differences data reported in clinical studies, there is a need to perform experiments in animals expressing anxiety induced by anxiogenic compounds, or by physiological or genetic manipulations.

The results of the present studies suggest that C57 mice are less anxious than C3H and Balb/c mice when exposed to the 3D maze. Unfortunately, it is not possible to draw direct comparisons between Balb/c and C3H as they were not tested in the same experiment, though our observations and the current data suggest that the former are less anxious than the latter. Numerous studies from our laboratory confirm the differences between these strains of mice in the 3D maze with C57 mice being less anxious than Balb/c and C3H while Balb/c are less anxious than C3H (data not shown). The consistencies of our results with these strains of mice in the 3D maze contrasts significantly with the inconsistencies of the results reported in the literature for the same strains of mice. In some reports, Balb/c mice seem more anxious than C57 while in other reports it is completely the opposite [11,23,48,79,108,144,150,181,202,251,271]. Whether these inconsistencies and contradictions arise from the use of the same test or from comparable tests aimed at anxiety, they clearly raise some doubts about the construct validity of these tests: are they measuring the same construct? In many reports the difference observed between different behavioural tests designed for anxiety and lack of correlations between these tests is too easily accounted for by a simplistic view that the tests are tapping into different states or forms of anxiety disorders [5,11,26,33,109,113,144,253]. The discriminant validity of these tests is left unchallenged, that these tests are not measuring other constructs such as fear inducing escape or avoidance responses rather than fear inducing anxiety.

Numerous methodological concerns have been raised following the accumulation, over two decades, of inconsistent and contradictory results with the current models of anxiety. Unfortunately, the focus of most critical reviews was limited to sensitivity, predictive validity and reliability of the measures of the tests [22,36,69,116,122,130,132,133,142,163,199]. There are no direct references to the construct validity of the respective behavioural tests. It seems that any test is assumed to be a valid model of anxiety whenever it demonstrates sensitivity to benzodiazepines or to newer subtype-selective $GABA_A$ receptor compounds [36,103,108,160,167,172,183,189,190,195,203, 212,219,225,248]. In fact, the current theory of anxiety [35,159] is almost exclusively based on the sensitivity of a wide range of behavioural responses to anxiolytic compounds which were provided by clinical studies. The validity of a behavioural test is considered a correlation between a set of symptoms and the predicted effects of a neurochemical, physiological or genetic manipulations. The learning and cognitive hypothesis of anxiety [16,20,65,66,75,145,139,140,153,156,165,168,261] is rarely mentioned in this context, which is surprising considering the well documented effects on cognition induced by benzodiazepines and GABAA agonist compounds [21,15,44,53,54,100,171,174,221,236,238,268]. GABA is widely distributed and utilized throughout the CNS and it is involved in major behavioural and cognitive functions. There is no experimental evidence excluding a primary action of these compounds on cognition rather than anxiety.

A behavioural test is developed to provide objective assessments of a specific behaviour, emotion or cognitive function it is designed to measure. This means that a particular behaviour, emotion or cognitive response is translated to parameters of a variable to be quantified and then analyzed within the definition attributed beforehand to the significance of a specific behaviour, emotion or cognitive function. A wide range of parameters are therefore made available in the development of a test such as frequency, number of correct or incorrect responses, duration and distance of crossings or transitions. These parameters are challenged for content validity, discriminant validity and internal consistency to support the construct validity of the test [4,61,96,118,149,192]. Unfortunately, in current anxiety studies, scientists attempt to deal ambitiously with

would reveal the presence or absence of activation in these brain structures.

A behavioural test can also provide the means to manipulate the variable that it is concerned with and modify its expression and therefore its consequence on the brain. For instance, the emotional responses of animals in the 3D maze can be manipulated through separate or combined use of anxiolytics compounds and the introduction of stressors such as increased light intensity, sounds and predator odours. These manipulations are not meant to contribute to the construct validity of a behavioural test; they are not part of the test. They are external variables that are introduced to the test to examine their effects on behaviour within the established settings of the test—a norm or a standard of reference. A behavioural test has to be validated behaviourally before any of these manipulations can be performed. Any claim of validity of a behavioural test through pharmacological, physiological or genetic manipulations is inappropriate and illogical. These manipulations can be associated with the validity of animal models of human psychopathology and neurological disorders and not to animal models of human behaviour.

In the 3D maze, we do not induce symptoms but provide the conditions under which these symptoms might be expressed and appreciated. C57 mice seem less anxious and visited all arms of the maze while Balb/c and C3H appears more anxious as they were more hesitant and reluctant to visit the bridges and arms of the maze. Therefore, C57 as well as C3H and Balb/c can be used to screen for anxiolytic and anxiogenic compounds. For instance, administration of anxiolytic drugs would be expected to modify the behaviour of C3H and Balb/c to align to that of C57 mice while an anxiolytic effect on the behaviour of C57 mice would increase the number of bridge entries to equal the number of entries to arms while reducing latencies between entries to arms and bridges without reducing the number of entries to bridges. An anxiogenic compound would reduce the number of entries to arms and may increase the number of entries to bridges. It should not decrease the number of entries to bridges as such an effect can be assumed to be non-specific. Repeated entries to bridges with less or no entries to arms would reflect an increase in the number of hesitations of animals to engage in the arms of the maze which can be considered an expression of an anxiety state.

When assessing the validity of our test, we included a variety of parameters of measurements for the crossing responses made by mice in the different areas of the maze. We tried to cover all relevant dimensions of the measured variable to achieve content validity and challenge the internal consistency of the parameters of the test. Most of these parameters provided concordant results within each experiment and consistent results between experiments. The correlation analyses confirmed furthermore the strong association between the different parameters of the test which were used to assess anxiety responses in animals. An interesting feature of our behavioural model is the very low variability between individuals that is demonstrated in most of the parameters used to assess anxiety despite the test being based on spontaneous exploratory activity. The issue of inter-individual variability has been a subject of attention and debates [45,69,255,270]. Most scientists view that the main purpose of standardization is to minimize variability and to facilitate the reproduction of the results, however Würbel [270] seems to object to standardization, raising concerns about experiments turning into case studies due to loss of inter-individual variability and consequently resulting in a loss of external validity. This view is supported by Calatayud et al. [45] who consider that "inter-individual variability... should be seen as the essence of behaviour" and propose "to maximise inter-individual variability". These critical comments are ill founded; they contradict the basic fundamental principles of a test that is to provide reliable measures of behaviour. An increase in inter-individual variability would prevent reliability and consistency between results. If a test promotes inter-individual differences then predictability becomes impossible. In fact an increase in inter-individual variability would result inevitability in step-by-step selection of cases that fit with the expectations of the experimenter. This is illustrated by an increased number of studies who failed to provide consistent results and now resort to arbitrary sub-group selection within a specific strain of mice and rats. These studies are expected to reveal pathological cases that are highly unlikely to be produced by a behavioural test in normal animals. Large inter-individual variability would question the validity of the measurements of a behavioural test. For instance, the latency of first entry to a bridge in the 3D maze produces larger inter-individual variability than other measurements and this accounts for the poor correlation of this measure with the remaining measures of emotional responses of mice (see Table 1). This inter-individual variability is often observed with the measure of latency of first entry to the centre in the open-field or to the lit compartment in the light–dark box [83,116]. It is mainly due to the difficulty in controlling and standardizing the release of animals in the apparatus. It can be reduced when animals are poured from a bucket onto the central platform instead of being released by hand but this still may not be sufficient to produce consistent results.

The 3D maze was used in the present studies as an open space model of anxiety in support of a previous report in which we suggested that the current models of anxiety involve separate open spaces and enclosed spaces and therefore would involve fear-inducing escape or avoidance rather than anxiety per se. In experiment 4 we tried to demonstrate that the presence of an enclosed space would encourage animals to remain in an enclosed central platform and would avoid entries to bridges and arms. Unfortunately, C57 mice were inappropriate for this demonstration as they were shown to be the least fearful strain of mice. Balb/c and C3H would have been much more appropriate for this purpose. However, the experiment still demonstrates that the presence of an enclosed space affects the behaviour of mice. C57 mice took longer time to make a first entry to an arm, made more visits to bridges before first entry to an arm and required longer time between re-entries to arms, spent longer time on the central platform and shorter time on arms compared to mice tested in an open central platform. They also made frequent re-entries to the centre and bridges compared to mice in the lowered arm with an open central platform. These results suggest that the presence of an enclosed space alongside an open space promotes avoidance responses.

In conclusion, the present report provides the first model of unconditioned behaviour which involves a conflict between exploration and fear inherent to the nature of the test. It demonstrates that animals released in an unenclosed central platform display clear differential anxiety responses dependent on sex and strains, providing an important new model for future investigation of the underlying neural systems involved in anxiety and screening for anxiolytic therapeutics. Through this report, we attempted to challenge the current models of anxiety that are used in animal studies and to trigger a wide ranging debate regarding basic methodological issues in clinical and laboratory studies, focusing on the construct validity of the techniques used to assess affective states.

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Research report

Detailed analysis of the behavior and memory performance of middle-aged male and female CD-1 mice in a 3D maze☆

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Abstract

Fifty percent of CD-1 mice from both sex die by the end of 2 years. The survival rate is higher in females than in males. This high mortality rate is associated to the high susceptibility of this strain of mice to some immuno-pathologies and the high incidence of systemic amyloidosis. It is therefore possible that premature cognitive deficits can be observed in CD-1 mice. In the present study, we describe a novel method for assessing emotional responses and memory performance of young (4 months) and middle-aged (12 months) CD-1 mice of both sexes in a 3D spatial navigation task. Animals are introduced to the maze without preliminary habituation and trained in a working memory test. As expected CD-1 mice have a low number of entries to arms on their first exposure to the maze which confirm our previous report on the anxious trait of this strain compared to C57/BL6 mice. The measure of arm/bridge ratio suggests that anxiety induced by exposure to the maze persists much longer in middle-aged male mice compared to middle-aged female mice and compared to both young male and female mice. The measure of memory revealed that young female mice made significantly less arm repeats and more unique arm visits before first arm repeat than middle-aged female and male mice. There are also significant differences between young female and young male mice with the former committing fewer errors than the latter.

