Zebrafish as a model for translational neurobiology: Implications for drug discovery and development

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Statement of Originality

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Abstract

Diseases which affect the central nervous system present a huge burden to sufferers and caregivers. In tandem with longevity, prevalences of age-related neurodegenerative diseases are increasing. However, despite the evident necessity for pharmaceutical interventions, there has been a distinct lack of drug development to combat these disorders. This is largely attributed to high financial costs of using rodent models. Thus the validation of a more cost-effective *in vivo* system would facilitate pharmaceutical screening. The work presented in this thesis addresses this issue by assessing the utility of zebrafish in two costly areas of translational neurobiology – lead identification and safety pharmacology.

An aversive classical conditioning assay was developed and automated as a behavioural screening method. This robust assay allows fast assessment of cognition and cognitive decline. The effect of neurotoxin treatment on aversive learning was then assessed using this assay, demonstrating its efficacy as a screening tool for neurodegeneration research.

Subsequently, a transgenic zebrafish line - expressing a mutated form of the Alzheimer's-associated human amyloid precursor protein - was assessed, demonstrating an age-related cognitive impairment. Additionally new genetic zebrafish lines were generated, which over-express genes (both endogenous and transgenic) related to Alzheimer's-like pathologies. Whilst these were not assessed within this thesis, they present promising tools for possible future investigations.

Regarding safety pharmacology, regulatory bodies require all CNS-penetrant drugs be assessed for abuse potential. Zebrafish display reward responses to several common drugs of abuse (e.g. amphetamine, cocaine, morphine). Thus, the latter sections of this thesis evaluated the utility of zebrafish for assessing human abuse potential. A CPP paradigm was utilised to test a range of drugs, with the sensitivity and specificity of zebrafish compared to previous reports using rodent. Additionally, the development of a zebrafish drug discrimination assay was attempted. However the paradigms utilised failed to develop an efficacious assay.

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Abbreviations

5-HT, 5-hydroxytryptamine (serotonin)
5-HT-R, 5-hydroxytryptamine receptor
ACh, acetylcholine
AChE, acetylcholinesterase
AD, Alzheimer's-like diseases
ADAM, a disintegrin and metalloproteinase
ADD, attention deficit disorder
ADHD, attention deficit hyperactivity disorder
AIF, apoptotic inducing factor
Al, aluminium
$AlCl_3$, aluminium chloride
ALS, amyotrophic lateral sclerosis
APAF, apoptotic protease activating factor
APH1, anterior pharynx-defective 1
ApoE, apolipoprotein E
APP, amyloid precursor protein
appb, amyloid precursor protein b (zebrafish)
Au, arbitrary unit
BACE1, β-site APP cleaving enzyme 1 (human)
bace1, β-site app cleaving enzyme 1 (zebrafish)
BBB, blood-brain-barrier
BChE, butyrylcholinesterase
blA, basolateral amygdala
Ca, calcium
CaMKII, calcium/calmodulin-dependent kinase II
CaN, calcineurin
CB_1, cannabinoid receptor type 1
CB_2, cannabinoid receptor type 2
CBD, cannabidiol
CDK5, cyclin-dependent kinase 5
ChAT, choline acetyltransferase
COX, cyclooxygenase
CNS, central nervous system
CPP, conditioned place preference
Cq, quantitation cycle

CHRNα7, cholinergic receptor, nicotinic α7 (subunit)
CS, conditioned stimulus
CSPG, condroitin sulphate proteoglycan
CTF, C-terminal fragment
D_2, dopamine receptor D_2
DA, dopamine
DAergic, dopaminergic
Dc, central zone of dorsal telencephalic area
Dl, lateral zone of the dorsal telencephalic area
Dm, medial zone of the dorsal telencephalic area, corresponds to the MP
DRN, dorsal raphe nucleus
EOAD, early-onset Alzheimer's-like diseases
EtOH, ethanol
ER, endoplasmic reticulum
FC, frontal cortex
Fe, iron
fMRI, functional magnetic imaging
GABA, γ-Aminobutyric acid
GABA-R, GABA-receptor
GI, gastrointestinal
GLU, glutamate
GLUergic, glutamatergic
GRP78, 78kDa glucose-regulated protein
GSK3β, glycogen synthase kinase 3β
hAPP, human amyloid precursor protein
$hAPP_{LON}$, hAPP containing the *London* mutation
$hAPP_{LON/SWE}$, hAPP containing both the *London* and *Swedish* mutations
$hAPP_{SWE}$, hAPP containing the *Swedish* mutation
HD, Huntington's disease
HSP, heat shock protein
HTT, Huntingtin
JUN3, c-Jun N-terminal kinase 3
lA, lateral amygdala
IL, interleukin
LP, lateral pallium (corresponds to the Dl)
IQR, interquartile range
LOAD, late-onset Alzheimer's-like diseases
LTP, long term potentiation
mA, medial amygdala
mAChR, muscarinic acetylecholine receptor
MAM, mitochondria-associated membranes
MAO, monoamine oxidase
MAP, microtubule-associated protein
MP, medial pallium, corresponds to the Dm
MPF, months post fertilisation
mPFC, medial pre-frontal cortex
MPTP, mitochondrial permeability transition pore
MRN, median raphe nucleus

mRNA, messenger RNA
MS, multiple sclerosis
NAcc, nucleus accumbens
nAChR, neuronal nicotinic acetylcholine receptor
NCT, nicastrin
NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells
NFT, neurofibrillary tangles
NHP, non-human primates
NMDA, *N*-methyl-D-aspartate
NMDA-R, NMDA-recptor
NO, nitric oxide
non-CS, non-conditioned stimulus
NSAID, non-steroidal anti-inflammatory drug
PaC, parietal cortex
PD, Parkinson's disease
PCP, phencyclidine
PCR, polymerase chain reaction
PEN2, presenilin enhancer 2
PFC, pre-frontal cortex
PHF, paired helical filament
PIN1, peptidyl-prolyl cis-trans isomerase NIMA-interacting 1
PNN, perineuronal net
PP1, protein phosphatase 1
PP2A, protein phosphatase 2A
PP2B, protein phosphatase 2B
PS1, presenilin 1
PS2, presenilin 2
PTN, posterior tuberal nucleus
qPCR, quantitative polymerase chain reaction
ROS, reactive oxygen species
RT-PCR, reverse-transcription PCR
SA, self-administration
sAPPβ, soluble APP N-terminal fragment
SCI, spinal cord injury
SNc, substantia nigra pars compacta
SNP, single nucleotide polymorphism
SOD1, superoxide dismutase 1
SSRI, selective serotonin re-uptake inhibitor
TBI, traumatic brain injury
THC, Δ^9-tetrahydrocannabinol
TNF, tumour necrosis factor
TU, Tubingen
UAS, upstream activation sequence
UPR, unfolded protein response
US, unconditioned stimulus
VGluT2, vesicular glutamatergic transporter 2
vP, ventral pallium
Vp, postcommissural nucleus of the ventral telencephalon

VTA, ventral tegmental area
Vd, dorsal nucleus of the ventral telencephalic area
Vv, venteral nucleus of the ventral telencephalic area

Chapter 1

General Introduction

1.1 The necessity for new therapeutics to treat *Alzheimer's-like diseases*

The failure of current Alzheimer's-like diseases therapies to effectively treat these diseases has become evident in recent years, particularly due to the increased longevity seen in western societies. It is argued in this thesis that much of this failure results from the variety of molecular dysregulations which result in similar pathologies, commonly termed '*Alzheimer's disease*'. However, treating these distinct pathologies as a single disease results in a generic approach, unspecific to the array of molecular dysregulations which may be aetiological in these conditions. Thus, a review of the prevalences, various mechanisms of molecular dysregulations and currently available treatments are presented and evaluated.

1.1.1 Prevalence of *Alzheimer's-like diseases*

Diseases affecting the central nervous system (CNS) are a growing concern within modern medicine. The World Health Organisation reported that neurological conditions present the highest burden ('*disability-adjusted life years*' - a measure of the number of years lost due to illness) of all disorders, including various infectious diseases (WHO 2006). Indeed, recent years have seen increases in the prevalence of many chronic neurological conditions, including Alzheimer's-like diseases (AD), attention deficit disorder (ADD), cerebral palsy, Huntinton's disease (HD), multiple sclerosis (MS), schizophrenia, and unipolar depression (Andersen et al. 2011; Barnett et al. 2003; Bray et al. 2006; Brookmeyer et al. 2007; Evans et al. 2013; Ferri et al. 2006; Vincer et al. 2006; Visser et al. 2010). Further, with increasing longevity, increases in the prevalences of *age-related* neurological conditions are becoming particularly evident, with the impact of on society ever more prominent (Joseph et al. 1998).

Many age-related CNS disorders include a neurodegenerative component, due to the lower cellular regeneration in comparison with other organs of the body. These complex diseases are characterised by aberrant apoptosis of CNS neurons. Depending on the regions affected, this may result in lasting impairment to a range of abilities, including cognitive, motor, sensory, olfactory and social functioning (e.g. Draganski et al. 2013; Wickremaratchi et al. 2011; Nolano et al. 2008; Mesholam et al. 1998; Zahn et al. 2009). These subsequently have devastating effects on quality

of life for both sufferers and carers (Janardhan and Bakshi 2002; Schrag et al. 2000; Aoun et al. 2010). The vast array of different molecular dysregulations that may lead to these disorders present extremely complex challenges for medical researchers to investigate. Indeed, even within the umbrella-term of '*Alzheimer's-like diseases*', a range of potential molecular candidates have been proposed to be aetiological in disease pathologies.

1.1.2 Complexity and diversity of the pathogenesis of Alzheimer's-like diseases

AD are the most prominent neurodegenerative disorders and the second most common of all neurological disorders (following cerebrovascular disease) (WHO 2006). Research indicates that the global instances of AD in 2006 was between 24 and 27 million, with cases doubling every 20 years and expected to treble by 2050 (Brookmeyer et al. 2007; Ferri et al. 2006).

Commonly, AD are divided into two catagories - *early-onset* (EOAD) and *late-onset* (LOAD); divisions characterised by the age at which symptoms begin to present (commonly, before or after 60-65 years; e.g. Bertram and Tanzi 2008; Filley et al. 1986). EOAD are estimated to account for less than 6% of total AD (Zhu et al. 2015). Despite this, research has paid more attention to this subdivision, with a greater understanding of the genetic components of EOAD than that of LOAD.

Mechanisms of neurodegeneration and variety of possible aetiologies

Both divisions of AD are characterised by extracellular amyloid plaques (formed by aggregates of Aβ peptides) and neurofibrillary tangles (NFT; resulting from hyperphosphorylation of tau proteins, causing an instability of microtubules) (see Figure 1.1; Krstic and Knuesel 2013; Schmechel et al. 1993; Schoonenboom et al. 2004; Small and Duff 2008).

'*Amyloid cascade*' hypotheses (see Figure 1.2) have been heavily implicated in EOAD; whereby dysregulated amyloid peptide production is considered aetiological and tau hyperphosphorylation a down-stream consequence (albeit with its own destructive consequences) (e.g. Haass and Selkoe 2007; Hardy et al. 1998;

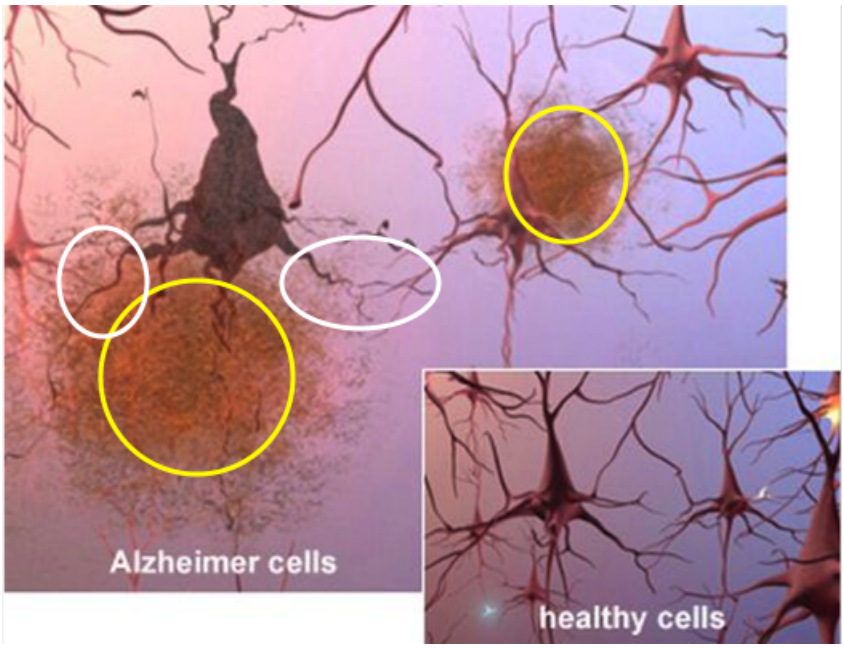
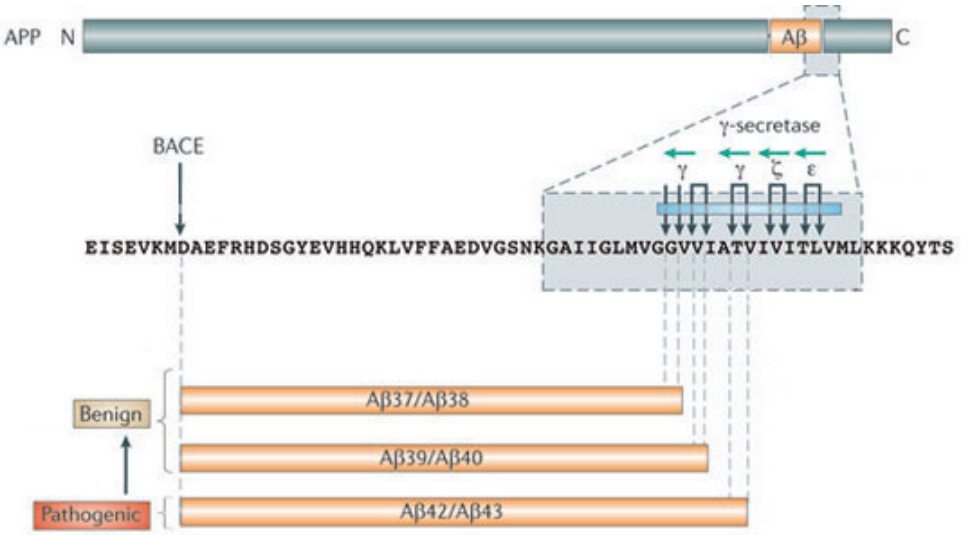


Figure 1.1: Hallmarks of Alzheimer's-like diseases. The two most prominent pathologies of AD are the extracellular aggregation of Aβ peptides, resulting in amyloid plaque deposition (yellow circles) and the hyperphosphorylation of tau, resulting in neurofibrillarly tangles (white circles). (Image adapted from Association® 2017.)