Keywords: Aging; Alzheimer's; Amyloid; Amnesia; Hippocampus; Amygdala; Benzodiazepines; Strains; Rats

1. Introduction

In the present study we examined whether emotional responses to novelty and open spaces and the acquisition of a working memory spatial navigation task are affected by age in CD-1 strain of mice. We examined both male and female CD-1 mice from 4 and 12 months of age in a 3D maze which is a modification of the eight arm radial maze. In this maze, animals need to cross a bridge to reach an arm which can be presented horizontally raised above or lowered below the level of a central platform. The arms can also be presented at the same level of the central platform as in the standard version of a radial maze. There are no enclosures surrounding the central platform, bridges or arms. In our laboratory, animals are introduced to the maze without prior habituation. They can be assessed for their emotional responses to novelty and open spaces in a single or a few sessions without food deprivation [1,2]. They can also be food-deprived and tested in a working memory paradigm in 12–16 sessions and the first few sessions are assessed for emotional responses [3]. In our view, the first sessions of exposure to the test are likely to trigger emotional responses induced by novelty and unfamiliarity to the open space of the maze that would subside over time after repeated exposures while learning takes place and consolidates. Learning and memory processes are involved from the first encounter of a novel or a familiar environment however anxiety would be predominant in the first phase of the test and it is unlikely to persist after several exposures. The acquisition of a working memory task is evident at some later stage of the training period; it is not in place on the first sessions as animals need to learn the rule of the task. Therefore, it is possible to assess emotional responses and working memory performance within the same experimental settings

☆ Animals used in the study were handled in accordance with approved use of animals in scientific procedures regulated by the Animals (Scientific Procedures) Act 1986, UK.

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and testing conditions. The introduction of animals to a learning and memory test without preliminary habituation can be useful in understanding the difference between strains and the consequence of a drug treatment on performance. Based on the current study and previous one, we have shown that different strains of mice require different number of exposures to habituate to the experimental settings. Furthermore, a drug treatment may extend the period of time required to habituate for some strains of mice. Therefore, in many published studies anxiety may still be present within the initial stages of the acquisition of a learning or a memory task which could influence the interpretation of drug effects.

A large number of CD-1 mice have been shown to display tumors, skin lesions and abscesses and die earlier than observed in other strains of mice [4–7]. A high incidence of systemic amyloidosis in CD-1 mice was reported [8,86]. Amyloidosis was observed in 50% of CD-1 mice of both sexes, beginning at 8 months in males and 12 months in females [8]. It was found to be higher than in other strains of mice and as one of the major factors contributory to death in aging CD-1 mice [9,94]. Numerous studies suggest that more than 50% of CD-1 mice from both sex die by the end of 2 years. The survival rate was higher in females than in males [4,5,7,10,11]. It is therefore possible that this strain of mice also develops premature cognitive deficits and would be useful as a model of aging and for screening for drug and therapeutic manipulations. A recent study from our laboratory demonstrates that middle-aged CD-1 female mice (12 months) made significantly more errors than young CD-1 female mice (3 months) in a 3D maze [3].

Very few studies have considered other parameters than the number of errors (or correct choices) in the radial maze. This is very unfortunate as performance of animals can be accounted for by other parameters than the number of repeated arm choices. The records and data analysis of several spatio-temporal parameters of a behavioral task contribute to its content validity and are very helpful in the interpretation of animal behavior [1,12]. In the present experiment, we recorded not only the number of visits to bridges and arms of the maze but also the latencies to bridges and arm entries, and duration of visits. Some parameters are related to anxiety responses while others are related to learning and memory. Measures of anxiety do not preclude an involvement of learning and memory processes. Therefore some measures are shared between measures of anxiety and memory while others are more specific to either one. Correlation analysis between the different parameters of our spatial navigation task is performed to demonstrate the concordances and consistencies of the results obtained in this experiment.

2. Methods

2.1. Animals

Thirty-three mice from CD-1 mice strains were obtained from our breeding colony. They were 8 young females (FY, 4 months), 10 middle-aged females (FA, 12 months), 8 young males (MY, 4 months) and 7 middle-aged males (MA, 12 months). The average animal weight was 42.32 ± 2.52 g at the start of the experiment. The colony room was held under a 12 h light/12 h dark cycle (light 07:00–19:00 h at 180 lx) and at 23 ± 1 °C. In order to avoid unequal light exposure, the upper shelf was occupied with empty plastic cages filled with sawdust. Mice were housed in a group of five mice per cage. Individual mice could be identified by their cage number and their ear tags. All mice had *ad libitum* access to food and water. During their stay in respective housing conditions, they were removed twice a week from their cages for cleaning the cages and renewing their food and water supply. Animals were food-deprived 24 h before the testing began; their body weights were subsequently adjusted and maintained to 85% of their normal free-feeding weights throughout the test. Animal treatment and husbandry were in accordance with approved use of animals in scientific procedures regulated by the Animals (Scientific Procedures) Act 1986, UK.

Fig. 1. 3D maze with a raised arm configuration. Note the narrowing of the entries to and exits from the bridges to prevent mice relying entirely on sequential arm choices.

2.2. Apparatus

The maze is made from grey PVC (5 mm thick). It consists of eight arms radiating from a central platform (Fig. 1). Each arm (51 cm × 11.2 cm) is made from two segments, extended from an octagonal shaped central hub (30 cm in diameter) and can be manipulated independently. The first segment of an arm (15.2 cm × 11.2 cm) directly attached to the central platform was tilted to form a 60° slope and constitutes a bridge that allows access to the second segment (35 cm × 11.2 cm) of the arm which is presented horizontally either at the same (flat arm configuration), below (lowered arm configuration) or above (raised arm configuration) the level of a central platform. In order to change the configurations of the maze, the central platform can be raised or lowered. Each entry to a bridge is narrowed with a short wiremesh wall (8 cm × 4 cm) either on the left or on the right side. The narrowing of the entries to and exits from the bridges are designed to prevent mice relying entirely on sequential arm choices. The end of each arm is extended with panels of identical size (20.2 cm × 11.2 cm). These panels are used for holding intra-maze cues made of distinctive pattern drawings designed on plastic adhesive material and attached to a PVC board (18 cm × 11.2 cm). Sidewalls, about 1 cm high, extended the length of each arm. There are no enclosures surrounding the central platform, bridges or arms. The maze is totally surrounded with a heavy beige-light colored curtain. A camera is centred 180 cm above the central platform. The ambient light at the surface of the central platform is 180 lx.

2.3. Testing procedures

Naïve food-deprived mice were introduced to the maze to acquire a spatial working memory task. They had no previous experience or habituation to the maze. They are placed in the central platform and let free to explore the maze until eight arm choices are made or 10 min elapsed. One food pellet is available

at the end of each arm. A first visit to an arm is a recorded as unique visit (correct choice) and any subsequent visit is considered as a repeated choice or incorrect choice. A mouse can make up to seven repeated visits to the same arm. Animals are tested one session a day on 16 consecutive days.

2.4. Tools and recording measures

During the test, mice were observed on a screen monitor connected to a video camera suspended above the test arena. The video record of the experiment is run on a computer screen monitor through a capture card. The experiment is also video taped, the experimenter can review the VCR tape and check the accuracy of the recording at any time. An in-house computer program is used to record the frequency of entries as well as the duration and latencies of entries of pre-defined areas of the maze.

2.5. Measurements and statistical analysis

Several measurements were considered. A session last until eight choices are made or 10 min elapsed. An entry to an arm or a bridge is recorded whenever a mouse crosses with all four paws the line that delimits these areas.

(1) *Number of bridge and arm entries*: An entry onto a bridge is recorded only once if a mouse enters a bridge and continues to an arm. A return from an arm to a bridge within the same visit is not recorded.
(2) *Arm/bridge ratio*: Number of arm entries divided by the number of bridges entries. A ratio close to 1 describes an animal that continued to an arm each time it visited a bridge. A ratio close to 0 describes an animal that made numerous visits to bridges before engaging onto an arm. In the former, the animal showed no hesitations or fewer hesitations to engage fully onto an arm whereas in the latter the animal demonstrated more hesitations before adventuring onto an arm.
(3) *Average number of entries onto bridges between arm visits*: This measure examines the average number of entries onto the bridges between successive visits to arms.
(4) *Number of bridge entries before first arm visit*: The total number of bridge entries is recorded for those animals that did not visit any arm.
(5) *Number of bridge entries before first repeated arm visit*: A repeated entry onto an arm is considered as an incorrect choice or an error (see *Number of repeated visits to arms*). In the first few sessions some animals visit no arms or a fewer number of arms (made less than eight choices). One bridge entry is recorded for animals that did not visit any arm (a penalty). The total number of bridge entries is recorded for those animals that made less than eight arm choices.
(6) *Number of arm entries before first repeated arm visit*: An arm that is visited only once during a session is considered as a unique entry onto that arm (see *Number of repeated visits to arms*). A mouse that did not visit any arm will be recorded as having made zero unique visits before first repeat. A mouse that has made less than eight choices with no repeat will be recorded as having made his first repeated choice after his last arm visit.
(7) *Number of repeated visits to arms*: This measure refers to incorrect choices or errors. An arm that is visited only once during a session is considered as a unique entry onto that arm (a correct choice). A session last until eight choices are made or 10 min elapsed. Each entry onto an arm is considered as one choice. An animal can make up to eight visits to the same arm. The first visit is considered a unique visit or a correct choice and subsequent visits are considered repeated choice or incorrect choices. In the first few sessions a mouse may visit a few arms or may not visit any arm. In this case, a mouse that did not visit any arm will receive a penalty of eight repeated visits. For a mouse that has made less than eight choices, the number of the remaining arm choices is added to their actual number of repeated arm visits. For example, a mouse that has made five choices and two repeat visits to arms will be recorded having made three unique visits and five repeated visited (the actual 2 repeats + the remaining arm choices 3). A mouse that has made five choices and zero repeats visits to arms will be recorded having made five unique visits and three repeated visited.

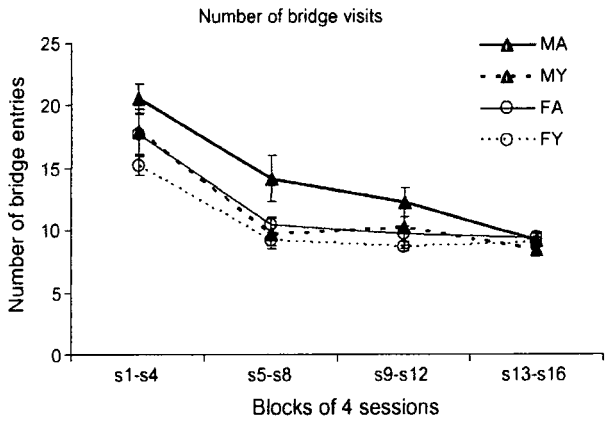


Fig. 2. In the second block of four sessions, young males (MY) and both young (FY) and middle-aged (FA) females compared to middle-aged males (MA), $p < 0.02$.

(8) *Number of sessions and unique arm visits to criterion*: A criterion is set to a total of five repeated choices maximum obtained in five consecutive sessions.
(9) *Latency of first entry onto a bridge*: The time spent by a mouse in the central platform before it enters for the first time onto one of the bridges with all four paws. A mouse that did not enter onto a bridge receives the highest score which is 10 min.
(10) *Latency of first entry onto an arm*: The time spent by a mouse in the central platform and bridges before it enters for the first time onto an arm. A mouse that did not enter onto any arm receives the highest score which is 10 min.
(11) *Latency of first repeated visit onto an arm*: The time spent by a mouse in a session before it makes a first repeat visit onto an arm. A mouse that did not enter onto any arm receives the lowest score which is 0 min.
(12) *Total latencies between exits from and entries onto arms*: The time is recorded when a mouse exits from an arm with all four paws until its next re-entry onto an arm (the same or another arm). A mouse that did not enter onto an arm receives the highest score which is 10 min.
(13) *Total duration of centre re-entries*: The time is recorded from the entry of a mouse with all four paws onto the central platform until it exits to a bridge.
(14) *Total duration of bridge entries*: The time is recorded from the entry of a mouse with all four paws onto a bridge until it exits to the central platform or onto an arm. If a mouse enters onto a bridge and continues to an arm, then the time on an arm is subtracted from the time spent on a bridge.
(15) *Total duration of arm entries*: The time is recorded from the entry of a mouse with all four paws onto an arm until it exits onto the bridge.
(16) *Session duration*: Animals are removed from the maze at the end of a session when 10 min elapsed or when animals return onto the central platform after eight choices were made. A choice can be any visit or repeated visit to an arm. It is highly recommended that animals are not removed from an arm but left to return to the central platform. Our experiments with a radial maze and a T-maze show that removing animals from the arms delay the acquisition of the test and can lead to some animals not learning the test.

Differences among group means for each of the above measurements were tested for significance with one-way ANOVA repeated measures. This was followed up with Newman–Keuls post hoc comparisons. Statistics were calculated using the statistical package Statistica for Windows (version 5.5). Results were considered significant when $p \leq 0.05$. When $p \leq 0.10$, it was reported and rounded up to the nearest value.

3. Results

(1) *Number of bridge and arm entries* (Fig. 2): One-way ANOVA repeated measures reveals significant differences between groups ($F3,29 = 3.44$, $p \leq 0.03$) on the number

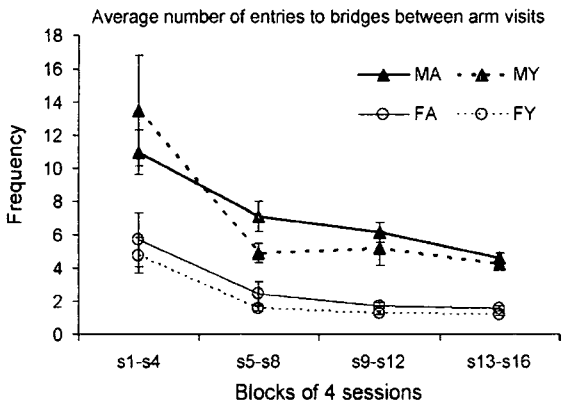


Fig. 3. FY and FA compared to MY and MA ($p < 0.03$) in each sessions' block.

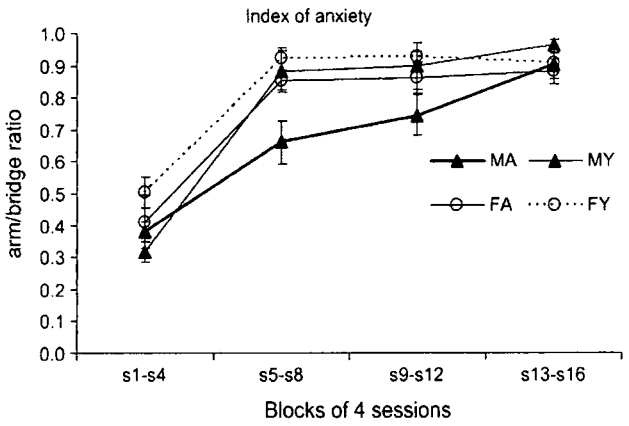


Fig. 4. In the second and third blocks of four sessions, MA compared to FA ($p < 0.002$ and <0.05, respectively), FY ($p < 0.001$ and <0.03, respectively) and MY ($p < 0.002$ and <0.05, respectively).

of bridge entries. Middle-aged males made more bridge entries than young ($p < 0.01$) and middle-aged ($p < 0.05$) females. They also made more entries to bridges than young males but these were not significant ($p < 0.08$).

There are also significant differences between sessions ($F3,87 = 81.02$, $p < 0.0001$) but no significant interaction between groups and sessions ($p > 0.10$) on the number of bridge visits. The number of bridge entries were significantly high in the first block of four sessions compared to the other three sessions' blocks ($p < 0.0001$) and they were significantly low in the last block of four sessions compared to the second ($p < 0.008$) and third ($p < 0.05$) sessions' block. Post hoc analysis shows that young males and both young and middle-aged females made less entries to bridges than middle-aged males ($p < 0.02$) in the second sessions' block.

The number of entries to arms shows significant differences between sessions ($p < 0.0001$) but not between groups ($p > 0.10$) and there are no significant interactions between groups and sessions ($p > 0.10$).

(2) *Arm/bridge ratio* (Fig. 4): There are significant differences between groups ($F3,29 = 3.30$, $p < 0.03$), differences between sessions ($F3,87 = 142.54$, $p < 0.0001$) and significant interactions ($F9,87 = 3.24$, $p < 0.002$). The arm/bridge ratio is significantly low in the first session block compared to all other sessions' blocks ($p < 0.0001$) and in the second block compared to the third block ($p = 0.003$) and the third block compared to the fourth block ($p < 0.03$).

Post hoc analysis shows that in the second and third session blocks middle-aged males had a lower arm/bridge ratio compared to middle-aged females ($p < 0.002$ and <0.05), young females ($p < 0.001$ and <0.03, respectively) and young males ($p < 0.002$ and <0.05).

(3) *Average number of bridge entries between arm visits* (Fig. 3): There are significant differences between groups ($F3,29 = 13.08$, $p \leq 0.0001$). Young and middle-aged females made significantly fewer entries on bridges between arm visits than young and middle-aged males ($p < 0.0007$).

There are also significant differences between session blocks ($F3,87 = 29.78$, $p \leq 0.0001$) but no significant interactions between groups and sessions' blocks ($F9,87 = 1.76$, $p \leq 0.09$). The average number of entries on bridges was significantly high in the first sessions' blocks compared to the other sessions' blocks ($p < 0.0001$).

Post hoc analysis shows significant differences between groups in each sessions' block ($p < 0.01$). Young and middle-aged females made less number of bridge entries than young and middle-aged males ($p < 0.03$).

(4) *Number of bridge entries before first arm visit* (Fig. 5): There are significant differences between groups ($F3,29 = 3.27$, $p < 0.04$). Young and middle-aged females entered a fewer bridges than middle-aged males ($p < 0.02$ and <0.05, respectively). There are no significant differences between young females and middle-aged females ($p > 0.10$) and between young male and middle-aged males ($p = 0.06$).

There are also significant differences between sessions ($F3,87 = 3.27$, $p < 0.0001$) but no significant interactions between groups and session blocks ($F9,87 = 0.84$, $p > 0.10$). The number of bridge visits is high in the first sessions' block compared to the other sessions' blocks ($p < 0.0001$).

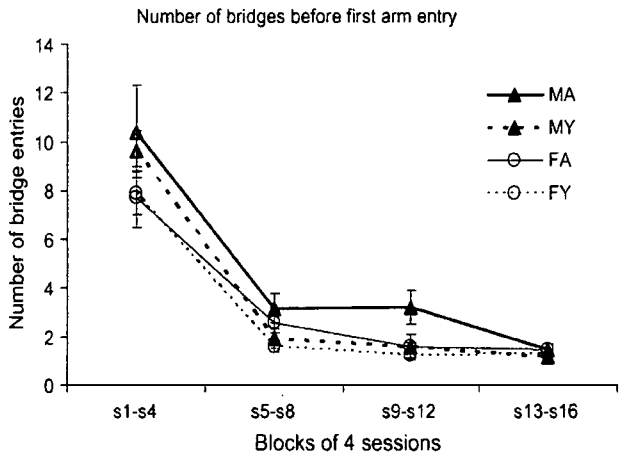


Fig. 5. In the third block of four sessions, FY and FA compared to MA ($p < 0.009$ and <0.02, respectively). MY compared to MA ($p < 0.009$).

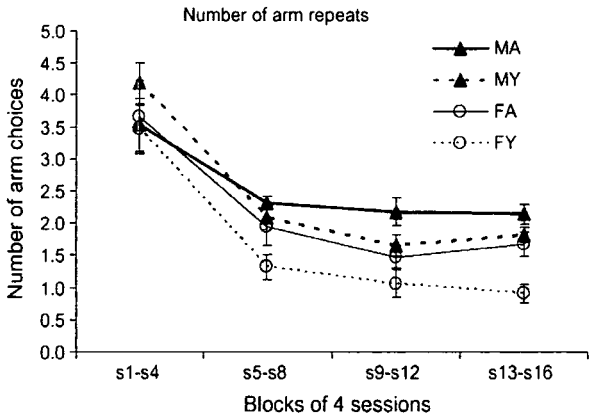


Fig. 6. FY compared to MA in the last three blocks of four sessions ($p < 0.02$). FY compared to MY in the second ($p = 0.06$), third ($p < 0.10$) and last ($p < 0.002$) sessions' blocks. FY compared to FA in the first ($p = 0.06$) and last ($p < 0.002$) sessions' blocks.

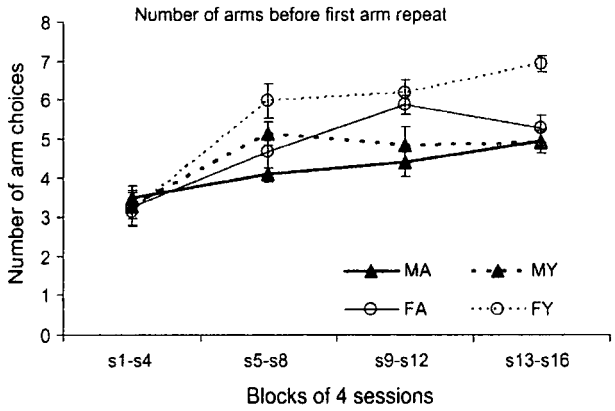


Fig. 7. FY compared to FA in the second ($p < 0.05$) and fourth ($p < 0.0008$) sessions' blocks. FY compared to MA in the last three blocks of four sessions ($p < 0.006$). FA compared to MY in the third sessions' block ($p < 0.04$).