Jin et al. 2011; Small and Duff 2008). A range of mutations within the amyloid precursor protein (APP) gene has been associated with dysregulated cleavage of Aβ peptides, resulting in extracellular aggregation and subsequent plaque formation (discussed in greater depth in Chapter 5, Section 5.1.1). However *amyloid cascade* hypotheses are somewhat limited in their explanation of certain findings.

For example, humans may develop both amyloid plaques and NFT in the absence of dementia (e.g. Mann et al. 1987b; Perry 1980; Sparks et al. 1993; Sparks et al. 1995; Tomlinson et al. 1968). The transgenic expression of mutated human APP (hAPP) in rodents fails to produce NFT, requiring an additional transgenic expression of mutant tau to achieve both of the characteristic hallmarks of AD (e.g. Lewis et al. 2001; Schwab et al. 2004; see Armstrong 2006; Reitz 2012). Interestingly, mutations to PS1 (part of the γ-secretase complex, which cleaves the Aβ peptides from APP) have been shown to induce tau phosphorylation, possibly by increasing its interaction with GSK3β (Pigino et al. 2001; Takashima et al. 1998). This suggests that amyloid and tau pathologies may share a common mechanism, but that it is up-stream of amyloid dysregulation.

A



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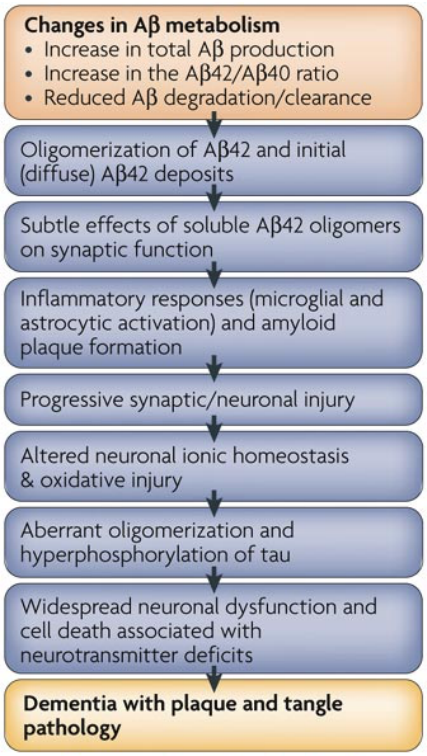

Figure 1.2: Amyloid cascade hypothesis. Amyloid cascade hypotheses regard dysregulation of amyloid metabolism as aetiological in AD. (A) APP metabolism by BACE1 and γ-secretase results in the cleavage of Aβ peptides, the length of which is determined by the site of γ-secretase action. (B) These theories suggest that increased Aβ peptides (particularly the Aβ_{42} species) result in amyloid plaque formation, initiating a 'cascade' of neurodegenerative responses, resulting in tau hyperphosphorylation, and ultimately the classic features of an Alzheimer's pathology (i.e. amyloid plaques and NFT). (Images adapted from Karran et al. 2011; Haass and Selkoe 2007; A & B, respectively.)

Additionally, a direct link between amyloid and tau suggests plaques and NFT would be distributed in the same brain regions; a supposition found not to occur in AD patients' brains (Armstrong et al. 1993; Armstrong 2006). Further, it has been reported that NFT can actually precede amyloid plaque formation (e.g. Duyckaerts 2004; Price et al. 1991b). Whilst this in itself does not disprove *amyloid cascade hypotheses* - it is possible that low levels of amyloid aggregation (undetectable as plaques in histological staining) trigger tau hyperphosphorylation to a greater level - the combination of these issues suggest that *amyloid cascade hypotheses* are insufficient to holistically explain the pathogenesis of AD (at least not as a single disease).

In addition to APP, mutations in the presenilin genes have been strongly associated with the pathogenesis of EOAD (e.g. Bertram and Tanzi 2008). These genes (PS1 and PS2) code for the catalytic subunits of the γ-secretase complex, facilitating its cleavage of APP (mechanism and mutations discussed in greater depth in Chapter 5, Section 5.1.1). As indicated above, PS1 mutations have been shown to increase tau phosphorylation (e.g. Takashima et al. 1998). A far greater number of AD-related mutations have been associated with PS1 than APP, demonstrating that function-altering mutations to APP-metabolising enzymes may have a greater impact than mutations to APP itself (MDB 2017; Alzforum 2017; Bertram and Tanzi 2008). In this regard, hypotheses which regard presenilin mutations as aetiological to (many types of) AD may be a viable alternative to *amyloid cascaded hypotheses*, suggesting that the dysregulation of up-stream (APP-metabolising) proteins results in amyloid plaque formation and tau hyperphosphorylation as down-stream consequences.

Whilst this perhaps provides a more inclusive aetiological understanding of AD, it is still impossible to regard them as a single disease (i.e. with a single molecular cause) as presenilin mutations do not explain the diseases of those patients without these SNPs. Indeed, it is not even sufficient to explain the cause of AD which do not result from amyloid mutations, as mutations to other up-stream genes have been associated with the induction of both pathological hallmarks (i.e. amyloid and tau). In this regard, the involvement of two proteins - a neuroprotective enzyme (PIN1) and it's inhibitor (DAPK1) - may provide an explanation for some of the diseases grouped under AD.

PIN1 has been demonstrated to exert multiple neuroprotective functions in relation to AD pathologies. It induces a conformational change in APP, resulting in decreased Aβ cleavage (Pastorino et al. 2006). In the presence of Aβ_{42} peptides

PIN1 is activated (dephosphorylated at Ser16), resulting in dephosphorylation of tau, as well as APP (which may inhibit β-site APP cleaving enzyme 1, BACE1, processing of APP and the subsequent Aβ secretion) (Bulbarelli et al. 2009; Lee et al. 2003; Maudsley and Mattson 2006). PIN1 also catalyses the isomerisation from *cis* to *trans* in both APP and tau, leading to dephosphorylation (Driver et al. 2014; Lu et al. 2003). Additionally, PIN1 has been demonstrated to inhibit GSK3β, believed to mediate amyloid-driven phosphorylation of tau (Ma et al. 2012; see Hooper et al. 2008; Kremer et al. 2011; Salcedo-Tello et al. 2011).

These effects on the key proteins associated with AD pathologies suggests that homeostatic dysregulation to PIN1 signalling may play a pivotal role in the pathogenesis of some manifestations of AD. The evidence for PIN1 mutations in AD patients is unclear, however sequence variants have been reported to be associated with disease in some patients (Maruszak et al. 2009). Thus, in combination with other known genetic factors (i.e. presenilin and APP mutations, ApoE ϵ4) PIN1 signalling may contribute to providing a more holistic understanding of the genetic basis for disease aetiologies (without regarding environmental factors).

Additionally, PIN1 is inactivated (via phosphorylation) by DAPK1 (Bialik and Kimchi 2011; Lee et al. 2011). A study by Kim and colleagues found that DAPK1 is up-regulated (at a protein level) in the hippocampi of LOAD patients, and that its expression caused tau phosphorylation *in vitro* (Kim et al. 2014). However another clinical study failed to find a significant up-regulation in the frontal cortices (FC), reporting high expression in controls as well as AD patients (Hainsworth et al. 2010). Again, these conflicting findings may be explained by the presence of DAPK1 dysregulation (over-expression) in combination with other AD-related genetic factors; however the evidence for these combinations of genetic risk-factors hypothesis is lacking.

Other than its inactivation of PIN1, DAPK1 signalling includes other mechanisms of neurodegeneration. DAPK1 interacts with NMDA-R's in rodent models of cerebral ischaemia, facilitating excitotoxicity; it's inhibition in these models is reported to facilitate regeneration and rescue (Tu et al. 2010; Won et al. 2014; Won and Hong 2016). Further, the excitotoxic interaction between DAPK1 and NMDA-R may somewhat explain the efficacy of *memantine* (a NMDA-R antagonist) in treating AD. With regard to gene variants, intronic SNPs in DAPK1 have been reported in LOAD patients, suggesting that a regulatory (rather than functional) dysregulation may be present (Li et al. 2006; Wu et al. 2011b).

LOAD are far more common than EOAD, with the apolipoprotein E (ApoE) $\epsilon 4$ allele consistently associated with *late-onset* disease manifestations (Bertram and Tanzi 2008). The involvement of this gene will be discussed in greater detail in Chapter 5 (Section 5.1.1). However, of interest, ApoE $\epsilon 4$ has been reported to modulate PIN1 and PS1, as well as SIRT1 (reported to be neuroprotective against Aβ toxicity) (Lattanzio et al. 2014). Additionally, it has been reported that the presence of $\epsilon 4$ allele alone is insufficient to describe the pathogenesis of LOAD, with mutations in the ApoE regulatory region possibly offering a more holistic explanation (Bullido et al. 1998; Morris et al. 2010a).

Neurotransmitter systems' sensitivities

It must be noted that the neurotransmitter systems appear to have differential sensitivities to AD pathologies. Cholinergic neurons are particularly vulnerable to amyloid toxicity, with serotonergic and glutamatergic (GLUergic) cells also showing high sensitivity; the dopaminergic (DAergic) and γ-Amynobutyric acid (GABA)ergic systems appear better preserved (Bowen et al. 1983; Martorana and Koch 2014; Nitsch 1996; Procter et al. 1988). Providing an in-depth description of the mechanisms of these degenerations would be somewhat of a digression from the theme of this thesis. However, in order to further emphasise the molecular variabilities of AD, a general description follows:

Cholinergic signalling is involved in a range of cognitive abilities; amongst these, attentional and memory functions are a prominent dysfunction in age-related dementias (Blokland 1995; Furey et al. 2000; Hasselmo 1999; Hasselmo 2006; Himmelheber et al. 2000; Klinkenberg et al. 2011; Perry 1980). Decreased acetylcholine (ACh) synthesis has been reported in various cortical regions of post-mortem AD brains, including the temporal lobes (Richter et al. 1980; Rossor et al. 1982). Additionally, acetylcholinesterase (AChE) activity has been shown to increase Aβ aggregation (Eubanks et al. 2006). Cholinergic functioning decreases (in the limbic system) during 'normal' aging, which likely contributes to the age-related manner of AD impairments (see Albuquerque et al. 2009). The mechanisms of cholinergic vulnerability appears to relate to the nAChR $\alpha 7$ & $\alpha 4$ subunits (see Figure 1.3). The number of neurons expressing $\alpha 7$ (at a protein level) in the temporal cortices has been reported to decrease by around 50% in AD patients (Banerjee et al. 2000). Decreases in total protein expression of both $\alpha 4$ and $\alpha 7$ have been reported in the hippocampi of AD patient (35% and 36%, respectively; see Guan et al. 2000). Interestingly, reductions in protein expression

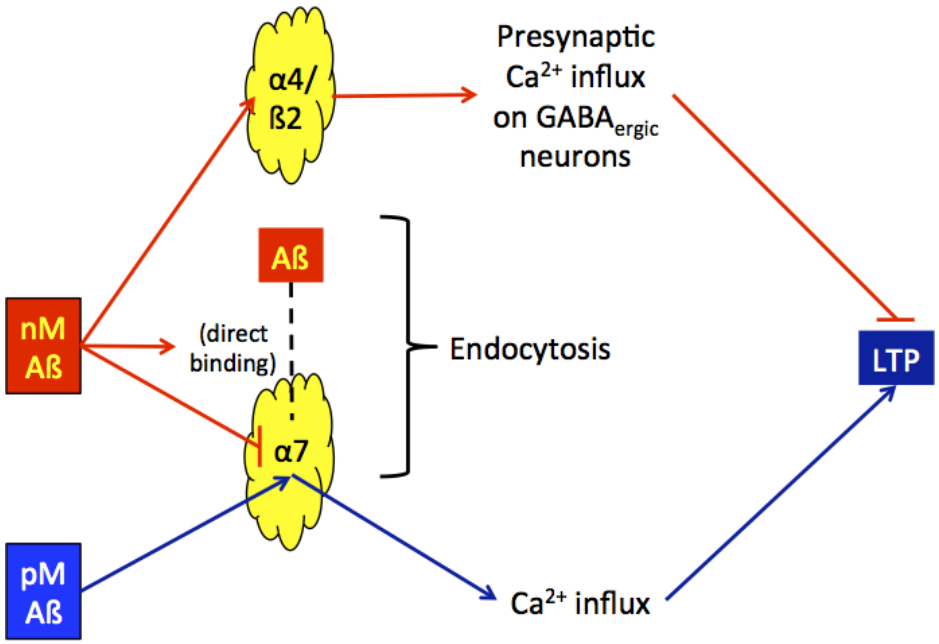

Figure 1.3: Nicotinic receptors in Alzheimer's-like diseases. $\alpha 4/\beta 2$-nAChRs facilitate pre-synaptic Ca^{2+} influx on GABAergic neurons, thus inhibiting synaptic transmission and LTP. This action is facilitated by high concentrations of Aβ peptides. $\alpha 7$-nAChRs facilitate Ca^{2+} influx at pre-synpatic excitatory neurons, as well as post-synaptically. Both of these actions are inhibited by high concentrations of Aβ peptides, whilst the pre-synaptic effect is facilitated by low concentrations.

have not been associated with changes in mRNA levels, demonstrating that these do not derive from transcriptional down-regulation (Wevers et al. 1999). Another study confirmed this finding with regard to the $\alpha 4$ subunit, but reported an increase in $\alpha 7$ mRNA (Hellström-Lindahl et al. 1999). It is possible that this finding may be explained by feedback from a reduction in protein load, however research to support this hypothesis is lacking. Behaviourally, this manifests primarily as cognitive deficits in memory functioning in AD patients (Coyle et al. 1983). Interestingly, nAChR $\alpha 7$ dysregulation has been implicated in traumatic brain injury (TBI), a condition which also includes amyloid deposition, as well as ApoE $\epsilon 4$ activity (Chu et al. 2005; D'Andrea and Nagele 2006; Hartman et al. 2002; Kelso et al. 2006; Parri et al. 2011; Pierce et al. 1996; Smith et al. 2003; Verbois et al. 2000; Verbois et al. 2003).

nAChR $\alpha 4$ subunits commonly form hetermeric receptor complexes with nAChR $\beta 2$ subunits (the most common nAChR formation). These receptors are expressed pre-synaptically on GABAergic neurons, facilitating their (inhibitory) signalling; an action facilitated by high (nM) concentrations of Aβ (Jürgensen and Ferreira 2010). Aβ peptides directly interact with the $\alpha 7$-nAChR (a homomeric receptor

which only contains the nAChR $\alpha 7$ subunit), inihibiting its activity (including its facilitation of Ca^{2+} influx at synaptosomes) (Lee and Wang 2003; Spencer et al. 2006; Wang et al. 2000a). Interestingly, the concentration of extracellular Aβ affects its modulation of $\alpha 7$-nAChR-mediated Ca^{2+} influx; low (pM) concentrations appear to facilitate pre-synaptic influx (and subsequently synaptic transmission), whilst higher (nM) concentrations inhibit post-synaptic influx (and subsequently long-term potentiation, LTP) (see Jürgensen and Ferreira 2010). Additionally, Aβ_{42} has been shown to bind $\alpha 7$-nAChRs with a greater affinity than Aβ_{40}, demonstrating one mechanism by which the longer peptide may facilitate neurotoxicity to a greater level than the shorter species (Lee and Wang 2003). Additionally, extracellular Aβ_{42} has been proposed to bind $\alpha 7$-nAChRs (expressed on the surface of neurons), and endocytosed to dysrupt intracellular mechanisms (D'Andrea and Nagele 2006).

Serotonergic pathways also play a role in memory functions, as well as anxiety and depression; dysfunctions which have been reported in clinical AD (Bowen et al. 1983; Chen et al. 1996; Cross et al. 1986; Devanand et al. 1996; Ferretti et al. 2001; Kepe et al. 2006; Meltzer et al. 1998; Palmer et al. 1987; Reinikainen et al. 1988; Teri et al. 1999; Wragg and Jeste 1989). An isoform of the 5-hydroxytryptamine $(5\text{-HT})_4$ receptor has been reported to regulate APP metabolism, reducing Aβ cleavage (Cho and Hu 2007; Lezoualc'h and Robert 2003). Whilst the exact nature of serotonergic dysregulation in AD is still unclear, this link between amyloid pathogenesis and depressive mood suggests it may present an important area of research for the welfare of patients.

Glutamatergic signals are excitatory, affect synaptic plasticity, and are involved in aspects of memory (Bashir et al. 1993; Bortolotto and Collingridge 1993; Liu et al. 2004; Malenka and Nicoll 1993; McEntee and Crook 1993; Nakanishi 1994; Tsien et al. 1996). Dysregulation in AD has been implicated in excitotoxicity, with an NMDA-R antagonist (memantine) prescribed as a treatment (Danysz and Parsons 2003; Hynd et al. 2004; Koutsilieri and Riederer 2007; Lipton 2006; Liu et al. 2007b). Interestingly, decreased glutamate, as well as aspartate, has been reported in the caudate nucleus of post-mortem AD brains, suggesting an impairment to decision making and reward-based association learning (Balleine et al. 2007; Haruno and Kawato 2006; Seidl et al. 2001). Further, Aβ inhibits NMDA-R functioning and causes loss of synapses (Shankar et al. 2007; Snyder et al. 2005).

Dopamine (DA) pathways facilitate reward signalling and fine motor control (Ikemoto 2007; Nestler and Carlezon 2006; Salamone 1992; Volkow et al. 1998; Wooten and Trugman 1989). DA irregularities have been reported in the ventral

tegmental area (VTA) and nucleus accumbens (NAcc) of AD patients, perhaps explaining dysfunctions observed in reward / motivational mechanisms (in addition to 5-HT dysregulation) (Mann et al. 1987a; Mitchell et al. 2011; Murray et al. 1995). nAChR $\alpha 7$ has been reported to mediate Aβ-induced DA secretion in the pre-frontal cortex (PFC), whilst muscarinic receptors facilitate an inhibition of NAcc DA, indicating a complex mechanism of indirect modulation (Preda et al. 2008; Trabace et al. 2007; Wu et al. 2007).

GABA functions as the primary inhibitory neurotransmitter of the developed brain (e.g. Davies 2003). Decreased GABA has been reported in temporal and occipital lobes, as well as the cerebellum of AD patients post-mortem (Rossor et al. 1982; Seidl et al. 2001). Receptor densities of both $GABA_A$ and $GABA_B$ are reduced in the hippocampi of post-mortem AD brains (Chu et al. 1987). In a rodent model of AD, reactive astrocytes (increased 'activated' astrocytes, a response to neuronal death and hallmark of AD; attenuation is associated with functional recovery) have been shown to synthesise GABA, with similar features reported in AD post-mortem brains (Eddleston and Mucke 1993; Jo et al. 2014; Ramírez et al. 2005). Additionally, Aβ_{40} has been shown to positively modulate $GABA_A$ $\alpha 6$ subunit (Zhan et al. 2014). This suggests that a shift in BACE1 cleavage of APP - to produce Aβ_{42} peptides (over Aβ_{40}) - would result in a decrease in (inhibitory) GABA signalling, leading to excitotoxicity.

1.1.3 Current therapies

Due to the underlying neurodegeneration in AD, it is logical that drug discovery would focus on *neuroprotection* and *neurogenesis*. 'Neuroprotection' is regarded in the current thesis as relating to mechanisms which attenuate dysregulated molecular signalling without necessarily having a direct '*anti-apoptotic*' action (i.e. inhibiting caspase signalling). By protecting existing neurons, or possibly replacing degenerating ones, the detrimental impacts of neurodgeneration may be somewhat attenuated. However the discovery and development of drugs to facilitate these phenomena have been distinctly lacking, with virtually all promising disease-modifying candidates failing clinical screening (Becker and Greig 2008; Becker and Greig 2010; Heemskerk et al. 2002; Schneider and Lahiri 2009). It is likely that these failures result from a failure to genotype patients in clinical development, often using a diagnosis of AD based on behavioural phenotypes (i.e. not distinguishing between the underlying aetiologies of these diseases).

In this regard, even attempting to treat the common mechanisms of neurodegeneration (i.e. caspase signalling) may be futile, as there is evidence from animal models that neuronal apoptosis occurs subsequent to cognitive impairment. For example, impaired *fear conditioning* has been reported in a double-transgenic mouse line (expressing hAPP/PS1) in the absence of neurodegeneration, with minimal (<1%) amyloid aggregation in the amygdala (which governs *fear conditioning* behaviour; see Section 3.1.2) (Knafo et al. 2009). Additionally, transgenic mice (expressing $hAPP_{SWE}$) show substantial amyloid deposition and plaque formation in the absence of neuronal apoptosis (Stein and Johnson 2002). Thus, the development of efficacious therapies appears to require pharmacological targetting of the specific molecular dysregulations which occur in different forms of AD. This approach has been overlooked in AD drug discovery, which has largely focussed on commonalities in these distinct pathologies.

There are currently only four drugs prescribed for AD, with the most recent being approved in 2003 (Cummings et al. 2014; Mangialasche et al. 2010). Of these current treatments, the primary action of three - *donepezil*, *galantamine* and *rivastigmine* - is the inhibition of cholinesterase, thereby prolonging the action of ACh (Ago et al. 2011; Cacabelos 2007; Polinsky 1998; Villarroya et al. 2007; Wilkinson 1999). These were developed in alignment with *cholinergic hypotheses* of AD (alluded to above in Section 1.1.2) - that dysfunction in cholinergic pathways are a major factor in AD (e.g. Coyle et al. 1983; Francis et al. 1999; Perry 1986; Shen 2004; Terry and Buccafusco 2003). The other drug - *memantine* - is a NMDA-R antagonist (Chen and Lipton 2005; Parsons et al. 2007). This was developed in light of the aforementioned findings of NMDA-related excitotoxicity in AD (Danysz and Parsons 2003; Olney et al. 1997). However in addition to these primary pharmacological targets, other (possibly beneficial) effects have been reported:

Donepezil is also a Sigma-1 receptor (σ_1; previously an orphan receptor, subsequently found to bind the endogenous hallucinogen *N,N*-dimethyltryptamine; Fontanilla et al. 2009) agonist, which is reported to protect against Aβ peptide toxicity, and may offer some level of neuroprotection against excitotoxicity, oxidative stress, ER stress, mitochondrial dysfunction and Ca^{2+} dysregulation (Griesmaier et al. 2012; Hayashi and Su 2007; Marrazzo et al. 2005; Maurice et al. 2006; Nguyen et al. 2015; Svensson and Nordberg 1998). The protection against Aβ toxicity has been shown to be indirectly mediated by α7-nAChR, but not $\alpha4\beta2$-nAChR (Arias et al. 2005). Donepezil has also been shown to inhibit GSK3β (indirectly, via protein kinase B), thus inhibiting tau phosphorylation (Hamano et al. 2013; Noh et al. 2013). Additionally, donepezil is a direct agonist of the

muscarinic M_1 receptor, suggesting an additional facilitation of memory functions, as well as modulation of axiogenic mechanisms (Roldán et al. 1997; Snape et al. 1999; Wall et al. 2001).

Galantamine, in addition to inhibition of AChE, exerts some level of butyrylcholinesterase (BChE) inhibition (Harvey 1995; Walsh et al. 2011). Inhibition of this non-specific cholinesterase has been shown to increase ACh, reduce Aβ peptide cleavage, and improve cognition (performance in maze assessment) and long-term potentiation in a transgenic mouse model (expressing $hAPP_{SWE}$ + PS1) (Greig et al. 2005). Galantamine has been reported to directly bind nicotinic (although not muscarinic) receptors ($\alpha3\beta4$, $\alpha4\beta2$, $\alpha6\beta4$), inducing a positive allosteric modulation (Samochocki et al. 2003). This has been shown to induce an increase in sAPPα (indicating an increase in α-secretase cleavage, in the 'non-amyloidogenic' processing pathway; see Chapter 4, Section 4.1.2) (Lenzken et al. 2007). *In vitro*, it has been found to protect against Aβ toxicity, mitochondrial dysfunction and ER stress (Arias et al. 2004; Liu et al. 2010). As with donepezil, galantamine's protection against Aβ toxicity has been shown to be mediated by α7-nAChR, but not $\alpha4\beta2$-nAChR (Arias et al. 2005). In comparison with donepezil, galantamine has been reported to exert greater long-term preservation of cognitive function (Wilcock et al. 2003).

Rivastigmine is less selective for AChE (over BChE) than donepezil and galantamine (Eskander et al. 2005; Farlow 2003; Ogura et al. 2000). Findings from AD post-mortem brains demonstrate greater BChE expression than AChE in amyloid plaques, indicating that targetting this enzyme may be more efficacious in the pathologies of AD (Eskander et al. 2005). Further, it has been found that AChE decreases with AD progression, whilst BChE increases (possibly as a compensatory mechanism for AChE), or else is unaffected (see Farlow 2003; Lane et al. 2006; Nordberg et al. 2013). Thus, drugs which specifically inhibit AChE may be less efficacious than BChE inhibition in later stages of the diseases. With regard to amyloid, rivastigmine has been shown to decrease Aβ production and increase sAPPα, indicating a shift from β- to α-secretase cleavage (Bailey et al. 2011; Ray et al. 2012; Yang et al. 2013). However, the neuroprotective effects of rivastigmine against Aβ are not mediated by α7-nAChR, in contrast to both donepezil and galantamine (Arias et al. 2005). Interestingly, via its affects on nAChRs, rivastigmine has been shown to somewhat attenuate 5-HT dysregulation following brain injury at a distant site (Islam et al. 2014).

Memantine (the NMDA-R antagonist) has been reported to protect neurons

Table 1.1: Pharmacology of current 'Alzheimer's disease' drugs. The primary pharmacological actions of the four current AD treatments are listed, as are known secondary secondary actions. The involvement of α7-nAChR in neuroprotective signalling is indicated.

Drug	'Primary' action(s)	'Secondary' action(s)	Mediated by α7-nAChR?
Donepezil	AChE (inhibitor)	σ_1R agonist (direct) GSK3β inhibitor (indirect) M_1 agonist (direct)	Yes
Galantamine	AChE (inhibitor) BChE (inhibitor)	nAChR ($\alpha3\beta4$, $\alpha4\beta2$, $\alpha6\beta4$) (positive modulator)	Yes
Rivastigmine	BChE (inhibitor) AChE (inhibitor)	Modulation of 5-HT-R expression	No
Memantine	NMDA-R (antagonist)	α7-nAChR antagonist	Yes

against glutamate excitotoxicity (Danysz and Parsons 2003; Lipton 2006; Tanović and Alfaro 2005). Interestingly, the mechanism of this excitotoxicity relates to the cellular location of NMDA activity. Indeed, activity at synapses has been shown to facilitate neuronal survival, whereas extrasynaptic NMDA transmission induces mitochondrial dysfunction and neurodegeneration (Hardingham et al. 2002; Hardingham and Bading 2010; Papadia and Hardingham 2007). In this regard, extrasynaptic NMDA-R are activated by glutamate, secreted from astrocytes in response to Aβ (a detrimental mechanism mediated by α7-nAChR; however memantine antagonises this receptor) (Banerjee et al. 2005; Talantova et al. 2013). The effect of Aβ on extrasynaptic NMDA-R impairs long-term potentiation and induces synaptic loss (Li et al. 2011; Rönicke et al. 2011). Interestingly, extrasynaptic NMDA-R activity has been shown to increase Aβ production, apparently forming a positive feedback loop, causing neurodegeneration (Bordji et al. 2010). Memantine (and its derivitive, nitromemantine) have been found to protect against Aβ-induced excitotoxicity, showing higher selectivity for extrasynaptic over synaptic receptors (Talantova et al. 2013; Xia et al. 2010).

Despite these potentially neuroprotective actions, the ability of these drugs to reduce atrophy is far from sufficient to halt disease progression. Indeed, despite a reduction (compared with untreated control subjects), donepezil patients still present a substantial level of hippocampal degeneration (Hashimoto et al. 2005). Another study found that, despite cognitive benefits, donepezil had no effect on disability progression or institutionalisation (Group 2004). A meta-analysis found

no effect for any of the cholinesterase inhibitors on disease progression (Ito et al. 2010). Additionally, methodological flaws in clinical studies have been highlighted, suggesting that positive findings for cholinesterase inhibition may be somewhat erroneously acclaimed (Kaduszkiewicz et al. 2005). Similarly, one study found that memantine reduced the rate of neurodegeneration specifically in the right hippocampus (although neither left hippocampus nor total brain showed any effect) (Weiner et al. 2011). However a longer-term (one-year) study failed to find significant effects on either total brain or hippocampal atrophy (Wilkinson et al. 2012).