Post hoc analysis shows that middle-aged males made more number of bridge visits compared to young males ($p < 0.009$) and compared to both young ($p < 0.009$) and middle-aged females ($p < 0.02$) in the third sessions' block. They also made more number of bridge visits compared to young females in the second sessions' block but this was not significant ($p < 0.06$).

(5) *Number of bridge entries before first arm repeat*: There are significant differences between sessions ($F3,87 = 36.89$, $p < 0.0001$) but no significant differences between groups ($F3,29 = 0.55$, $p > 0.10$) and no significant interactions ($F9,87 = 0.82$, $p > 0.10$). The number of bridge entries is significantly high in the first session block compared to the other blocks ($p < 0.0001$).

(6) *Number of repeated visits to arms* (Fig. 6): One-way ANOVA repeated measures revealed significant differences between groups ($F3,29 = 4.72$, $p \leq 0.008$). Young female mice made significantly a fewer errors compared to young male mice ($p < 0.01$) and to both middle-aged male ($p < 0.007$) and female ($p < 0.05$) mice.

There are also significant differences between sessions ($F3,87 = 56.47, p < 0.0001$) but there is no significant interaction between groups and sessions ($p > 0.10$). The number of errors in the first block of four sessions is significantly higher than any other session ($p < 0.0001$).

Post hoc analysis shows that the number of repeated visits to arms is significantly low in young females compared to middle-aged males ($p < 0.02$) in the last three sessions' blocks. It is also low in young females compared to young males but this is significant only in the last ($p < 0.002$) but not in the second ($p = 0.06$) and third ($p < 0.10$) sessions' blocks. Young female made less repeated visits to arms than middle-aged females in the first ($p = 0.06$) and last ($p < 0.002$) sessions' blocks.

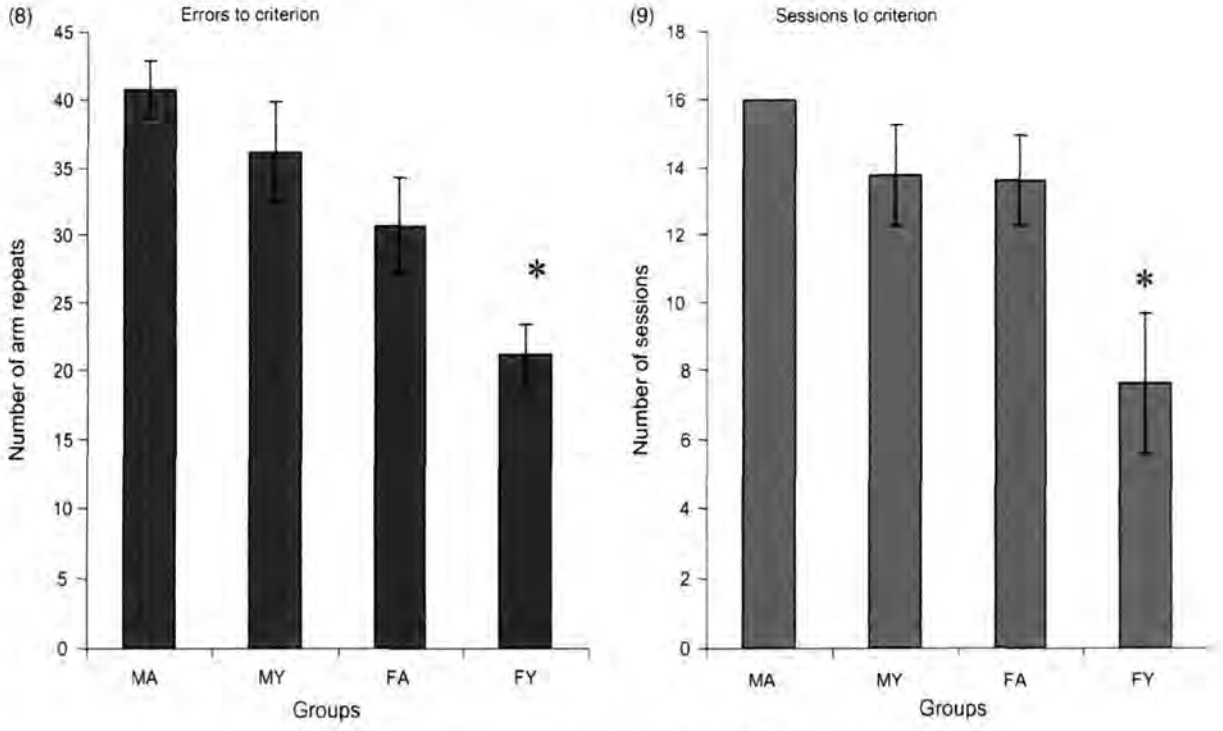
(7) *Number of arm entries before first arm repeat* (Fig. 7): One-way ANOVA reveals significant differences between groups on errors ($F3,29 = 5.43$, $p < 0.004$), between sessions ($F3,87 = 45.50$, $p < 0.0001$) and significant interactions between groups and sessions ($F9,87 = 45.50$, $p < 0.0008$).

The group differences are between young female and middle-aged female ($p < 0.03$) and between young female and both young ($p < 0.01$) and middle-aged males ($p = 0.002$). Young females made more arm entries before making their first arm repeat. The session differences are between the first block of four sessions and the remaining other blocks ($p < 0.0001$) and between the second and the last ($p < 0.04$) sessions' blocks. The second sessions' block is not significantly different from the third sessions' block ($p < 0.10$).

Post hoc analysis shows significant differences between groups on the second to the third blocks of four sessions ($p < 0.01$) but not on the first block ($p > 0.10$). The number of arm visits before first arm repeat is significantly high in young female compared to middle-aged female in the second ($p < 0.05$) and fourth ($p < 0.0008$) sessions' blocks. It is also significantly high in young females compared to middle-aged males in the last three blocks of four sessions ($p < 0.006$) and in middle-aged females compared to young males in the third sessions' block ($p < 0.04$).

(8) *Number of arm repeats* (Fig. 8) *and sessions to criterion* (Fig. 9): One-way ANOVA reveals significant differences between groups on the number of arm repeats ($F3,29 = 6.59$, $p < 0.002$) and sessions to criterion ($F3,29 = 5.50$, $p < 0.004$). Post hoc comparisons shows that young females made a few arm repeats to criterion and a few number of sessions to criterion compared to middle-aged females ($p < 0.04$), and to both young and middle-aged males ($p < 0.02$). Female middle-aged made less number of arm repeats than male middle-aged but this is not significant ($p < 0.09$).

(9) *Latency of first entry to a bridge*: There are significant differences between sessions ($F3,87 = 5.81$, $p \leq 0.001$), but no significant differences between groups ($p > 0.10$) and no significant interactions between groups and session blocks ($p > 0.10$). The latency of first entry to a bridge is significantly high in the first session block compared to the other blocks ($p < 0.02$).



Figs. 8 and 9. FY compared to FA ($p < 0.04$), and to both MY and MA ($p < 0.02$).

(10) *Latency of first entry to an arm*: There are significant differences between sessions ($F3,87 = 90.68, p \leq 0.0001$), but no significant differences between groups ($p > 0.10$) and no significant interactions between groups and session blocks ($p > 0.10$). The latency of first entry to an arm is significantly high in the first session block compared to the other blocks ($p < 0.0001$).

(11) *Latency of first repeated visit to an arm* (Fig. 10): There are significant differences between sessions ($F3,87 = 44.92$, $p \leq 0.0001$) and significant interactions between groups and session blocks ($F9,87 = 1.98$, $p \leq 0.05$) but no significant differences between groups ($F3,29 = 2.39$, $p \leq 0.09$). The latency of first repeated visit to an arm is significantly high in the first block of four sessions compared to the other blocks ($p < 0.0001$) and in the second block compared to third ($p < 0.04$) and fourth ($p < 0.001$) blocks. There is no significant difference between the third and fourth blocks ($p = 0.10$).

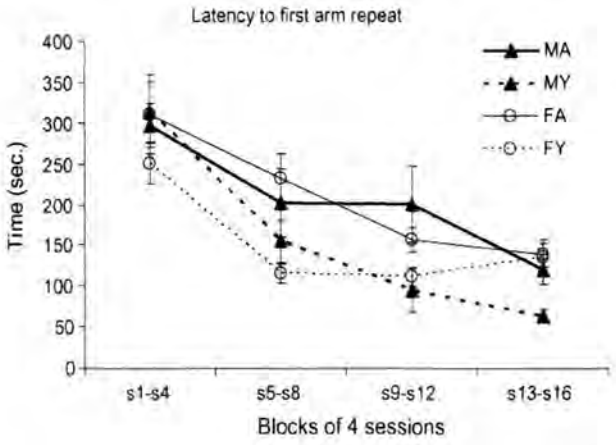


Fig. 10. FY compared to FA in the second sessions' block ($p < 0.03$) and to MA in the third sessions' block ($p = 0.05$). FY compared to MY ($p < 0.01$) in the last sessions' block. MY compared to MA in the third sessions' block ($p < 0.04$) and compared to both MA ($p < 0.02$) and FA ($p < 0.01$) in the last sessions' block.

Post hoc analysis shows that the latency of first repeated visit to an arm is shorter in young females compared to middle-aged females in the second block of four sessions ($p < 0.03$) and to middle-aged males in the third sessions' block ($p = 0.05$). It is, however, longer in the last sessions' block in young females compared to young males ($p < 0.01$). The latency of first repeated visit to an arm is significantly shorter in young males compared to middle-aged males in the third sessions' block ($p < 0.04$) and compared to both middle-aged males ($p < 0.02$) and females ($p < 0.01$) in the last sessions' block.

(12) *Total latencies between exits from and entries to arms* (Fig. 11): There are significant differences between groups ($F3,29 = 4.19$, $p \leq 0.01$). Middle-aged males spent significantly longer time between entries to arms than young males ($p < 0.005$) and middle-aged females ($p < 0.01$).

There are also significant differences between session blocks ($F3,87 = 195.05$, $p \leq 0.0001$) and significant interactions between groups and session blocks ($F9,87 = 3.51$, $p \leq 0.0009$). The latency of re-entry to arms is significantly high in the first session block compared to the other blocks ($p < 0.0001$) and in the second session block compared to the third ($p < 0.01$) of fourth session block ($p < 0.05$).