Thus, the current AD therapies may provide some symptomatic / functional benefits. However no convincing effect on halting the underlying neurodegeneration has been consistently reported. In this light, it is clear that the discovery of new pharmacological interventions is imperative to sufferers of AD.

1.2 Relationship to abuse potential

Amongst the myriad problems associated with drug discovery is the area of *safety pharmacology*; *"a discipline which uses principles of pharmacology ...to generate data to inform risk / benefit assessment"* (Pugsley et al. 2008, p.1382). This is especially pertinent to CNS-acting drugs, as the ability of compounds to affect mood / emotional state may lead to abuse, having dire consequences for patients.

For example, a drug which facilitates signalling within the reward pathways (mesocorticolimbic system) may prove to have high *abuse potential* (i.e. a high likelihood that it will be abused by humans). Additionally, compounds may have psychologically depressive effects on patients, thus diminishing *quality of life* in order to extend *length of life*. In this light, there is a clear necessity to screen drugs which cross the blood-brain barrier (BBB) for their abuse potential.

A range of drugs with neuroprotective efficacy (in relation to some form of CNS insult) is abused in human society (discussed in Chapter 6, Section 6.1.2). With specific regard to AD, the assessment of abuse potential is particularly pertinent due to common comorbidity with depression and anxiety, which have been proposed as early risk factors for AD development (Caraci et al. 2010; Kessing and Andersen 2004; Ownby et al. 2006; Robert et al. 2002; Taft and Barkin 1990; Tune 1998). Indeed, the prescription of antidepressants is common in the treatment of

AD; with improved outcomes being reported for combined treatment with AD drugs (Thompson et al. 2007). However, the administration of a drug with anxiogenic properties would likely compound the disease-induced depression. Thus, the concept of altered mood in AD highlights the necessity to assess the effects of novel drugs on this manner of psychological state.

1.2.1 Reinforcing and anxiolytic side-effects

Donepezil agonises σ_1 receptor, exerting an anti-depressant effect on rodents (Maurice et al. 2006). Indeed, the agonistic action of abused drugs (e.g. cocaine, mephamphetamine) on σ_1 receptor suggests that this pharmacological mechanism may have significant abuse-related consequences (Narayanan et al. 2011; Nguyen et al. 2005; Sharkey et al. 1988). Of concern, σ receptor agonists have been shown to substitute cocaine in a rodent *self administration* assay (Hiranita et al. 2010). This suggests that donepezil may substitute for cocaine, either in rodent self-administration, or in human drug abusers. However, as yet, these specific questions have not been addressed in the literature.

In addition to 'reinforcing' drugs (i.e. those which facilitate signalling in the mesocorticolimbic system), anxiolytic drugs have a high tendency to be abused in human society (although those which act on the 5-HT system may have lower abuse potential; Wit and Griffiths 1991; Lader 1994). In this regard, donepezil, rivastigmine and memantine have all been shown to exert anxiolytic properties, with the cholinesterase inhibitors reported to be antidepressant (by reversal of chronic stress-related behaviours) in rodents; an effect the authors linked to increased cholinergic signalling (Papp et al. 2016). Indeed, another cholinesterase inhibitor - an extract from *Salvia officinalis* (commonly termed *garden sage*) - has also been reported to produce anxiolytic effects on humans (Kennedy et al. 2006). Additionally, nicotine itself has been shown to produce much the same effect (Brioni et al. 1993; Picciotto et al. 2002; Salín-Pascual et al. 1996; Tizabi et al. 1999). Importantly for the current thesis, the phenomenon of nicotine-induced anxiolytic behaviour has been observed in zebrafish using '*tank diving*' assessment (Levin et al. 2007).

The primary action of memantine, as mentioned above, is antagonism of NMDA-Rs. Two drugs with this same primary pharmacological function are ketamine and phencyclidine (PCP) - general anaesthetics with significant human

abuse potential (Dotson et al. 1995; Pradhan 1985). Interestingly, both of these drugs have been found to exert some level of neuroprotection (discussed in Chapter 6, Section 6.1.2), suggesting two commonalities between NMDA-inhibiting compounds (namely, neuroprotection and anxiolysis).

Interestingly, the administration of antidepressants to AD patients has been associated with a reduction in cognitive decline (Mossello et al. 2008). This may suggest that depression itself facilitates this loss of cognitive faculties; thus inhibiting the depressive state somewhat attenuates the cognitive impairments. Alternatively, it is possible that the antidepressant drugs exert some level of pharmacological nootropic / neuroprotective effect. In this light, the antidepressant (SSRI) citalopram has been shown to inhibit both AChE and BChE; co-administration with galantamine has been reported to increase cognitive preservation (Walsh et al. 2011).

1.2.2 High costs of drug development

Over a decade ago, the development of a new drug was estimated to cost over $802 million (USD); this figure has been rising, now believed to exceed $2.5 billion (Adams and Brantner 2006; DiMasi et al. 2003). This is even higher for CNS-acting drugs, causing many prominent pharmaceutical companies to reduce their endeavours in these fields (Pankevich et al. 2014; Wegener and Rujescu 2013).

Much of these costs are incurred due to the number of identified leads which fail in drug development (DiMasi et al. 2003). It has been estimated that 30% of drug failures are due to their being ineffective, whilst an additional 30% are due to safety concerns, demonstrating a clear necessity for improving pre-clinical safety screening in an economical manner (Kola and Landis 2004). In this regard, the abilities of zebrafish to perform basic cognitive tasks suggests that this organism may present a useful, economical tool for pre-mammalian drug screening.

Larval zebrafish has been highlighted as a possible model for early toxicity assessment (Redfern et al. 2008). However, in assessing some of the more complex aspects of safety pharmacology, it seems that adult zebrafish may also present an important candidate in this area of drug development.

1.3 Current animal models

Within basic neurobiology, there is a tendency to utilise rodents to model human diseases. This is largely due to the high level of neuroanatomical and genetic conservation between these mammals and humans (estimated at 99.5% of disease-related genes; Huang et al. 2004). Similarly, the ability to observe and empirically measure complex behavioural outcomes in rodents has led to their extensive utilisation.

1.3.1 Models of Alzheimer's-like diseases

Following high-throughput *in vitro* identification of potential compounds, drug discovery processes rely heavily on rodent models of disease (Hughes et al. 2011; Van Dam and De Deyn 2006). Generally, the non-transgenic models of AD (i.e. 'spontaneous', chemically induced and lesioned models) may induce degeneration anatomically related to AD and mimic cognitive deficits, however they lack amyloid and tau hallmarks (see Van Dam and De Deyn 2006).

With regard to transgenic rodents, several strains have been generated to model aspects of AD pathologies. The rodent models which do not express hAPP do not show amyloid pathologies, as murine Aβ does not form plaques (Johnstone et al. 1991; Van Dam and De Deyn 2006). By expressing mutant forms of hAPP, pathologies including Aβ plaque formation and cognitive deficits have been modelled (Games et al. 1995; Hsiao et al. 1996; Sturchler-Pierrat et al. 1997). However, these lack NFT, casting doubt over the concept of amyloid as a single causative factor in AD pathogenesis (as discussed above in Section 1.1.2).

The double-transgenic expression of hAPP with PS1 or tau develop increased amyloid pathology (and tau pathology for the tau-expressing double-transgenic); however the neuroanatomical distribution of AD hallmarks is inconsistent with 'typical' AD (Götz et al. 2004; McGowan et al. 2006; Van Dam and De Deyn 2006). Thus, in order to induce a more holistic representation of '*typical*' AD pathology, *triple transgenic* expression has been required thus far (Janelsins et al. 2005; Oddo et al. 2003a; Oddo et al. 2003b; Rhein et al. 2009; Van Dam and De Deyn 2006). Whilst the development of this model (expressing mutated versions of hAPP, PS1 and tau) appears to represent the pathologies, the requirement of three mutated transgenes leaves questions as to its relevance with

the aetiology of the diseases.

In light of these difficulties with current AD models, it is clear that the generation of new genetically manipulated lines is necessary. Indeed, the strong association of amyloid and tau with these diseases, coupled with the insufficienct pathologies caused by mis-expression of these genes, suggests that up-stream signalling proteins may play a more aetiological role in some of these disease pathologies. In this regard, the aforementioned functions of PIN1 and DAPK1 may present efficacious targets for both basic and translational research.

Behavioural measures

In the assessment of behavioural outcomes, a range of rodent assays has been designed and implemented to assess AD-related cognitive declines:

Maze tests involve training an animal to navigate to a specific location, assessing visuo-spatial learning and memory retrieval competencies (e.g. Drapeau et al. 2003; Morris 1984; Olton et al. 1977; Vorhees and Williams 2006). However these often require multiple training session, reducing the throughput when used in screening. Additionally, physical exertion is required in water mazes, possibly compromising investigations aimed at neurotransmitter systems involved in motivation or motor control.

Operant conditioning paradigms (e.g. '*Skinner box*') assess non-spatial memory (e.g. Nelson et al. 1997; Tonkiss et al. 1988; Van Dam and De Deyn 2006). These assays require multiple training sessions to an even greater extent than maze tests, again reducing the throughput. Additionally, the utilisation of food reward may affect investigations in which neuroanatomical structures involved in appetite or motivation are affected, or where experimental intervention has an impact on the size of an animal (as with hAPP transgenic mice; see Huitrón-Reséndiz et al. 2002).

Fear conditioning also assesses non-spatial learning, however the minimal training (conditioning) required means that throughput is much higher than other behavioural paradigms; thus it is more optimal for development as a screening assay (e.g. Arvanitis et al. 2014; Van Dam and De Deyn 2006). However assays typically involve measurement of 'freezing' behaviour, the assessment of which is difficult in rodents (Van Dam and De Deyn 2006). Thus, the development of a robust screening assay would require empirical measurement of an alternative

index of learning (e.g. preference for a conditioned stimulus, CS).

1.3.2 Reward and addiction research

Addiction is a complex phenomenon, assessed by the extent to which drug seeking behaviours become detrimental to the subject. It is defined as *"behaviour ...employed in a pattern characterised by (1) recurrent failure to control behaviour and (2) continuation of the behaviour despite significant negative consequences"* (Goodman 1990, p.1403). In this light, the assessment of 'addiction' is problematic in model organisms.

Indeed, the ability to motivate an animal to change it's drug-seeking behaviour is only possible using an alternative (non-drug) reward; thus the perseverance of drug-seeking would only be indicative of the relative strengths of the rewards (drug vs non-drug). Some have attributed the persistence of operant responding in the absence of drug delivery to 'addiction' (e.g. Deroche-Gamonet et al. 2004). However there is a distinct lack of motivation to cease responding, other than the absence of reward. Thus, it is difficult to associate this behaviour with the motivational influences involved in human cessation.

With regard to negative consequences, it is possible to administer a negative stimulus (e.g. an electric shock) in addition to drug delivery, following operant responding (Deroche-Gamonet et al. 2004). However the ethical considerations of administering punishment obviate the preference for developing assays in which this is not required. Further, findings that relatively few rodents display this level of persistent drug-seeking behaviour suggest a high number of animals are required to model human addiction (Deroche-Gamonet et al. 2004). Thus, the utility of assays which indicate 'drug reinforcement' (measured as drug consumption or drug-seeking behaviour in the absence of negative consequences), rather than 'addiction' appear to have higher efficacy in assessing the human abuse liability of novel compounds. Indeed, short-term reinforcement appears a more sensitive index of human abuse potential (which would often follow prolonged exposure) than attempting to induce short-term addictive behaviour in model organisms.

The investigation of reward signalling and drug-seeking behaviours commonly involves wild-type animals (e.g. Baker et al. 1998; Shi et al. 2004). However, rodents carrying mutations - often to receptor targets of a drug - have been utilised to

assess the genetic basis of reward and addiction (e.g. Caine et al. 2002; Changeux 2010; Chiamulera et al. 2001; Cunningham et al. 2000; Epping-Jordan et al. 1999). Additionally, selective breeding has lead to the generation of rodent strains with high sensitivity to reinforcing compounds (e.g. alcohol-prefering rats; see Bell et al. 2006; McBride et al. 2014; Vengeliene et al. 2003).

Behavioural measures

The assessment of reward and drug-seeking behaviours has commonly revolved around two assays - self administration (SA) and conditioned place preference (CPP). Additionally, drug discrimination has been used to assess *abuse liability* in comparison to a drug with known abuse potential. These will be discussed in greater detail later in this thesis (see Chapters 6 and 7). However brief descriptions of typical paradigms are provided here:

Self administration assays involve the conditioned pairing of an operant behavioural response (i.e. presses on a lever) with the administration of a drug, typically delivered via a transcranial cannula (e.g. Ettenberg et al. 1982; Roberts et al. 1996; Roberts et al. 1999; Shaham et al. 1993). The response rate (number of drug administrations by lever presses in a given time) is then used as a measure of the reinforcing properties of the drug.

CPP assays involve the pavlovian conditioning of a drug with a neutral environmental stimulus (CS). Following conditioning, the animal's preference for the CS (in the absence of drug administration) is compared to basal preference, as a measure of drug-seeking behaviour.

Drug discrimination utilises operant conditioning paradigms, in which the animal receives a food reward following an operant behaviour (i.e. lever presses). Multiple training sessions are required, whereby presses on one lever are reinforced with food following drug administration, whilst presses on the alternative lever are reinforced following saline administration. A novel compound may then be administered and the animal placed in the conditioning chamber without any food reinforcement. A significantly higher number of presses on the drug-paired lever indicates that the novel drug is producing a '*state*' similar to that of the trained

drug. It thus indicates the specific pharmacology of a drug in relation to a drug with known pharmacological actions.

1.4 Using zebrafish to refine current animal models

The zebrafish (*Danio rerio*) has been utilised in molecular biological research for a number of years. The sequencing of the zebrafish genome has facilitated it's utility in molecular genetic investigations, as well as other basic molecular biological applications (Howe et al. 2013). Further, the organism is estimated to have conserved orthologues of 70% of human genes, and 80% of disease-related genes (Howe et al. 2013; Howe et al. 2017). This high level of genetic conservation highlights the potential efficacy of using zebrafish for both forward and reverse genetic screens, as well as pharmacological target validation and lead identification studies.

Within the field of molecular neurobiology, the zebrafish is an unique organism. The larvae remain transparent until approximately five days-post-fertilisation, and pigmentation can be inhibited after this stage using phenylthiocarbamide (PTU). This allows fluorescent tags to be fused to genes of interest, and imaged in vivo (e.g. Higashijima et al. 2000; Lawson and Weinstein 2002; Parinov et al. 2004). Further, an 'albino' ('*casper*') mutant line has been developed, in which pigmentation is inhibited throughout life, allowing fluorescent imaging in the adult fish (White et al. 2008).

1.4.1 Neuroanatomical conservation and molecular signalling

As a vertebrate organism, many neuroanatomical regions of the mammalian brain have been reported to have orthologues in zebrafish. Whilst this will be discussed in greater detail in subsequent chapters of this thesis, key conserved structures (as relative to the current research) have been reported to include the amygdala (medial zone of the dorsal telencephalic area, Dm), hippocampus (lateral zone of the dorsal telencephalic area, Dl), VTA (posterior tuberal nucleus, PTN), NAcc (dorsal nucleus of the ventral telencephalic area, Vd), and PFC (central zone of the

dorsal telencephalic area, Dc) (see Figures 3.2 & 6.2) (Klee et al. 2012; Mueller et al. 2011; Parker et al. 2012b; Rink and Wullimann 2001; Rupp et al. 1996).

1.4.2 Current AD-related transgenic zebrafish

Despite the significant utility of hAPP expression in rodent models of AD, transgenic zebrafish lines have paid more heed to tauopathies. The transgenic expression of human mutated tau has been shown to present hyperphosphorylation and neurodegeneration, whilst the inclusion of a fluorescent tag allows assessment of tau expression *in vivo* (Paquet et al. 2009; Paquet et al. 2010). Other transgenic mutant tau lines have been reported to present microtubule dysfunction representative of NFT (Bai et al. 2007; Tomasiewicz et al. 2002). However there is, as yet, no reports of a stable transgenic zebrafish amyloidogenic model.

With regard to the direction of the current research, despite the aforementioned uncertainties about the aetiological role of amyloid, a significant proportion of transgenic mice used in AD drug discovery present amyliodopathies. Thus, in order for zebrafish to prove efficacious as a refinement on rodent models, an observable amyloid-induced phenotype must be identified. Therefore, this thesis will assess cognition in an already-established (but unpublished) transgenic zebrafish (expressing $hAPP_{LON/SWE}$; see Chapter 5). Subsequent to this, the generation of new genetically manipulated zebrafish lines may be viewed as viable alternatives to the development of rodent strains in translational neurobiology.

1.4.3 Behavioural competencies

Despite their long utilisation in molecular biological investigations, the extent to which zebrafish are efficacious in behavioural research has only recently come to light. For example, they reliably perform simple choice discrimination tasks, in both appetitive and aversive conditioning paradigms (Bilotta et al. 2005; Valente et al. 2012). They have also been demonstrated to be capable of more complex behavioural tasks, including 5-choice discriminations, reversal learning and set-shift discrimination (Parker et al. 2014; Parker et al. 2012a).

It has also been reported that zebrafish display drug-seeking behaviour in CPP paradigms, suggesting their efficacy as a model for human drug reward (Collier et

al. 2014; Mathur et al. 2011). Importantly, the conservation of neuronal structures and pathways which govern many reward mechanisms have been demonstrated in zebrafish (mentioned above and discussed in Chapter 6). Whilst this implies their efficacy as a model in basic research, it also suggests their utility in industrial safety pharmacological settings; by implementing zebrafish screens prior to rodent studies it may be possible for pharmaceutical companies to reduce both financial and ethical costs associated with drug discovery. However, the extent (i.e. drug range) to which zebrafish respond to compounds abused in human society is currently unknown.

As mentioned above, anxiolytic / anxiogenic behaviours are observable in zebrafish using 'tank diving' assays (e.g. Levin et al. 2007). Whilst this specific assay is not pertinent to the current thesis, it demonstrates the conservation of these behavioural phenomena, which are imperative to the experience of reward and aversion.

1.5 General aims

The aims of this thesis are to investigate the utility of zebrafish in translational neurobiology, as a refinement on rodent models currently used This will be undertaken through the following objectives:

1. To develop an automated behavioural paradigm to assess *classical aversion learning* in zebrafish
2. To assess neurotoxin (aluminium, Al) treatment as a model of neurodegeneration in zebrafish, testing the hypothesis that *classical aversion* will be impaired
3. To assess the effects of transgenic hAPP expression in zebrafiish, as a model of AD-related molecular dysregulation, testing the hypothesis that *classical aversion* will be impaired
4. To determine the utility of a zebrafish CPP paradigm as a model of human reward pharmacology
5. To develop a zebrafish *drug discrimination* paradigm, testing the hypothesis that zebrafish can be used to predict human *abuse liability*

Chapter 2

General Methodology

2.1 Animal maintenance

Zebrafish (*Danio rerio*) of the Tubingen wild-type strain were used in all experimentation, unless stated otherwise. All fish were housed on a circulating aquarium system, at 28^{o}C, using a 14-hour : 10-hour light:dark cycle. Fish were fed a commercial, general-purpose pellet food in the morning, and live (hatched) brine shrimp (*artemia*) in the afternoon.

Breeding was conducted by 'pairing' fish (typically one male with with female) in breeding tanks (containing inserts with perforated floors) in the evening. The following morning, after the room lights were switched on, the water in the breeding tanks was changed and the fish were not fed until the afternoon. Eggs were collected at approximately midday, placed in clean aquarium water, and incubated (in Petri dishes) at 28^{o}C until five days post fertilisation. Following this, fry were fed a commercial fry food until ten days post fertilisation, then added to the circulating aquarium system.

2.2 Total RNA extraction from zebrafish brain tissue

Zebrafish were sacrificed by decapitation and brain tissue removed by dissection on a dry Petri dish lid, using micro-dissection forceps. Immediately following dissection, tissue was placed in a 'freezer block' (-20^o) for transportation. If the samples were not processed on the same same day, they were stored at -80^{o}C until processing.

RNA was extracted from each brain individually, using a Trizol-chloroform method. 500μl of Trizol (Ambion®, Thermo Fisher) was added to each brain and homogenised by trituration through pipette tips, in descending sizes (P1000, then P200, then P10). Following trituration, a further 500μl of Trizol was added, the combined 1mL samples were mixed (by pipetting six times) and then incubated at room temperature for 5 minutes.

200μl of chloroform was then added, and the sample agitated vigorously (by shaking) for 15 seconds. Following agitation, the samples were incubated at room temperature for 3 minutes, before centrifugation at $\geq$x17,000g for 15 minutes, at 4^{o}C.

The upper aqueous phase was then removed to a clean sample tube, mixed with isopropanol (1:1, mixed by pipetting six times), and incubated on ice for 10 minutes. The mixture was then centrifuged at $\geq$x17,000g for 30 minutes, at 4^{o}C.

The resultant supernatant was discarded. The remaining pellet was washed with 70% ethanol and centrifuged at $\geq$x17,000g for 5 minutes, at 4^{o}C.

The resultant supernatant was discarded. The remaining pellet was then air-dried, re-suspended in 30μl ddH_2O, and incubated at 55^{o}C for 10 minutes. Samples were stored at -20^{o}C short-term (up to 2 weeks) or at -80^{o}C for longer-term storage.

2.3 Quantification of nucleic acids

Nucleic acid concentrations (both DNA and RNA) were determined using 2.5μl drops on a Nanodrop 2000 (ThermoScientific), as per the manufacturer's recomendations. Absorbance was read at 260nm.

2.4 DNAse I treatment and cDNA synthesis

In order to remove any genomic DNA, the RNA extracts were treated with DNAse I (New England Biolabs). Frozen RNA samples were thawed on ice. The following reaction was then set up:

Total RNA	x μl (1μg)
Protoscript II Reaction Mix (2x)[1]	6.5μl
DNAse I	0.1μl
ddH_2O	to 12.5μl
Total reaction volume	**12.5μl**

[1]Part of the New England Biolabs *Protoscript II First Strand cDNA Synthesis Kit*, which was subsequently used to reverse-transcribe RNA into cDNA.

The reaction mix was incubated at 37^{o}C for 10 minutes, then at 75^{o}C to heat-inactivate the DNAse I enzyme.

The DNAse-treated RNA was then reverse transcribed using the Protoscript II First Strand cDNA Synthesis Kit (New England Biolabs) in the following reaction:

(DNAse I-treated RNA)	(12.5μl)
Protoscript II Reaction Mix (2x)	3.5μl
Protoscript II Enzyme Mix (10x)	2μl
Random Primer Mix	1μl
OligoDT Primer Mix	1μl
Total reaction volume	**20**μl

The reaction was incubated at 25^{o}C for 5 minutes (to facilitate primer binding), 42^{o}C for 60 minutes (temperature for enzymatic reaction), the enzyme was heat-inactived 85^{o}C for 5 minutes.

2.5 Agarose gel electrophoresis

Agarose was added to TAE buffer (40mM Tris, 20mM glacial acetic acid, 1mM EDTA) at the specified concentrations (between 1% - 2%) and heated in a microwave oven, on full power, until dissolved. peqGREEN dye (VWR International.; item 732-3196) was added to the solution as per the manufacturer's instructions (5μl in 100mL) and mixed by gentle agitation. The solution was then poured into a mould, a comb added, and allowed to set by cooling at room temperature.

The comb was then removed, the solidified gel was placed in an electrophoresis tank, and immersed in TAE buffer (described above) to cover the wells. DNA / RNA fragments were mixed with 'Loading Dye' (NEB; item B7025S) as per the manufacturer's instructions (1:6 dye:sample), and added to the wells of the gel. A DNA ladder (NEB; item N3200S) was added in the same manner to a well. The lid was then placed on the electrophoresis tank, and the nucleotide fragments separated by running the tank at 120V for approximately 25 minutes. The gel was then imaged on a '*Fusion Solo S*' imaging machine (Vilber Lourmat), using Vision Capt software.

2.6 Preparation of LB-broth

LB-broth (Miller's) was dissolved in ddH_2O, as per the manufacturer's instructions (25g/L). The solution was autoclaved, then allowed to cool to room temperature before antibiotics were added at 100μg/mL.

2.7 Preparation of agar plates

LB-agar was dissolved into ddH_2O as per the manufacturer's instructions (17.5g into 500mL). The solution was then autoclaved. Immediately following autoclaving, the agar was placed in a water bath, pre-heated to 50^{o}C.

Once the water bath temperature had returned to 50^{o}C, the relevant antibiotic was added to the agar at 100μg/mL. If blue/white screening was required, X-gal and IPTG was also added to the agar (final concentrations of 40ng/μl & 0.1mM, respectively). Plates were then poured immediately, using approximately 25mL agar per sterile Petri dish.

Plates were left to set for at least 30 minutes before plating cells. If not used immediately, plates were wrapped in parafilm and stored at 4^{o}C for a maximum of 4 days.

2.8 Preparation of competent cells

All procedures described in this section were conducted using aseptic technique (i.e. in the presence of a Bunsen burner flame).

One aliquot (50μl) of Top10 Competent Cells (Thermo Fisher) was thawed on ice and then streaked onto a LB-agar plate, containing streptomycin. The plate was then incubated up-side-down at 37^{o}C, overnight, in a temperature-regulated room.

The following morning the plate was removed from the heated room and stored at 4^{o}C, wrapped in parafilm. Single (isolated) bacterial colonies were later selected from the plate and cultured in 5mL LB-Broth containing streptomycin. Cultures

were then incubated at 37^{o}C in a shaking incubator, overnight, to generate 'starter cultures'.

The following morning, 1mL of the 5mL starter culture was added to 100mL fresh LB-broth (containing streptomycin). The larger culture was then incubated at 37^{o}C in a shaking incubator, for approximately 2 hours. The cells were then cooled on ice for approximately 15 minutes.

The 100mL culture was divided into 2x 50mL Falcon tubes and centrifuged at 500rpm for 10 minutes, at 4^{o}C. The supernatant was removed by decanting and the pellets re-suspended in ice-cold TfbI (see below), at a combined volume of 50mL. The re-suspended cells were inoculated on ice for approximately 15 minutes.

The cell suspension was centrifuged at 500rpm for 10 minutes, at 4^{o}C, the supernatant removed by decanting, and the cells were re-suspended in 4mL ice-cold TfbII (see below). The cells were then aliquotted at 100μl and stored at -80^{o}C until use.

Reagents for TfbI solution

Potassium acetate	0.295g
$MnCl_2$*$4H_2O$	0.989g
KCl	0.745g
CaCl*$2H_2O$	0.147g
Glycerol	15mL
ddH_2O	(to 100mL)

→pass through sterile 0.22μm filter

Reagents for TbfII solution

MOPS	0.209g
CaCl*$2H_2O$	1.1g
KCl	0.075g
Glycerol	15mL
ddH_2O	(to 100mL)

→pass through sterile 0.22μm filter

2.9 Transformation and culturing of competent cells

All procedures described in this section were conducted using aseptic technique (i.e. in the presence of a Bunsen burner flame).

Competent cells were thawed on ice for at least 10 minutes, then aliquotted into pre-chilled sample tubes at 50μl per transformation. 50-100ng of plasmid DNA was added per transformation and mixed by 'flicking' the tube six times. The samples were incubated on ice for 20 minutes, mixing again after approximately 10 minutes.

Samples were 'heat-shocked' at 42^{o}C for 60 seconds then incubated on ice for 2 minutes. 950μl LB-broth (without antibiotics) was added to the transformed cells and the culture incubated at 37^{o}C in a shaking incubator for 1 hour.

Cultures were then centrifuged at $\geq$x17,000g on a bench-top microcentrifuge for 2 minutes. The supernatant was removed by decanting and the cell pellet re-suspended in approximately 100μl LB-broth (without antibiotics). The re-suspended cells were plated on LB-agar plates (prepared as described in Section 2.7) containing 100μg/mL antibiotic (usually ampicillin).

The cultured plates were left to dry for approximately 10 minutes and then incubated up-side-down at 37^{o}C, overnight.

The following day the plates were removed from incubation, wrapped in parafilm, and stored at 4^{o}C for the duration of the day. Individual (isolated) colonies were then selected using a sterile P10 pipette tip and cultured in 5mL LB-broth containing antibiotics at 100μg/mL (usually ampicillin). These cultures were then incubated at 37^{o}C, in a shaking incubator, overnight.

2.10 Generation of glycerol stocks of transformed cells

All procedures described in this section were conducted using aseptic technique (i.e. in the presence of a Bunsen burner flame).

Following transformation (as described in Section 2.9), the 5mL 'Starter cultures' were removed from incubation. 500μl of each culture was added to 500μl of autoclaved 60% glycerol, vortexed at maximum speed for approximately 10 seconds, then stored at -80^{o}C.

2.11 Gel extraction

DNA was extracted from agarose gels (following electrophoresis) using the *QIAquick Gel Extraction* kit (QIAGEN), as per the manufacturer's instructions.