Post hoc analysis shows that the latencies between arms were significantly low in young females compared to young males ($p < 0.04$) in the first session block and compared to middle-aged males in the second ($p < 0.002$) and

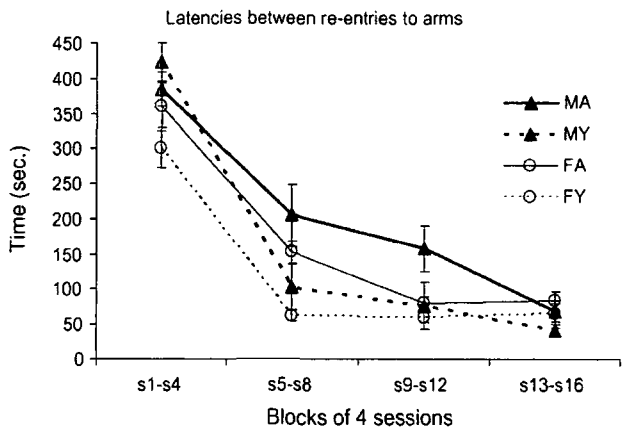


Fig. 11. FY compared to MY ($p < 0.04$) in the first session block and compared to MA in the second ($p < 0.002$) and third ($p < 0.01$) session blocks. FY compared to FA in the second block ($p < 0.04$). MY compared to MA in the third session block ($p < 0.01$).

third ($p < 0.01$) session blocks. Young females spent significantly less time than middle-aged females in the second block ($p < 0.04$) and young males spent significantly less time than middle-aged males in the third session block ($p < 0.01$).

(13) *Total duration of centre re-entries* (Fig. 12): There are significant differences between groups ($F3,29 = 3.36$, $p < 0.03$). Young females spent significantly less time on the central platform compared to middle-aged female ($p < 0.03$) but not significant compared to young ($p = 0.10$) and middle-aged ($p < 0.07$) males.

There are also significant differences between sessions ($F3,87 = 159.61$, $p < 0.0001$) and significant interactions between groups and sessions' blocks ($F9,87 = 3.86$, $p < 0.0004$). The time spent on the central platform is significantly high in the first sessions' block compared to the other sessions' blocks ($p < 0.0001$) and in the second sessions' block compared to the third ($p < 0.02$) of fourth ($p < 0.003$) sessions' blocks.

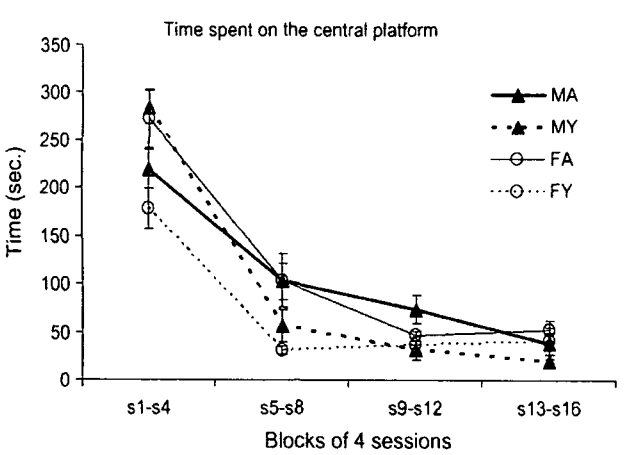


Fig. 12. FY compared to FA and MY in the first sessions' block ($p < 0.03$) and compared to both FA and MA in the second sessions' block ($p < 0.04$). MA compared to MY and FY ($p < 0.03$) and to FA ($p < 0.05$) in the third sessions' block.

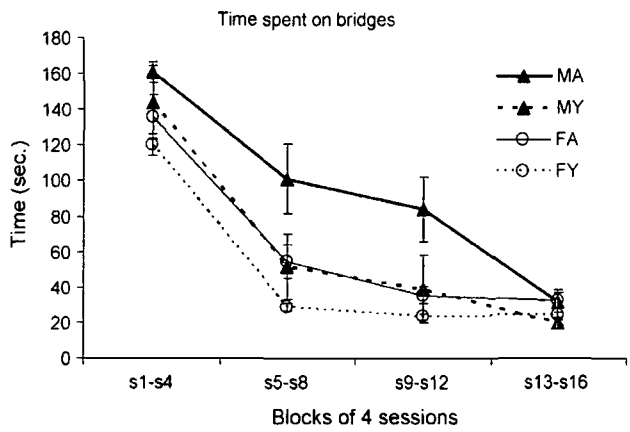


Fig. 13. MA compared to MY ($p < 0.04$) and to both FY ($p < 0.01$) and FA ($p < 0.03$) in the second and third sessions' blocks.

Post hoc analysis show that young females spent less time on the central platform compared to young males and middle-aged females in the first sessions' block ($p < 0.03$) and compared to both middle-aged males and middle-aged females in the second sessions' block ($p < 0.04$). In the third sessions' block middle-aged males spent significantly more time on the central platform compared to young males and young females ($p < 0.03$) and compared to middle-aged females ($p < 0.05$).

(14) *Total duration of bridge entries* (Fig. 13): There are significant differences between groups ($F3,29 = 4.78$, $p < 0.008$). The time spent on the bridges is significantly high in middle-aged males compared to middle-aged females ($p < 0.02$), and compared to both young males ($p < 0.04$) and young females ($p < 0.003$).

There are also significant differences between sessions ($F3,87 = 118.21$, $p < 0.0001$) but no significant interactions between groups and sessions' blocks ($F9,87 = 1.86$, $p < 0.07$). The time spent on the bridges is high in the first sessions' block and decreases significantly between each subsequent sessions' block ($p < 0.005$).

Post hoc analysis shows that the above differences between groups are observed in the second and third sessions' blocks. Middle-aged males spent longer time on the bridges compared to young males ($p < 0.04$) and to both young ($p < 0.01$) and middle-aged ($p < 0.03$) females.

(15) *Total duration of arm entries* (Fig. 14): There are significant differences between groups ($F3,29 = 2.86$, $p < 0.05$), between sessions' blocks ($F3,87 = 2.99$, $p < 0.04$) and significant interactions between groups and sessions' blocks ($F9,87 = 2.74$, $p < 0.007$).

The overall analysis shows that the time spent on the arms is significantly high in middle-aged females compared to young males ($p < 0.05$). The time spent on the arms is significantly high in the second sessions' block compared to the last sessions' block ($p < 0.04$). It is low in the first sessions' block compared to the second sessions' block but is not significant ($p = 0.06$).

Post hoc analysis shows the difference between middle-aged females and young males on the second and last

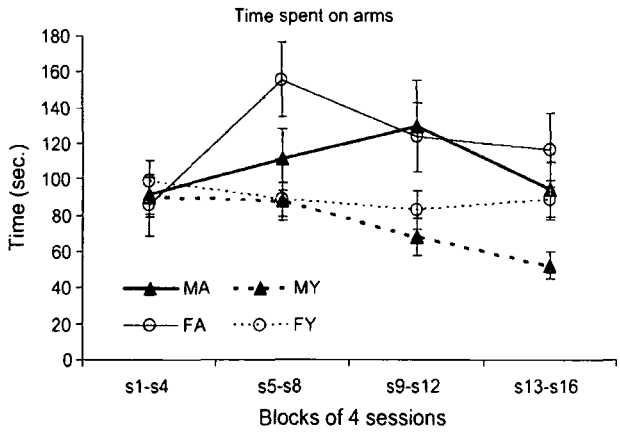


Fig. 14. FA compared to MY in the second and last sessions' blocks ($p < 0.03$). FA compared to MA in the second sessions' block ($p < 0.06$) and compared to MY in the third sessions' block ($p < 0.09$). MA compared to MY in the third sessions' block ($p < 0.09$).

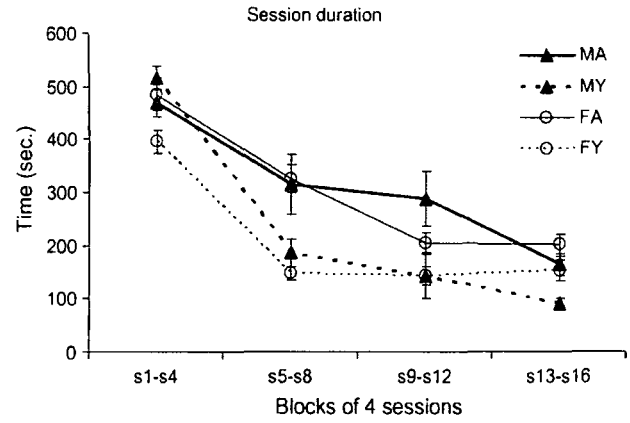


Fig. 15. FY compared to FA and MA in the first ($p < 0.07$) and second ($p < 0.002$) sessions' blocks. FY compared to MY in the first ($p < 0.02$) and fourth ($p < 0.03$) sessions' blocks and compared to MA ($p < 0.02$) in the third sessions' block. MY compared to MA in the second ($p = 0.007$), third ($p < 0.01$) and fourth ($p < 0.03$) sessions' blocks. MY compared to FA in the second ($p < 0.01$) and fourth ($p = 0.002$) sessions' blocks.

sessions' blocks ($p < 0.03$). Middle-aged females spent more time on the arms compared to middle-aged males in the second sessions' block ($p < 0.06$) and compared to young males in the third sessions' block ($p < 0.09$) but this is not significant. Middle-aged males spent also longer time on the arms compared to young males in the third sessions' block but this is not significant ($p < 0.09$).

(16) *Total duration of unique visits to arms*: There are no significant differences between groups ($F3,29 = 2.70$, $p = 0.06$) and no significant differences between sessions' blocks ($F3,87 = 2.23$, $p = 0.09$) but significant interactions between groups and sessions' blocks ($F9,87 = 2.38$, $p < 0.01$).

(17) *Total duration of repeated visits to arms*: There are no significant differences between groups ($F3,29 = 1.85$, $p > 0.10$) but significant differences between sessions' blocks ($F3,87 = 3.73$, $p < 0.01$) and no significant interactions between groups and sessions' blocks ($F9,87 = 1.85$, $p < 0.07$).

(18) *Session duration* (Fig. 15): There are significant differences between groups ($F3,29 = 5.75$, $p < 0.003$). Young males and females made significantly less time to perform eight choices in the maze than middle-aged males and middle-aged females ($p < 0.03$).

There are also significant differences between sessions' blocks ($F3,87 = 160.57$, $p < 0.0001$) and significant interactions between groups and sessions' blocks ($F9,87 = 5.12$, $p < 0.0001$). The time spent on the maze is high in the first sessions' block and decreases significantly between each subsequent sessions' block ($p < 0.008$).