All centrifugation steps were conducted on a bench-top microcentrifuge at maximum speed (∼17,000xg), at room temperature.

Following electrophoresis through agarose gels, bands corresponding to the correct gene fragment sizes were excised from the gels using a sharp scalpel. The gel slices were weighed, and *Buffer QG* was then added at 3x v/w for 1% gels, or 6x v/w for 2% gels. The gels were dissolved in the buffer by incubating in a 50^{o}C waterbath for 10 minutes (vortexing occasionally).

DNA was then precipitated by adding isopropanol at 1x v/w gel volume mixed by vortexing. The samples were then placed in QIAquick spin columns and centrifuged for 60 seconds.

Flow-through was discarded by decanting, 500μl *Buffer QG* added to the spin columns and then centrifuged for 60 seconds.

Flow-through was discarded by decanting, 750μl *Buffer PE* added to the columns and then centrifuged for 60 seconds.

Flow-through was again discarded and the column re-centrifuged (dry) for 60 seconds.

The columns were then removed and added to a clean microcentrifuge tube. 30μl ddH_2O was added to the centre of each column, left to stand at room temperature for 3 minutes, then centrifuged for 60 seconds.

2.12 Miniprep purification of plasmid cultures

Plasmid DNA from 'Starter cultures' (5mL) was purified using the *Quicklyse Miniprep* kit (QIAGEN), as per the manufacturer's instructions. All centrifugation steps were conducted on a bench-top microcentrifuge at maximum speed (∼17,000xg), at room temperature.

Following generation of glycerol stocks (as described in Section 2.10), 1.5mL of each starter culture was added to a 2mL sample tube and centrifuged for 60 seconds. Supernatant was removed by decanting and the cells re-suspended in 400μl ice-cold *Complete Lysis Solution* by vortexing at maximum speed, for approximately 30

seconds. Samples were incubated at room temperature for 3 minutes (to allow cell lysis). The lysates were then added to *QuickLyse spin columns* and centrifuged for 60 seconds.

The column was washed with 400μl *Buffer QLW* (containing ethanol) and centrifuged for 60 seconds. The flow-through was discarded and the column re-centrifuged (dry) for 60 seconds.

The columns were then removed and added to a clean microcentrifuge tube. 30μl ddH_2O was added to the centre of each column, left to stand at room temperature for 3 minutes, then centrifuged for 60 seconds.

2.13 Maxiprep purification of plasmid cultures

For applications that required large quantities of plamid DNA, 100μl of 'Starter cultuers' (5mL) or 200μl glycerol stocks were inoculated into 100mL LB-broth and cultured at 37^{o}C, in a shaking incubator, overnight. Plasmid DNA was then purified using the *QIAfilter Maxi* kit, as per the manufacturer's instructions.

Cells were harvested by centrifugation at 6,000xg, at 4^{o}C, for 15 minutes. Supernatants were discarded by decanting and the cell pellets re-suspended in 10mL *Buffer P1* by vortexing at maximum speed.

10mL *Buffer P2* was then added to the cell suspension and the sample mixed by vigorous inversion approximately 6 times. Mixed samples were then incubated at room temperature for 5 minutes to lyse cells.

10mL *Buffer P3* was added to the lysates, the samples mixed by vigorous inversion approximately 6 times, and the samples added to the barrels of *QIAfilter Cartridges* (with caps attached to the outlet nozzle). The samples were incubated in the cartridges at room temperature for 10 minutes (without the plunger inserted).

During the incubation period, *QIAGEN-tip 500* tips were equilibrated by application of 10mL *Buffer QBT*, and allowing the buffer to pass through the tip by gravity flow.

The caps were then removed from the *QIAfilter Cartridges*, the plungers inserted into the cartridges, and the samples were filtered into the equilibrated *QIAGEN-tip 500* tips. The samples were allowed to pass through the tips by gravity flow.

The tips were washed with 30mL *Buffer QC* (x2), which were allowed to pass through the tip by gravity flow. DNA was then eluted from the tips by adding 15mL *Buffer QF* and allowing it to pass through the tips, into a clean 15mL Falcon tube, by gravity flow.

DNA was precipitated by addition of 10.5mL isopropanol to eluted DNA and mixed by vortexing at maximum speed. Samples were then centrifuged at $\geq$15,000xg, at 4^{o}C, for 30 minutes.

The supernatant was discarded by decanting, the DNA pellet washed with 5mL 70% ethanol and centrifuged at $\geq$15,000xg for 10 minutes.

The supernatant was discarded and the DNA pellet allowed to air-dry for approximately 10 minutes. DNA was then re-suspended in 100μl ddH_2O.

2.14 PCR purification

Polymerase chain reaction (PCR) products were purified using the *QIAquick PCR Purification* kit (QIAGEN), as per the manufacturer's instructions. All centrifugation steps were conducted on a bench-top microcentrifuge at maximum speed ($\sim$17,000xg), at room temperature.

Buffer PB was added to the PCR product at a ratio or 5:1 (buffer:sample - i.e. 125μl buffer to 25μl PCR product) and mixed by pipetting. Mixed samples were added to QIAquick spin columns and centrifuged for 60 seconds.

Flow-through was discarded, 750μl *Buffer PE* added to the columns, and centrigued for 60 seconds. Flow-through was again discarded and the columns re-centrifuged (dry) for 60 seconds.

The columns were then removed and added to a clean microcentrifuge tube. 30μl ddH_2O was added to the centre of each column, left to stand at room temperature for 3 minutes, then centrifuged for 60 seconds.

2.15 Ethanol precipitation

DNA / RNA was purified by ethanol precipitation in the following manner. All centrifugation steps were conducted on a bench-top microcentrifuge at maximum speed ($\geq$17,000xg), at 4^{o}C.

Sodium acetate (3M, pH5.2) was added to nucleic acid at 1/10$^{\text{th}}$ volume (i.e. 1μl sodium acetate to 9μl sample). The samples were mixed thoroughly by pipetting.

Pre-chilled ethanol was then added at 2.5x volume of mixed samples (i.e. 25μl added to 10μl sample). The samples were mixed thoroughly by pipetting, then incubated at -20^{o}C for at least 20 minutes.

Following incubation, the samples were centrifuged at $\geq$x12,000g for 15 minutes. Supernatants were discarded by decanting. The pellet was then washed by adding 70% ethanol and mixing by inversion (6x). Samples were centrifuged at $\geq$x12,000g for 2 mins.

The supernatant was discarded by decanting. The pellet was allowed to air-dry, then re-suspended in an appropriate volume of ddH_2O or buffer.

2.16 Cloning into pGEM®-T Easy

PCR products with adenosine overhangs (i.e. from Taq polymerase) were cloned into the pGEM-®-T Easy vector (Promega) - which contains complementary thymidine overhangs - as per the manufacturer's instructions. The following reactions were set up:

Ligation buffer (2x)	5μl
pGEM®-T Easy vector (50ng)	1μl
PCR product (insert)	$x\mu$l*
T4 DNA ligase	1μl
ddH_2O	(to 10μl)
Total	**10μl**

*(3:1 insert:plasmid molecular ratio)

Table 2.2: Primer sequences used to generate *in situ* hybridisation probes. All primer sequences were designed using the NCBI *Primer Blast* tool. Annealing temperatures were initially calculated using the New England Biolabs Tm calculator tool (NEB 2016) and optimised if needed.

Gene	Primer sequence	Fragment size	Annealing temperature
apoeb	Forward: AACACACAAACTGACGGCAT Reverse: CTCCAAGACAGGCAAGTTGA	978 bp	50^{o}C
appb	Forward: TGGGACAACGATGAAGACGG Reverse: TGGCCTGGTTAAAGCTCTCG	1,000 bp	51^{o}C
bace1	Forward: AGCGGGGATTATGGAGATGTG Reverse: TGCAATCTCTGCATAGGCCA	805 bp	50^{o}C
gsk3β	Forward: TGCGTCAGCACAGAATGATA Reverse: GCTTTACATTGAGGGTTCGG	1,135 bp	48^{o}C
pin1	Forward: GCTTCTCAGGGCGTGTGTAT Reverse: TTAGCCGTGCCGGATTAAGG	672 bp	51^{o}C
M13	Forward: GTAAAACGACGGCCAGT Reverse: GGTCATAGCTGTTTCCTG	-	44^{o}C

Reactions were mixed by pipetting, then incubated at room temperature for 2 hours.

Ligated DNA (2μl) was then transformed into competent cells (as described in Section 2.9), then plated on ampicillin plates using blue/white selection (as described in Section 2.7).

2.17 Primer design for *in situ* hybridisation probes

cDNA sequences were derived from the Ensembl project website (Flicek et al. 2014). All primers (see table 2.2) were designed using the NCBI *Primer Blast* tool (Ye et al. 2012) and purchased from Eurofins Genomics. Where possible, primers were designed to amplify a sequence that includes an intron in genomic DNA.

2.18 Generation of *in situ* hybridisation probes from cDNA

Total RNA was extracted, DNAse-treated and reverse transcribed into cDNA, as described in Sections 2.2 and 2.4. Gene fragments were then PCR-amplified from cDNA (using the primers specified in Table 2.2, in the following reaction (reagents from New England Biolabs):

Reaction mix:

Thermopol Buffer (10x)	2.5μl
dNTPs (10mM)	0.5μl
Forward primer (10μM)	0.5μl
Reverse primer (10μM)	0.5μl
cDNA	2μl
Taq polymerase	0.125μl
ddH_2O	18.375μl
Total	**25μl**

Thermocycles:

Initial denaturation	95^{o}C	30 seconds	
Denature	95^{o}C	30 seconds	x35 cycles
Anneal	(variable)	1 minute	
Extension	68^{o}C	1.5 minutes	
Final extension	68^{o}C	5 minutes	
Hold	4^{o}C	∞	

The resulting gene fragments were electrophoresed through a 1% agarose gel. Bands of the correct size were extracted from the gel as described in Section 2.11.

Extracted gene fragments were then cloned into pGEM®-T Easy, transformed into competent cells with blue/white screening, and cultured as described in Sections

2.16, 2.9 and 2.7. *Starter Cultures* were Miniprepped as described in Section 2.12 (following glycerol stock generation, described in Section 2.10). Minipreps were sequences (by Eurofin Genomics) to confirm insertion of the correct gene fragment and to check orientation ($5' \rightarrow 3'$) in pGEM®-T Easy.

The gene fragments were then amplified from the pGEM®-T Easy plasmid using M13 primers (to include both the SP6 and the T7 polymerase sites), in the following reaction (reagents from New England Biolabs):

Reaction mix:

Thermopol Buffer (10x)	10μl
dNTPs	2μl
M13 Forward primer	2μl
M13 Reverse primer	2μl
DNA (Miniprep)	1μl
Taq polymerase	0.52μl
ddH_2O	82.48μl
Total	**100μl**

Thermocycles:

Initial denaturation	95^{o}C	30 seconds	
Denature	95^{o}C	30 seconds	x35 cycles
Anneal	44^{o}C	1 minute	
Extension	68^{o}C	1.5 minutes	
Final extension	68^{o}C	5 minutes	
Hold	4^{o}C	∞	

The linear fragments were PCR purified (as described in Section 2.14), and then *in vitro* transcription of the riboprobes was set up in the following reactions (using Roche SP6/T7 Transcription Kit):

Transcription Buffer (10x)	1μl
Linear DNA template	$x\mu$l (1μg)
NTPs (Dig-labelled U)	1μl
RNAse inhibitor	0.5μl
RNA polymerase*	1μl
ddH_2O	(to 10μl)
Total	**10μl**

*(*Either SP6 polymerase or T7 polymerase, depending on gene orientation.)*

Samples were mixed by pipetting, then incubated at 37^{o}C for 3 hours. Riboprobes were purified by ethanol precipitation (as described in Section 2.15) and re-suspended in 83.3μl buffer (containing 25% formamide and *5*x *SSC Buffer* (75mM sodium citrate, 750mM NaCl)).

2.19 *In situ* hybridisation

In situ hybridisation was conducted as described previously (Carleton et al. 2014):

Tissue preparation

Zebrafish were sacrificed by decapitation. Whole brains were dissected on a dry Petri dish lid, placed in OCT compound and immediately frozen on dry ice. Time between sacrificing and freezing did not exceed 10 minutes. Tissue was stored at -80^{o}C until further processing.

Tissue was then cut in -30^{o}C in 10μm sections and mounted onto charged ('*Superfrost*') slides (VWR). Slides were then stored at -20^{o}C until further processing.

Slides were fixed for exactly 5 minutes in 3% *Fixation Buffer* (3% paraformaldehyde, 0.3% NaH_2PO_4*H_2O, 1.6% $NaHPO_4$, + 3 drops NaOH per 700mL). Slides were then dehydrated in an ascending ethanol series (70%, 95%, 100%) for 2 minutes each.

If slides were not immediately processed further, they were stored at -80^{o}C until needed.

In situ probing

Slides were acetylated in freshly prepared *Acetylation Solution* (0.675% triethanolamine, 0.15% acetic anhydride) at room temperature for 10 minutes. Slides were then rinsed in *2*x *SSPE Buffer* (1.753% NaCl, 0.276% NaH_2PO_4*H_2O, 0.074%, pH 7.4), twice. Sections were dehydrated in an ascending ethanol series (70%, 95%, 100%) for 2 minutes each. They were then air-dried for ∼10 minutes.

120μl *hybridisation solution* (50% deionised formamide, *2*x *SSPE Buffer*, 2μg/μl yeast tRNA, 250ng/mL riboprobe) was pipetted on to each slide. A strip of parafilm placed on top and the slides incubated in a humidity chamber at 65^{o}C overnight.

The following morning, slides were immersed in cold *2*x *SSPE Buffer* and the parafilm peeled off using forceps. Samples were then washed in *Post Hybridisation Wash Buffer I* (*2*x *SSPE Buffer*, 50% formamide) at 65^{o}C for 70 minutes (agitating every ∼10 minutes), followed by two washes in *Post Hybridisation Wash Buffer II* (0.1% *SSPE Buffer*) at 65^{o}C for 30 minutes each (agitating every ∼10 minutes). Slides were then placed in *TNT Buffer* (100mM Tris-HCl pH 7.5, 100mM NaCl, 0.3% Triton-X 100) for at least 10 minutes.

TNB Blocking Buffer (100mM Tris-HCl pH 7.5, 100mM NaCl, 0.36% *Blocking Reagent* (Invitrogen)) was filtered through a 0.22μm syringe filter and 1% skimmed milk added. 150μl was then pipetted on to each slide and parafilm placed on top. The samples were incubated in a humidity chamber at room temperature, for 30 minutes.