Post hoc analysis shows that young females finished the test faster than middle-aged males and middle-aged females in the first sessions' block ($p < 0.07$) and this is significant in the second sessions' block ($p < 0.002$). Young females are also significantly faster than young males in the first ($p < 0.02$) and fourth ($p < 0.03$) sessions' blocks and than middle-aged males in the third sessions' block ($p < 0.02$). Young males were faster than middle-aged males in the second ($p = 0.007$), third ($p < 0.01$) and fourth ($p < 0.03$) sessions' blocks. They were also faster than middle-aged females in the second ($p < 0.01$) and fourth ($p = 0.002$) sessions' blocks.

3.1. Correlation analyses

Table 1A describes the correlations between all parameters of the tests for the total number of 16 sessions. It shows that the memory indices (number of arm repeats, number of unique arm visits before first arm repeats, number arm repeats to criterion and sessions to criterion) are strongly correlated to each other ($r = 0.58$, $p < 0.0004$ to $r = 0.92$, $p < 0.0001$). Number of arm visits before first arm repeat correlates negatively with number of arm repeats ($r = -0.85$, $p < 0.0001$), with arm repeats to criterion ($r = -0.84$, $p < 0.0001$) and sessions to criterion ($r = -0.79$, $p < 0.0001$), with duration in bridges ($r = -0.42$, $p < 0.02$), with number of bridges before first arm visit ($r = -0.38$, $p < 0.03$), with number of bridges ($r = -0.36$, $p < 0.04$) and with central platform entries ($r = -0.36$, $p < 0.04$). Number of arm repeats correlates positively with number of arm repeat to criterion ($r = 0.91$, $p < 0.0001$) and sessions to criterion ($r = 0.58$, $p < 0.0004$), latency to first entry on an arm ($r = 0.56$, $p < 0.0008$), number of bridge before first arm visit ($r = 0.50$, $p < 0.003$) and it correlates negatively with number of bridges before first arm repeat ($r = -0.34$, $p < 0.05$) and number of arm visits ($r = -0.54$, $p < 0.001$). Number of arm repeats to criterion correlates negatively with number of arms before first arm repeat ($r = -0.84$, $p < 0.0001$) and it correlates positively with number of arm repeats ($r = 0.91$, $p < 0.0001$), number of sessions to criterion ($r = 0.76$, $p < 0.0001$), number of bridge visits ($r = 0.41$, $p < 0.02$), latency to first arm visit ($r = 0.36$, $p < 0.04$), and number of bridge before first arm visit ($r = 0.44$, $p < 0.01$). Number of sessions to criterion correlates negatively with number of arm before first arm repeat ($r = -0.79$, $p < 0.0001$) and it correlates positively with number of arm repeats ($r = 0.59$, $p < 0.0003$), with number of arm repeats to criterion ($r = 0.76$, $p < 0.0001$).

Table 1A–C
Correlations between the different measures of the test based on the average values of 16 sessions (A), first block of 4 sessions (B) and last block of 4 sessions (C).

A	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
2	0.05																	
3	**-0.89**	0.24																
4	**0.60**	**-0.41**	**-0.68**															
5	**0.80**	0.24	**-0.72**	*0.29*														
6	**-0.36**	0.18	*0.31*	**-0.38**	0.20													
7	0.18	**-0.54**	-0.27	**0.50**	**-0.34**	**-0.85**												
8	0.27	*-0.31*	-0.27	**0.44**	-0.23	**-0.84**	**0.91**											
9	0.26	0.15	-0.14	0.15	-0.15	**-0.79**	**0.58**	**0.76**										
10	*-0.31*	**-0.35**	0.05	0.01	**-0.36**	-0.06	0.25	0.19	0.11									
11	0.11	**-0.81**	**-0.42**	**0.60**	-0.12	-0.25	**0.56**	**0.36**	0.00	**0.55**								
12	**0.63**	0.04	**-0.59**	0.21	**0.61**	-0.13	0.07	0.17	0.18	0.18	0.19							
13	0.27	0.12	*-0.31*	-0.11	**0.49**	0.20	-0.18	-0.10	-0.05	0.25	0.09	**0.80**						
14	0.28	**-0.52**	**-0.45**	0.27	0.17	-0.17	*0.32*	0.20	0.06	**0.52**	**0.67**	**0.71**	**0.54**					
15	**0.86**	-0.07	**-0.82**	**0.46**	**0.65**	**-0.42**	*0.33*	**0.41**	*0.31*	-0.09	0.24	**0.78**	**0.53**	**0.46**				
16	-0.13	0.16	0.10	*-0.33*	0.03	0.12	-0.11	-0.05	0.01	0.21	-0.06	*0.31*	**0.72**	0.14	0.19			
17	-0.13	0.09	0.07	-0.32	0.09	0.24	-0.20	-0.16	-0.13	0.20	-0.01	**0.34**	**0.77**	0.20	0.18	**0.97**		
18	-0.06	*0.34*	0.15	-0.19	-0.18	**-0.37**	0.24	**0.39**	**0.49**	0.10	-0.21	0.05	0.18	-0.16	0.18	**0.61**	**0.41**	
19	**0.40**	-0.20	**-0.49**	0.14	**0.35**	-0.19	0.23	0.25	0.18	**0.35**	**0.39**	**0.81**	**0.86**	**0.74**	**0.72**	**0.67**	**0.68**	*0.31*

B	1	2	3	4	5	6	7	10	11	12	13	14	15	16	17	18
2	0.04															
3	**-0.51**	**0.70**														
4	**0.38**	**-0.50**	**-0.59**													
5	**0.70**	**0.36**	-0.12	-0.07												
6	-0.12	**0.58**	**0.47**	**-0.49**	**0.36**											
7	0.00	**-0.88**	**-0.62**	**0.56**	**-0.40**	**-0.72**										
10	**-0.54**	**-0.38**	0.02	0.19	**-0.63**	-0.26	**0.45**									
11	-0.15	**-0.84**	**-0.56**	**0.66**	**-0.50**	**-0.52**	**0.83**	**0.52**								
12	0.23	-0.13	-0.25	-0.04	**0.37**	*0.31*	0.06	-0.03	-0.01							
13	-0.03	0.24	0.23	**-0.39**	**0.40**	**0.60**	-0.26	-0.12	-0.25	**0.71**						
14	-0.10	**-0.75**	**-0.54**	0.25	*-0.29*	-0.22	**0.62**	**0.50**	**0.69**	**0.49**	0.20					
15	**0.62**	-0.28	**-0.56**	0.10	**0.45**	-0.16	*0.32*	*-0.32*	0.06	**0.49**	0.27	0.24				
16	**-0.53**	**0.59**	**0.78**	**-0.48**	-0.18	**0.47**	**-0.46**	0.20	**-0.34**	0.04	**0.41**	-0.27	**-0.50**			
17	**-0.59**	**0.49**	**0.72**	**-0.46**	-0.21	**0.54**	**-0.46**	0.23	-0.26	0.14	**0.51**	-0.11	**-0.49**	**0.94**		
18	0.03	**0.46**	*0.31*	-0.18	0.06	-0.05	-0.18	-0.08	**-0.35**	-0.27	-0.19	**-0.54**	-0.16	**0.42**	0.09	
19	-0.04	**-0.57**	**-0.44**	0.08	-0.15	-0.08	**0.52**	**0.39**	**0.52**	**0.68**	**0.45**	**0.91**	**0.43**	-0.05	0.07	**-0.39**

C	1	2	3	4	5	6	7	10	11	12	13	14	15	16	17	18
2	-															
3	**-0.99**	-														
4	**0.81**	-	**-0.78**													
5	**0.62**	-	**-0.65**	**0.34**												
6	-0.04	-	-0.01	-0.23	**0.74**											
7	0.01	-	0.05	0.24	**-0.63**	**-0.87**										
10	0.05	-	-0.08	-0.11	0.11	0.07	-0.03									
11	**0.58**	-	**-0.57**	**0.53**	*0.32*	-0.10	0.16	**0.75**								
12	**0.64**	-	**-0.64**	**0.53**	**0.45**	0.00	0.06	**0.54**	**0.81**							
13	**0.42**	-	**-0.44**	*0.33*	**0.64**	**0.42**	-0.25	**0.41**	**0.57**	**0.73**						
14	**0.45**	-	**-0.46**	**0.36**	**0.37**	0.06	-0.01	**0.65**	**0.76**	**0.96**	**0.71**					
15	**0.88**	-	**-0.84**	**0.77**	**0.47**	-0.17	0.25	0.04	**0.60**	**0.69**	**0.50**	**0.46**				
16	0.10	-	-0.10	0.12	0.21	0.16	-0.07	-0.02	0.10	0.13	**0.63**	0.06	0.25			
17	0.10	-	-0.11	0.09	0.27	0.23	-0.17	0.00	0.08	0.11	**0.65**	0.06	0.19	**0.98**		
18	0.09	-	-0.05	0.20	-0.09	-0.22	**0.37**	-0.06	0.14	0.16	*0.31*	0.04	**0.40**	**0.68**	**0.54**	
19	**0.45**	-	**-0.45**	**0.40**	**0.42**	0.12	-0.01	*0.31*	**0.55**	**0.67**	**0.90**	**0.60**	**0.59**	**0.82**	**0.79**	**0.61**

(1) Number of bridge entries; (2) number of arm entries; (3) ratio arm/bridge entries; (4) number of bridge entries before first arm visit; (5) number of bridge entries before first repeated arm visit; (6) number of arms before first repeated arm entry; (7) number of repeated arm entries; (8) number of repeated arm entries to criterion; (9) number of sessions to criterion; (10) latency of first bridge entry; (11) latency of first arm entry; (12) latency between arm entries; (13) latency of first repeated arm entry; (14) time spent on the central platform; (15) time spent on the bridges; (16) time spent on the arms; (17) time spent on unique arm visits; (18) time spent on repeated arm visits; (19) session duration. Grayed cells bold fonts ($r = 0.34$, $p \leq 0.05$) and shaded cells italic fonts ($r = 0.29$, $p \leq 0.10$).

There are no correlations between the memory indices and session duration, latency to first entry on a bridge, latency to first arm repeat.

Table 1B and C shows the correlations between the different parameters of the test on the first and last blocks of four sessions. It demonstrates that there are no significant correlations between measures of anxiety and measures of memory in the last session block ($p > 0.10$). The number of arm entries before first arm repeat and the number of arm repeats are significantly correlated to each other in both session blocks (respectively, $r = -0.72$ and -0.87, $p < 0.0001$). They do not correlate to any other parameters in the last session block except that number of arm entries before first arm repeat correlates with latency of first repeated arm entry ($r = 0.42$, $p < 0.02$) and that number of arm repeats correlates with time spent on repeated arm visits ($r = 0.37$, $p < 0.04$); both parameters correlate with number of bridge entries before first repeated arm visit ($r = 0.74$ and -0.63, $p < 0.001$).