Blocking Buffer was then discarded and 150μl *anti-digoxigenin-AP antibody* (Roche) was added (1:500 in fresh *TNB Blocking Buffer*). Samples were incubated with the antibody in a humidity chamber at room temperature, for 2 hours.

Antibody solution was then discarded and slides washed in *TNM* (100mM Tris-HCl pH 9.5, 100mM NaCl, 5mM $MgCl_2$), agitating, for 15 minutes, twice.

NBT/BCIP solution (Roche) was filtered using a 0.22μm syringe filter. The

Table 2.3: Primers sequences used for qPCR. All sequences were designed using the NCBI *Primer Blast* tool. Annealing temperatures are those optimised in Section 2.21

Gene	Primer sequence	Fragment size	Annealing temperature
appb	Forward: TCGTCCCAGATAAGTGCAAG Reverse: ACGGCAACAGCATACCATAA	138 bp	65^{o}C
bace1	Forward: TTACCACCGCTCACTCTCCT Reverse: CAGGGGACTGACACCACATC	114 bp	68^{o}C
gapdh	Forward: TGAGCTCAATGGCAAGCTTACTGGT Reverse: TCAGCTGCAGCCTTGACGACT	135 bp	60^{o}C
pin1	Forward: TATTTGCTTCTCAGGGCGTGT Reverse: GCGTGTGATGTTCTCCTCTCT	184 bp	60^{o}C
rpl13α	Forward: TCTGGAGGACTGTAAGAGGTATGC Reverse: TCAGACGCACAATCTTGAGAGCAG	150 bp	51^{o}C
β-actin2	Forward: GCCCCACCTGAGCGTAAATA Reverse: AGTTTGAGTCGGCGTGAAGT	171 bp	65^{o}C

slides were incubated in filtered *NBT/BCIP* overnight, or until signal became evident.

Slides were then washed in ddH_2O for at least 1 hour, air-dried and cleared in xylene. Coverslips were mounted using non-aqueous mounting media (either Histomount DPX).

2.20 Primer design for qPCR

cDNA sequences were derived from the Ensembl project website (Flicek et al. 2014). All primers (see table 2.3) were designed using the NCBI *Primer Blast* tool (Ye et al. 2012) and purchased from Eurofins Genomics. Where possible, primers were designed to amplify a sequence that includes an intron in genomic DNA.

2.21 qPCR primer optimisation

All quantitative polymerase chain reaction (qPCR) experiments were conducted using *absolute quantification* (standard curve) methodology (e.g. Parker et al. 2016).

In order to generate standard curves, qPCR primers were used to amplify the correct fragments from cDNA (synthesised as per Section 2.4), in a standard PCR reaction:

Reaction mix:

Thermopol Buffer (10x)	2.5μl
dNTPs (10mM)	0.5μl
Forward primer (10μM)	0.5μl
Reverse primer (10μM)	0.5μl
cDNA	2μl
Taq polymerase	0.125μl
ddH_2O	18.375μl
Total	**25μl**

Thermocycles:

Initial denaturation	95^{o}C	30 seconds	
Denature	95^{o}C	30 seconds	x35 cycles
Anneal	(variable)	1 minute	
Extension	68^{o}C	1.5 minutes	
Final extension	68^{o}C	5 minutes	
Hold	4^{o}C	∞	

PCR products were run on 2% agarose gels and bands of the correct size were extracted, as described in Section 2.11.

Following gel extraction, DNA concentration was determined as described in Section 2.3. The total number of fragments was calculated using the Avogadro constant, then diluted to an appropriate concentration (usually 10^{11}). This sample was then serially diluted to produce a standard curve of 10^6, 10^5, 10^4, 10^3, and 10^2 fragments per 2μl.

Initially, standard curves were set up for each gene, in the following manner:

Reaction mix:

*2*x *SYBR Green I Mastermix* (Bio-Rad)	5μl
Forward primer (10μM)	0.22μl
Reverse primer (10μM)	0.22μl
ddH_2O	4.06μl
Total	**9.5μl**

The standard curve samples were loaded, in triplicates, directly into the bottom of a 96-well plate, at 2μl per sample. In the same manner, 2μl of each test sample cDNA (reverse-transcribed from 1μg RNA) was loaded in triplicate, directly into the bottom of the wells. 9μl of the above reaction mix was then loaded (by reverse pipetting) on the side of each well. An adhesive film strip was placed over the top of the plate, and the plate centrifuged at full speed, at 4^{o}C, for 2 minutes.

The plates was run through thermocycles on a Bio-Rad CFX Connect qPCR machine, in the following protocol:

Thermocycles:

Initial denaturation	95^{o}C	5 minutes	
Denature	95^{o}C	10 seconds	x45 cycles
Anneal	(variable)	6 seconds	
Extension	72^{o}C	6 seconds	
		(*plate read*)	
Denature	95^{o}C	30 seconds	
Melt curve	x^oC - 99^{o}C*	30 seconds	
Hold	4^{o}C	∞	

(**temperature increased in 0.5^{o}C increments, from annealing temperature*)

Annealing temperatures were optimised for each primer set. Temperatures that produced a single product size, with *Efficiency* between 90%-110% and R^2 of >0.98 were used for experiments qPCR trials.

2.22 Total protein extraction from zebrafish brain tissue

Zebrafish were sacrificed by decapitation. Full brain tissue was dissected out and immediately stored at -20^{o}C.

RIPA buffer (50mM Tris-HCl pH 7.6, 150mM NaCl, 1% IGEPAL-CA630, 0.5% sodium desoxycholate, 0.1% SDS) containing cOmplete Protease Inhibitor Cocktail (Roche), was added to frozen tissue (100μl/brain) and allowed to thaw on ice. Tissue was homogenised in buffer using a small mechanical homogeniser, then sonicated using a probe sonicator on full power, for 10 strokes.

The samples were centrifuged at 700 xg, at 4^{o}C, for 5 minutes. Supernatants were removed to a clean sample tube, *6x Laemmli Sample Buffer* (recipe for final working concentrations - 62.5mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.01% Bromophenol blue, 1.25% β-mercaptoethanol) added (1:6), and then boiled at 95^{o}C, for 10 minutes.

If not processed immediately, protein extracts were stored at -80^{o}C until use.

2.23 Western blot antibody optimisation

10% Resolving gel:

H_2O	1.9mL
30% acrylamide mix	1.7mL
1.5M Tris-HCl (pH 8.8)	1.3mL
10% SDS	0.05mL
10% ammonium persulfate	0.05mL
TEMED	0.002mL

Stacking gel:

H_2O	1.4mL
30% acrylamide mix	0.33mL
1M Tris-HCl (pH 6.8)	0.25mL
10% SDS	0.02mL
10% ammonium persulfate	0.02mL
TEMED	0.002mL

10% SDS-PAGE gels were cast (see above). Proteins were loaded and run through the gel at 120V for, using running buffer (25mM Tris, 192mM glycine, 10% SDS), for 70 minutes, on ice.

Proteins were then blotted on to pure nitrocellulose membrane (Perkin Elmer) using a Trans-Blot Semi-Dry Transfer Cell (Bio-Rad), at 10V, for 45 minutes.

Blots were blocked with 10% skimmed milk + 5% BSA in PBS-T (PBS + 0.02% tween), at 4^oC, overnight. Membranes were washed with PBS-T for 5 minutes (x5), then probed with either chicken anti-Aβ (Abcam, ab17473) or rabbit anti-APP C-terminal (Millipore, 171610), as specified, in 3% BSA (1:2,000), at room temperature, for 2 hours. The antibody was removed (and stored with sodium azide at 4^oC) and the membranes washed with PBS-T for 5 minutes (x5). Normalisation antibody (mouse anti-acetylated tubulin, Sigma T6793), was added in 3% BSA (1:2,000) and incubated at room temperature, for 10 minutes.

Blots were washed with PBS-T for 5 minutes (x5). Infra-red fluorescent secondary antibody (Abcam, ab175787 and ab175775) were diluted in 3% BSA (1:10,000) and incubated at room temperature for 40 minutes. Membranes were again washed with PBS-T for 5 minutes (x5), rinsed in ddH_2O, and stored in ddH_2O. Blots were then imaged on an *Odyssey CLx* system (LI-COR). Quantification was conducted using *Image Studio Lite* software (LI-COR).

2.24 TUNEL assay

The *TUNEL assay* (Millipore) was performed as per the manufacturer's instructions, except that nickel enhancement was used to increase signal:

Zebrafish were sacrificed by decapitation and whole brain tissue dissected out. Tissue was immediately immersed in *OCT Compound* (Tissue-Tek) and frozen on

dry ice. If not processed immediately, samples were then stored in *OCT Compound* at -80^{o}C.

Tissue was cut on a cryostat at -20^{o}C in 10μm sections and mounted on charged ('*Superfrost*') slides. If not used immediately after sectioning, slides were stored at -20^{o}C.

Sections were fixed in 1% paraformaldehyde (in PBS, pH 7.4) at room temperature for 10 minutes, then washed in PBS for 5 minutes (x2). They were then post-fixed in pre-chilled ethanol: acetic acid (2:1) at -20^{o}C, for 5 minutes. Sections were again washed in PBS for 5 minutes (x2).

Following this, endogenous peroxidases were quenched with 3% hydrogen peroxide (in PBS) at room temperature, for 5 minutes. Sections were again washed in PBS for 5 minutes (x2).

Excess liquid was removed from the slides and *Equilibration Buffer* added for $\sim$3 minutes. Working concentration of TdT enzyme (in *Reaction Buffer* was added to the slides, a plastic coverslip placed over the liquid, and incubated at 37^{o}C, in a humidified chamber, for 1 hour. Coverslips were then removed, excess liquid poured off, and the slides immersed in working strenght *Stop/Wash Buffer* (agitating for 15 seconds, then incubating for 10 minutes, at room temperature).

Slides were washed with PBS for 1 minute (x3), *Anti-Digoxigenin Peroxidase Conjugate* added to each slide and a plastic coverslip placed on top. Slides were then incubated in a humidified chamber for 30 minutes, at room temperature.

Slides were washed with PBS for 2 minutes (x4), then incubated in working-strength peroxidase substrate (0.05% 3,3′-diaminobenzidine, 0.05% nickel ammonium sulfate, 0.015% H_2O_2, in PHB, pH 7.2) at room temperature, for up to 20 minutes.

Slides were washed in dH_2O for 1 minute (x3), followed by a 5 minute incubation in dH_2O. They were then dipped in 100% n-butanol ten times (x2) and incubated in 100% n-butanol for 30 seconds.

Finally, sections were dehydrated in 100% xylene for 2 minutes (x3), before coverslips were mounted using DPX mounting media.

2.25 Fluoro-Jade® B staining

Fluoro-Jade® B was purchased from Millipore. Staining protocol was as per previously reported (see Laflamme et al. 2016):

Zebrafish were sacrificed by decapitation and whole brain tissue dissected out. Tissue was immediately immersed in *OCT Compound* (Tissue-Tek) and frozen on dry ice. If not processed immediately, samples were then stored in *OCT Compound* at -80^{o}C.

Tissue was cut on a cryostat at -20^{o}C in 10μm sections and mounted on charged ('*Superfrost*') slides. If not used immediately after sectioning, slides were stored at -20^{o}C.

Sections were fixed in 4% paraformaldehyde (+50mM NaOH, in PBS) at room temperature for 20 minutes, then washed in KPBS (22mM K_2HPO_4, 3.3mM KH_2PO_4, 138.6mM NaCl) for 2 minutes. They were dehydrated in an ascending ethanol series (50%, 70%, 100%) for 2 minutes each, then rehydration in a descending ethanol series (70%, 50%) for 2 minutes each, and incubated in KPBS for 2 minutes.

Slides were then incubated in 0.06% $KMnO_4$ (in ddH_2O) for 5 minutes at room temperature, and rinsed in ddH_2O for 1 min.

Sections were stained by incubation in 0.0004% Fluoro-Jade® B (+ DAPI, in ddH_2O) for 10 mins, rocking (agitating) at room temperature. They were then rinsed in ddH_2O for 1 minute (x3) and left to dry overnight.

The following day, slides were cleared in xylene for 2 minutes (x2), and coverslips mounted using DPX.

2.26 Cloning into pCS2+

pCS2+ plasmid (Adgene) was transformed into competent cells and Maxiprepped (as described in Sections 2.9 and 2.13). The resultant DNA was digested using EcoRI restriction enzyme (New England Biolabs), in the following reaction (NB:

desired genes - previously cloned into pGEM®-T Easy, as described in Section 2.16 - were digested in a parallel reaction):

Cutsmart®Buffer	5μl
Plasmid DNA	$x\mu$l (1μg)
EcoRI-HF	1μl
ddH_2O	(to 10μl)
Total	**10μl**

Samples were mixed by pipetting and incubated at 37^{o}C for ∼1 hour. The EcoRI restriction enzyme was then heat-inactivated by incubation at 65^{o}C for 20 minutes.

Linearised pCS2+ plasmid and gene sequences were electrophoresed through 1% agarose gels, and extracted as described in Section 2.11. Genes were then inserted into the linear pCS2+ by the following ligation reaction:

Reaction Buffer	2μl
Vector DNA	$x\mu$l (50ng)
Gene insert	$x\mu$l*
ddH_2O	(to 20μl)
T4 DNA Ligase	1μl
Total	**20μl**

*(*3:1 insert:plasmid molecular ratio*)

Samples were mixed by pipetting, then incubated at room temperature for 2 hours. Ligated gene fragments-pCS2+ were transformed into competent cells and Maxiprepped as described in Sections 2.9 and 2.13, respectively.

2.27 Cloning into Tol2 expression vector

Following cloning into pCS2+ (as described in Section 2.26), gene-pCS2+ constructs were sequenced (Eurofins Genomics) to confirm 5′ →3′ orientation. Gene fragments were PCR-amplified off the plasmid, in the following reaction:

Reaction mix:

Thermopol Buffer (10x)	2.5μl
dNTPs (10mM)	0.5μl
SP6 primer (forward) (10μM)	0.5μl
M13 reverse primer (10μM)	0.5μl
cDNA	2μl
Taq polymerase	0.125μl
ddH_2O	18.375μl
Total	**25μl**

Thermocycles:

Initial denaturation	95^{o}C	30 seconds	
Denature	95^{o}C	30 seconds	x35 cycles
Anneal	43^{o}C	1 minute	
Extension	68^{o}C	1.5 minutes	
Final extension	68^{o}C	5 minutes	
Hold	4^{o}C	∞	

The gene sequences was then isolated by electrophoresis through a 1% agarose gel and gel extraction (described in Section 2.11). The isolated genes were digested with NotI (at 3′ of gene) in the following reaction:

Cutsmart®Buffer	5μl
Linear DNA	$x\mu$l (1μg)
NotI-HF	1μl
ddH_2O	(to 10μl
Total	**10μl**

Following restriction digestion, DNA was again isolated by electrophoresis through a 1% agarose gel, before extraction as described in Section 2.11.