4. Discussion

The present study examined the behavior of young and middle-aged CD-1 mice of both sexes in a raised arm configuration of a 3D maze. Mice were introduced to a working memory test without prior habituation to the apparatus and the testing environment. This testing procedure provides a chance to examine the emotional responses to novelty and open spaces on first exposure to the test in addition to learning and memory performance which continues throughout the successive testing sessions.

Examination of the first sessions of exposure to novelty in the 3D maze revealed that young females spent significantly less time on the central platform compared to young males and they spent less time between arm visits compared to young males and middle-aged females. They also finish the test session faster than middle-aged females and than both young and middle-aged males. There is however no significant differences between groups on the number of entries to arms. CD-1 mice did not make all eight arm choices on the first sessions (average from all animals on the first block of four sessions 5.71 ± 0.29). The low number of entries to arms suggests that CD-1 mice express some anxiety responses in the 3D maze which confirms previous studies from our laboratory where CD-1 were compared to C57/BL6 and Balb/c mice [1,2]. We demonstrated consistently that CD-1 male mice are more anxious than C57/BL6 mice and less anxious than Balb/c mice in a 12 min exposure to the 3D maze. CD-1 mice make a very few number of arm visits on their first exposure to the maze and required more than two sessions to make eight or more arm visits in a session compared to C57/BL6 mice which visit more arms on a first exposure to the maze and make eight or more arm visits on a second exposure. CD-1 mice however are less anxious than Balb/c mice which make a very few or no visit onto arms on their first exposure to the maze and require several sessions to make eight arm visits. In the present study, it is not possible to rely on the number of arm entries to assess anxiety as it is limited to a maximum of eight arm visits which are achieved by all CD-1 mice on the fourth session and maintained through the remaining test sessions. Instead, the index arm/bridge ratio is used to assess the difference in anxiety between sex and age groups. Analysis of the arm/bridge ratio confirms the lack of significant effect between groups on the first four sessions but revealed significant differences between groups on the second and third sessions blocks. There were no significant differences between groups on the last session block. The arm/bridge ratio was significantly low in middle-aged males compared to middle-aged female mice and compared to both young male and female mice. This index arm/bridge ratio may reflect a prolonged anxiety in middle-aged males that subsided after three sessions' blocks. It cannot be accounted for by hesitation responses associated to cognitive processes as the arm/bridge ratio did not correlate with measures of learning and memory (number of arm before first arm repeat, number of arm repeats, errors and sessions to criterion, see Table 1A). This lack of correlations was observed in each of the last three sessions' blocks (only the last session block shown in Table 1C). Furthermore, in middle-aged male mice the latency of first entry onto a unique arm, the latency of first repeated arm visit, the number of arm repeats, number of arm visits before first arm repeat, number of errors and session to criterion were comparable to that of middle-aged female mice. Further experiments are required to confirm the presence of anxiety in middle-aged male CD-1 mice after repeated exposure to a behavioral test. CD-1 middle-aged male mice need to be compared to a strain of mice of the same age that expresses lower anxiety trait.

Examination of the behavior of CD-1 mice during the acquisition and memory performance of the spatial navigation task shows that both young males and young females required significantly less time to perform eight arm choices in the maze than middle-aged males and middle-aged females. However, only young females were able to make more correct arm choices. They made significantly fewer arm repeats, more unique arm entries before making first arm repeat, fewer arm repeats to criterion and fewer number of sessions to criterion compared to young male mice and to both middle-aged male and female mice. The main difference between young females and middle-aged females, in addition to session duration and the memory indices, is the time spent on the central platform which was shorter in young females compared to middle-aged females. The main difference between young males and middle-aged males, in addition to session duration and arm/bridge ratio, is the time spent on the bridges and latencies between re-entries to arms which were shorter in young males compared to middle-aged males. The main difference between young females and young males, in addition to memory indices, is the number of re-entries to the centre which were significantly lower in females than in males. The main difference between middle-aged females and middle-aged males is the arm/bridge ratio, the number of entries to bridges, duration of entries to bridges, number of bridges before first arm visit and number of entries to the central platform which were significantly lower in females than in males.

The central platform and the bridges are the two places were a decision can be made about the choice of an arm. A high number of entries to bridges reflect the hesitations or reluctance of

mice to engage onto an arm while a lengthy duration on the central platform may reflect the hesitation and reluctance of mice to engage onto the bridges. Our results suggest that females made their decision on the central platform while for males the decision were made on the bridges. This hypothesis is supported by the fact that female mice made a fewer bridge entries, entered a fewer bridges before first arm visit and spent less time on the bridges than middle-aged males. They also made significantly a fewer returns to the central platform between arm visits than young males and middle-aged males. This means that most of the time female mice enter the bridges and continue to arms while males move back and forth between the central platform and bridges before adventuring onto an arm—younger males being faster than middle-aged males. Hesitations of rats at a choice point before committing to a choice were originally described as vicarious trials-and-errors (VTEs) by Tolman [13,14] and Muenzinger [82]. These are discrete responses that are not easy to record manually and time consuming if examined over 12–16 sessions. Bimonte and Denenberg [15] proposed to record a VTE when the tip of a rat's snout did not cross a line that was set approximately 11 cm into the 27.9 cm of arm length and that the rat "was clearly oriented toward the end of an arm, and was accompanied by a distinct stop in motion". Under this criterion, female rats were reported to demonstrate more VTEs than male rats which conflict with our observations. There is however some differences between the two experimental settings in addition to the species differences. The total length of an arm (including the bridge) in the 3D maze is 51 cm, the width is 11.2 cm and the diameter of the central platform is 30 cm while in Bimonte and Denenberg's radial maze the length of an arm is 38.9 cm, the width 12.7 cm and the diameter of the central platform is 48.3. The length of a bridge of the 3D maze constitutes only 30% (15 cm) of the total length of an arm while in Bimonte and Denenberg's radial maze the VTE zone constitutes 39% (11 cm). Considering the size of a rat, there is a very short distance left to reach the end of an arm (28 cm) while in the 3D maze mice have still 2/3 (35 cm) left to cover. In addition, in a very large central platform with wide separation between arms, animals are likely to follow sequential choices which would reduce the amount of VTEs (see below). In our view, hesitations or VTEs take place on the central platform at the point of entry to an arm and also on the first third part of an arm—this third should be long enough to contain at least the full length of an animal.

There are only a few studies devoted to age-related memory performance in CD-1 mice. In a recent study, a premature deficit in the acquisition of a working memory test in a 3D maze was observed in middle-aged (12 months) female CD-1 mice in comparison to young (3 months) female CD-1 mice [3] which confirms our present findings. In another study [10], 13 weeks old male and female CD-1 mice were tested every 2 weeks over 65 weeks in a T-maze spontaneous exploration task. Performance decreased with age in both male and female mice but females performed better and showed a greater exploratory activity than males. Exploration time was longer in young and old males than in young and adult females. In recent study [85], adult (5 months) vs. middle-aged (13 months) CD-1 male mice were examined in a water maze. The authors report that middle-aged mice show deficit in the acquisition of the test as they displayed longer latencies to find the platform location than adults, showed longer cumulative distance from both the acquisition quadrant and platform location area than adults, and tended to spend more time in the outer area. They also report no age differences in swimming path or swimming speed. Similar deficits were reported in C57BL/6Nia mice at age 10 months [93], 15 months [99], 22 months [16] and 24–26 months [93,17]. A deficit in the water maze is also observed in aged C57BL/6J mice (18–20 months old) [18,100,101] but middle-aged (10–12 months old) mice were comparable to 3–4 months old mice [18]. These results from the water maze are difficult to compare with the 3D maze or the standard radial maze which involve choices between various alternatives and are based on food reward and not an aversive reinforcer. In addition, in the standard radial maze and the 3D maze, memory performance is inferred from the type of choices made by animals during a session whereas in the water maze they are inferred from latencies, duration and distance which can be confounded with alteration in motor activity. In the 3D maze, the radial maze and the T-maze, a choice of an arm is a specific response made by animals that can be measured independent of the latency and duration of its occurrence and independent of the length or the surface of the maze covered for its occurrence. The latency of entry to an arm, distance traveled to an arm and time spent on an arm can be used to account for memory performance (paramètres explicatives) but do not measure or provide valid measures of memory; they cannot be used as memory indices (paramètres indicatives); they are very often contaminated by confounding factors. In our experiment, the memory parameters of the test (number of arm repeats, number of unique arm visits before first arm repeats, number arm repeats to criterion and sessions to criterion) are strongly correlated to each other ($r = 0.58$, $p < 0.0004$ to $r = 0.91$, $p < 0.0001$). They all revealed consistently that young female mice performed better than young males and than both middle-aged male and female mice. These parameters do not correlate with latency between entries to arms, time spent on the arms or the central platform, time spent on unique arm visits, latency to first arm repeat, latency to first entry to bridge and sessions' duration ($p > 0.10$). Only the latency to first arm visit correlates significantly with number of arm repeats ($r = 0.56$, $p < 0.0008$) and with number arm repeats to criterion ($r = 0.36$, $p < 0.04$), and time spent on the bridges correlates significantly with number of arm visits before first repeat ($r = 0.42$, $p < 0.02$) and with arm repeats to criterion ($r = 0.41$, $p < 0.02$). However, latency to first arm visit revealed no significant differences between groups while time spent on the bridges show significant differences between middle-aged males compared to middle-aged females and compared to both young male and female mice. The results from the latency of first arm visit and time spent on bridges are not consistent with the results observed with the memory indices. Measures of latencies, duration and distance are not specific indices of memory function. They are likely to reflect a physical or physiological state of animals as well as the exploratory strategy or pattern of responses adopted by animals.

There are numerous reports on sex differences in cognitive processes and these are becoming increasingly inconsistent and conflicting both in humans and animals ([15,19–34,91]. However, these inconsistencies and contradictions need to be carefully analyzed as one needs to consider the nature of the behavioral test and the parameters of the test that have been affected, the age of the subjects, and in animals the strains differences and estrus cycle. Sex differences in spatial learning and memory tasks have been reported for humans and animals, with males often performing better than females [20,31,34–48]. There are other studies which demonstrate the opposite [10,15,19,22,29,49] or no sex differences [15,91].

Sex differences in age-related cognitive decline have been reported for humans and animals but here too the results are inconsistent and conflicting. In some studies, a rapid age-related decline was reported in females than in males [17,37,50,51–55,84,96,98] while other studies suggest the opposite, a rapid decline in males than in females [43,56–61]. No sex difference is also reported in other studies [34,35,37,62–64].

It has been reported that motor activity in animals [65–68] and motor and psychomotor skills in humans [69,70] decline with age. Therefore it is possible that in our experiment aged mice have impaired motor activity which is reflected by a longer time to perform the test and this may account for their poor memory performance. However, if this were so, this argument would also apply to young males which also did not perform well in the test. In addition, the latency of first entry to an arm was comparable between all groups in all sessions. In our experiments, all mice of different strains and ages are able to climb the bridges to reach the raised arms. In fact, mice seem to prefer climbing, and the raised arm configuration appears more attractive to mice than the flat or lowered arm configurations. In addition, the bridge floors are covered with wiremesh to provide mice with further grip on the floor though our experiments demonstrate that mice can climb the bridges tilted by 60° without wiremesh.

The design and the physical characteristics of a test apparatus can contribute to or interfere with learning and memory performance in animals which are encouraged to adopt behavioral choice patterns or responses strategies [71–77]. For instance, with narrow arms that are about 12 cm wide, some aged rats are too large to be able to turn back when at the end of an arm and are forced to withdraw rear-first to the central platform facing once again the same arm which may constrain their subsequent choice to one of the two adjacent arms. In contrast, young rats will return head-first from an arm to the central platform facing all remaining arms. In addition, wide angles between adjacent arms can lead animals to learn movement sequences which result, through habit, in a smooth body movement swerving from one arm to the next. In our maze, the width of the bridges and arms is sufficient for three mice to run comfortably side by side. The exits from the bridges to the central platform are narrowed on either side of the bridge entries in a semi-random arrangement to prevent mice following a sequential strategy. At any time, an animal can miss the next one or two adjacent arms as it cannot rely on automatic body movement, and would be forced to rely on its memory of the previous visited arms.

In human studies, cognitive functions have been reported to decline with age, these include episodic memory [97], working memory [83] and spatial memory [92,78,79]). Aging has been reported to affect information processing speed in human [90,95] and animals [88]. Older subjects were found to require more time to solve a 3D spatial navigation task and to acquire spatial information in a novel environment compared to younger adults [69,70,89]). The speed with which animals and humans perform a cognitive task is not an indicator of memory performance. Latencies between arm entries and session durations do not correlate with any of the measures of memory performance in the 3D maze. Making a fast decision or choice does not guarantee an accurate or a correct response, and taking longer time to make a decision or a choice can be equivalent to increasing a memory retention interval leading to confusion between choices. However, it is also possible that performing a memory task at a slower more considered pace can benefit performance in the aged subject as shown with non-thermal near infra-red light treatment in female middle-aged mice [3].

CD-1 mice demonstrate a high incidence of spontaneous neoplastic and non-neoplastic lesions [4,5,8,80] and record a high mortality rate at about 24 months of age (32.6% and 28.6% at 83 weeks of age and 66.4% and 63.3% at 109 weeks of age for male and females, respectively [4]). Amyloidosis is considered one of the major factors that contribute to death in aging CD-1 mice [9,94]. Amyloid deposits represent frequent histological findings in CD-1 strains of mice [87] but their observation was limited to peripheral organs; there are no reports on histological brain tissues. It is possible that the premature deficit in the acquisition of a working memory task observed female mice in this and a previous experiment in the 3D maze [3] can be due to a high level of circulating amyloid or possibly to a natural occurrence of amyloidosis in the brain of CD-1 mice. This issue is currently investigated in our laboratory. The poor performance of young male CD-1 mice which compare to that of middle-aged male CD-1 mice in the acquisition of a spatial working memory task is supported by an early study from Mizumori et al. [81] in the validation of the radial maze for mice. Mizumori et al. [81] report that 3 months old male CD-1 mice were unable to acquire a working memory task in a continuous choice procedure. Our results and those of Mizumori et al. [81] suggest that male CD-1 mice present cognitive deficit very early in life and that this deficit is delayed in female. Examination of working memory performance in a radial maze of younger male CD-1 mice of 1 month and up to 3 months of age is required to investigate whether male CD-1 mice would perform a working memory task better than 4-months old CD-1 rats. A study comparing middle-aged female CD-1 mice with other strains of mice is also needed to corroborate our finding of the premature cognitive deficit observed at 12 months of age.

Disclosure statement

A preliminary account of some of the present findings has been submitted in an abstract to the Society for Neuroscience

2007. None of the authors has any actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations within 3 years of beginning the work submitted that could inappropriately influence (bias) our work. We also do not have contracts relating to this research through which ours or any other organization may stand to gain financially now or in the future. Our Institutions do not have a financial interest in this work.

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Research report

Are benzodiazepines really anxiolytic? Evidence from a 3D maze spatial navigation task

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Abstract

The effects of diazepam and chlordiazepoxide were assessed in a 3D maze which is a modification of an 8-arm radial maze. Each arm of the maze is attached to a bridge radiating from a central platform. Animals exposed for the first time to the maze do not venture beyond the line that separate a bridge from an arm. The prime criteria set for an anxiolytic effect is whether mice would increase the frequency of entries onto arms and increase arm/bridge entries ratio. C57 mice readily cross the line on first exposure and make more than 8 arm visits onto arms on second exposure, while other strains (CD-1 and Balb/c) hold back and rarely cross the line on first exposure and require more sessions to make more than 8 arm entries. An anxiolytic drug is expected to encourage intermediate (CD-1) and high (Balb/c) anxiety mice to adventure onto the arms of the maze and make more visits to the arms to comparable levels seen with low anxiety c57 mice. In the present report, administration of different doses of diazepam (0.625, 1.25, 2.5 and 5 mg kg^{-1} i.p.) and chlordiazepoxide (5, 10 and 15 mg kg^{-1} i.p.) did not reduce anxiety in animals, with the lowest dose of diazepam increasing motor activity in Balb/c and increasing anxiety in c57 mice while the highest doses of both diazepam (2.5 and 5 mg kg^{-1} i.p.) and chlordiazepoxide (15 mg kg^{-1} i.p.) induced mild sedation. Our results raise some concerns about the methodological foundations in the current assessment of anxiety and anxiolytic compounds both in animal and human studies.

Keywords: GABAA; Benzodiazepines; Spatial navigation; Construct validity; Strains; Rats

1. Introduction

Benzodiazepines (BZDs) are the first-line drugs for the treatment of anxiety disorders, acting at the GABAA receptors which remain primary targets for novel anxiolytic compounds. They are held up as the prototypical anxiolytic agent despite major central and peripheral side effects which undermine their use and conflict with their therapeutic purposes. The apparent calming effect of BZDs reported in the clinic through interviews and questionnaires may actually result from a combination of mild sedation, cognitive dampening and/or peripheral myorelaxation. BZDs are indeed prescribed to induce anxiolysis but also sleep and anesthesia. They are also amnesic, and are routinely used in a perioperative setting for this purpose, to ensure that patients forget their experience in surgery [58,63,88,91,121,129]. Furthermore, long-term BZD use induces tolerance and dependence. Numerous basic research studies have been devoted, for more than half a century, to follow up the clinical findings of the perceived anxiolytic effects of benzodiazepines. Vast amounts of resources are currently invested in animal studies, mainly rats and mice and, in the development of new GABAA compounds without the side effects of benzodiazepines.

Current animal tests for anxiety have proved unreliable for the development of new anxiolytic drugs. They lack construct validity which is *sine qua none* to any other validity issue, producing often very inconsistent and contradictory results [3,16,73,109,139, see 138]. These tests are mostly based on a choice between two options, aversive and non-aversive stimuli. Animals are free to avoid an aversive stimulus; this is not an option in an anxiety state. In anxiety "*fear is compounded with a tendency to approach the source of potential threat—producing an approach-avoidance conflict*" [45]. In the current two-choice paradigms, BZDs did not demonstrate consistently an increase in

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Emotional responses and memory performance of middle-aged CD1 mice in a 3D maze: Effects of low infrared light

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Abstract

Non-thermal near infra-red (IR) has been shown to have many beneficial photobiological effects on a range of cell types, including neurons. In the present study, a pretreatment with a daily 6 min exposure to IR1072 for 10 days yielded a number of significant behavioral effects on middle-aged female CD-1 mice (12-months) tested in a 3D-maze. Middle-aged mice show significant deficits in a working memory test and IR treatment reversed this deficit. Interestingly, the IR treated middle-aged group despite making less memory errors than sham middle-aged group spent longer time in different parts of the maze than both the young group (4-months) and sham-middle-aged group (12-months). Young mice appeared more anxious than middle-aged mice in the first sessions of the test. Exposure to IR appeared to have no significant effects upon exploratory activity or anxiety responses. However, it elicited significant effects on working memory, with the IR middle-aged mice being more considerate in their decision making, which results in an overall improved cognitive performance which is comparable to that of young CD-1 mice. The present study describes a novel method for assessing emotional responses and memory performance in a 3D spatial navigation task and demonstrates the validity of our new all-in-one test and its sensitivity to ageing and non-invasive beneficial IR treatment.

Keywords: Aging; Ageing; Amnesia; Amyloid; APP; Alzheimer; Hippocampus; Rats

1. Introduction

Sunlight is the most important and universal source of non-ionising radiation essential for life on Earth. Non-thermal near infra-red (IR) (700–2000 nm) light has high penetrability of biological tissues, with low degrees of light scatter, and has been shown to have a wide range of therapeutic benefits, for example, in the treatment of musculoskeletal disorders (Ceylan, Hizmetli, & Silig, 2003), healing indolent wounds (Karu, 2003; Vinck, Cagnie, Cambier, & Cornelissen, 2001; Whelan et al., 2003) and reducing pain (Honmura, Ishii, Yanase, Obata, & Haruki, 1993). IR improved recovery from ischemic injury of the heart (Kawasuji et al., 2000), prevents the development of oral mucositis in pediatric bone marrow transplant patients (Whelan et al., 2002), and attenuates the developmental toxicity of dioxin in chicken embryos (Yeager et al., 2005). It can also generate significant biological effects including cellular proliferation, collagen synthesis, and the release of growth factors from cells (Conlan, Rapley, & Cobb, 1996; Karu, 1999; Leung, Lo, Siu, & So, 2002).

A growing number of reports have shown effects of IR on the nervous system. Low power laser irradiation has been shown also to improve retinal function and attenuate degeneration of injured optic nerves induced by methanol-derived formic acid in rat models (Eells et al., 2003) and has also been shown to alter gene expression in retinal neurons (Eells et al., 2004) and olfactory ensheathing cells *in vitro* (Byrnes, Wu, Waynant, Ilev, & Anders, 2005). Furthermore, effects upon neurotransmission has been described, modulating cerebral blood flow and changes in energy metabolism, including selective rises in ATP levels (Moc-

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