The double-UAS Tol2 vector was a kind gift from Masa Tada (UCL). This was transformed into competent cells and Maxiprepped (as described in Sections 2.9 and

2.13). The resultant plasmid was double-digested using EcoRV and NotI restriction enzymes (New England Biolabs), in the following reaction:

Cutsmart®Buffer	5μl
Plasmid DNA	$x\mu$l (1μg)
EcoRV-HF	1μl
NotI-HF	1μl
ddH_2O	(to 10μl)
Total	**10μl**

Samples were mixed by pipetting and incubated at 37^{o}C for 1 hour. The EcoRV and NotI restriction enzymes were then heat-inactivated by incubation at 65^{o}C for 20 minutes. The linearised plasmid was then isolated on a 1% agarose gel and extracted as described in Section 2.11.

The gene sequences (blunt-ended at 5′, NotI sticky-ended at 3′ was ligated into the linearised Tol2 vector (EcoRV blunt-ended at 3′, NotI sticky-ended at at 5′) in the following reaction:

Reaction Buffer	2μl
Vector DNA	$x\mu$l (50ng)
Gene insert	$x\mu$l*
ddH_2O	(to 20μl)
T4 DNA Ligase	1μl
Total	**20μl**

*(*3:1 insert:plasmid molecular ratio*)

Samples were mixed by pipetting, then incubated at room temperature for 2 hours. Ligated gene-Tol2 constructs were transformed into competent cells and Maxiprepped as described in Sections 2.9 and 2.13, respectively. Constructs were then ethanol precipitated (as described in Section 2.15) prior to injection into embryos.

2.28 Generation of transposase mRNA

pCS2-TP was linearised using NotI restriction enzyme (New England Biolabs) in the following reaction:

Cutsmart®Buffer	5μl
Plasmid DNA	3μl (1μg)
ddH_2O	9μl
NotI-HF	1μl
Total	**50μl**

The reaction was mixed by pipetting and incubated at 37^{o}C for 1 hour. The digested plasmid was then PCR-purified, as described in Section 2.14.

Transposase mRNA was generated using a *mMessage mMachine SP6 Transcription Kit* (Ambion), according to the manufacturer's instructions. Linearised pCS2-TP was transcribed from the up-stream SP6 polymerase site, in the following reaction:

NTP/CAP (2x)	10μl
Nuclease-free ddH_2O	2.8μl
Reaction Buffer (10x)	2μl
Linear DNA template	3.2μl
Enzyme Mix	2μl
Total	**20μl**

The reaction was mixed by pipetting, then incubated at 37^{o}C for 2 hours. Following this, RNA was purified via phenol:chloroform extraction (reagents part of the *mMessenge mMachine SP6 Transcription Kit* from Ambion).

115μl nuclease-free ddH_2O and 15μl *Ammonium Acetate Stop Solution* were added to the 20μl reaction, and mixed by pipetting. 150μl phenol:chloroform (1:1) was added to the sample and mixed by pipetting. An additional 300μl chloroform was added and mixed by pipetting. The aqueous phase was removed to a clean sample tube.

RNA was precipitated using 1x volume isopropanol, mixed by pipetting, and incubated at -20^{o}C for 15 minutes. The sample was then centrifuged (full speed on

a benchtop centrifuge) at 4^{o}C for 15 minutes. The supernatant was discarded by decanting and the RNA pellet re-suspended in 40μl ddH_2O.

RNA concentration was determined as described in Section 2.3. RNA was then aliquotted and stored at -80^{o}C (long-term) or -20^{o}C (short-term).

2.29 Injection calculations and methodology

Transgenic zebrafish lines were generated by combined injection of *transgene*-Tol2 constructs and transposase mRNA (both at 20ng/μl) in *Danieau Buffer* (58mM NaCl, 0.7mM KCl, 0.4mM $MgSO_4$, 0.6mM $Ca(NO_3)_2$, 5mM HEPES, pH 7.6) (e.g. Kajita et al. 2010). A line expressing GAL4 in CNS neurons (driven by s1101t) was a kind gift from Dr Tom Hawkins (UCL). The '*pan neuronal*' expression of GAL4 in this line was used to drive expression of the UAS-promoted transgene constructs (generated as described in Section 2.27), as described previously (Arrenberg et al. 2010; Nevin et al. 2010).

A *Picospritzer II* (Parker) was used to control injection volumes, which were calculated with a stage micrometer; a drop of mineral oil ($\sim$5μl) was placed on the scale of a the stage micrometer, and the *Picospritzer* pressure adjusted to produce 0.5nl per injection.

Adult zebrafish were 'paired' the evening before injection (usually 2x males with 1x female), with a barrier separating the male and female fish. The following morning, approximately 30 minutes after the room lights came on, the barriers were lifted and the fish allowed to breed. Fertilised eggs were collected in a sieve (to remove debris), aligned along an histology slide, and injected with 2x *Picospritzer* injections (for a total injection volume of 1nl).

Following injections, embryos were placed in clean aquarium water and incubated at 28^{o}C. At approximately 3-dpf, carriers of the transgene were identified by fluorescent expression, as described in Section 2.30.

2.30 Identification of germ-line transgene carriers

Injected embryos were identified by expression of the mCherry at between 3- and 5-DPF. This demonstrated the insertion of the transgene into the genome. In order to determine whether the transgene was expressed in the germ-line, these were then crossed with the wild-type (s1101t-GAL4); those which produced fluorescent offspring were thereby identified as expressing the transgene. The progeny of these mCherry-expressing crosses were then reared and crossed to establish the line.

2.31 Microscopy

Imaging of Fluoro-Jade® B staining was conducted on a Leica DMRA2 fluorescent microscope, using Velocity® 6.3 software (Perkin Elmer).

Imaging of fluorescent embryos was conducted on a Lieca FluoIII™, using IM50 1.20 software.

Chapter 3

Development of an Aversive Classical Conditioning Assay

3.1 Introduction

In order to facilitate lead identification (focussing on AD-related cognitive impairment), the development of a behavioural paradigm was conducted. Due to the high costs incurred using current rodent models and assay systems, the intention of this development is for utility as a pre-mammalian screen in the pharmaceutical industry. Thus, the behaviour must be robustly observed in zebrafish, and relate to an impairment reported in clinical AD. This assay will then be validated using neurotoxic and genetic models of neurodegeneration (see chapters 4 and 5, respectively).

3.1.1 Neurodegeneration-related cognitive impairments

A range of behavioural impairments has been observed in AD patients. These include cognitive deficiencies in episodic memory, visuospatial processing, choice discrimination, reversal learning, and attention (e.g. Bäckman et al. 2001; Freedman and Oscar-Berman 1989; Hodges and Patterson 1995; Mormino et al. 2009; Nasreddine et al. 2005; Ober et al. 1986; Prvulovic et al. 2002; Sahakian et al. 1988). Whilst these behavioural deficits can themselves be related to specific neuroanatomical structures and pathways, the identification and full characterisation of pathogenic molecular dysregulations requires investigation using genetically-manipulated animal models. Similarly, the subsequent screening of pharmacological interventions requires the implementation of these animal models in robust assays.

The transgenic expression of AD-related genetic mutations in rodents has been shown to result in a range of cognitive deficits (discussed in Chapter 5). However the measurement and assessment of these clinically-relevant behaviours in animal models requires simplification of behavioural tasks to generate robust assays. For example, spatial dysfunction is often assessed clinically by asking patients to draw common objects (i.e. the *clock drawing test*; see Agrell and Dehlin 1998; Brodaty and Moore 1997; Esteban-Santillan et al. 1998; Sunderland et al. 1989). However animal assays typically involve training, and the subsequent location and discrimination of visuospatial stimuli (e.g. Cracchiolo et al. 2007; Huitrón-Reséndiz et al. 2002; Nagahara et al. 2009). Whilst parallels can clearly be drawn between certain aspects of research assays and clinical assessments, the functional differences imply a disparity between the cognitive processes which govern these

behaviours.

Additionally, the behavioural training in animal models of cognitive decline often involves appetitive reward (e.g. Deacon et al. 2008; McDonald et al. 1994; McDonald et al. 1996; Semina et al. 2015; Teather et al. 2002). This may present a problem specific for models of AD developed around *amyloid cascade hypotheses*, due to findings of lower body weight in transgenic mice expressing hAPP (Huitrón-Reséndiz et al. 2002). Thus, the implication of reduced appetite in these models suggests a reduction in incentive to perform in appetitive tasks. Further, findings that Aβ expression alters olfactory circuitry suggests impairment to the sensory detection of food reward (Cao et al. 2012). Thus it is possible that this impairment may overshadow or accentuate cognitive deficits in transgenic amyloid animals, when performing appetitive learning tasks.

In addition to lower body weight, it has been reported that both young and old hAPP-expressing mice have lower core body temperatures (Huitrón-Reséndiz et al. 2002). Interestingly, both body weight and core body temperature have been associated with cholinergic function in the hypothalamus, which additionally receives olfactory neuronal inputs (e.g. Grossman 1962; Henry 2007; Jo et al. 2005; Meister 2007; Myers and Yaksh 1969; Price et al. 1991a; Yamada et al. 2001). Thus it is possible that transgenic hAPP expression causes early hypothalamic dysfunction. Whilst the concept of hypothalamic dysfunction is an interesting area of research in itself, it renders inferences of subtle cognitive dysfunction based on appetitive reward assays difficult to interpret. Thus, simplistic cognitive measures (based on non-appetitive tasks) may provide assays with higher *validity* than their more complex counterparts.

However there are concerns as to the extent to which these measures actually reflect cognitive function, with deficits not consistently reported between different research groups (Karl et al. 2012). The superior performance of rats over mice in water mazes has been attributed to their swimming ability (Lipp and Wolfer 1998). Thus, conclusions drawn from these studies must take into regard the possibility that experimental intervention may affect motor function and swim ability.

It must be noted that various non-cognitive abilities have been found to be impaired in animal models of AD, including locomotion (see Harkany et al. 1998; Pugh et al. 2007; Walker et al. 2011; Zheng et al. 1995), somatosensory ability (*adhesive removal / sticky-tape* assay; see Sughrue et al. 2006; Xing et al. 2014; Zhang et al. 2011), and exploration (*open field* assays; see Deacon et al. 2008;

Harkany et al. 1998; Pugh et al. 2007; Semina et al. 2015). The lack of cognition involved in these tasks reduces the time required for analysis, as no training / conditioning is required. However, in the absence of a specific cognitive impairment, it is difficult to associate these measures with a specific neurological condition. For example, locomotor activity is affected in a rodent model of schizophrenia, and has commonly been used as a measure of anxiety (Dawson et al. 1995; Hikida et al. 2007). Adhesive removal assays have demonstrated somatosensory impairment in rodent models of PD and cerebral ischaemia (Goldberg et al. 2003; Komotar et al. 2007). Open field assays have also been used as a measure of anxiety (Crawley 1985; Prut and Belzung 2003). In this regard, the non-cognitive behavioural paradigms described above lack phenotypic specificity to AD-related neurodegenerative pathologies.

Thus, the development of an efficacious drug-screening assay for neurodegeneration requires assessment of a simplistic cognitive behaviour which is conserved in zebrafish; the behaviour must be governed by well-defined structures reported to be dysfunctional in neurodegenerative diseases, and require little conditioning to achieve robust empirical measurement. In this regard, cued fear conditioning appears to present the optimal candidate.

3.1.2 Neuroanatomy of classical fear conditioning

Cued fear conditioning can be divided into two sub-categories - “delay” and “trace”. Both of these involve the pairing of a conditioned stimulus (CS) with an unconditioned stimulus (US); however *delayed fear conditioning* involves the co-termination of the CS and US, whilst *trace fear conditioning* involves a “trace” period between the presentation of the two sitmuli - following CS but before US (see Figure 3.1) (Curzon et al. 2009). Importantly, the insertion of a *trace* interval results in the recruitment of additional neuroanatomical structures (see Figure 3.2).

fMRI studies have demonstrated the involvement of the amygdala in both *delayed* and *trace* fear conditioning, in both rodents and humans (Büchel et al. 1999; Cheng et al. 2003; Kochli et al. 2015). Further, research has implicated the lateral (lA) and basolateral (blA) nuclei of the amygdala in fear conditioning, with the medial (mA) nucleus playing a role in unconditioned fear responses (LeDoux et al. 1990; Li et al. 2004; Quirk et al. 1995; Walker et al. 2005; see Fendt and Fanselow 1999).

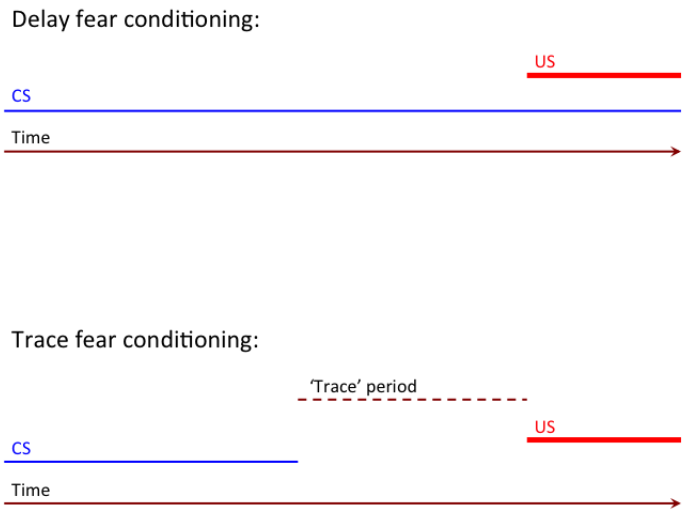

Figure 3.1: Delay vs Trace fear conditioning. During *delay conditioning*, the US presented at the end of CS presentation, with both stimuli terminating together. During *trace conditioning*, there is a 'trace' period between CS and US presentation, during which no stimulus is presented

Additionally, the amygdala has been implicated in extinction of fear conditioning (Knight et al. 2004).

However, whilst the amygdala is involved in both variations of cued fear conditioning, hippocampal activity has been reported only in *trace fear conditioning* (as well as non-cued *contextual fear conditioning*), in both experimental animals and humans (Büchel et al. 1999; Clark and Squire 1998; Marschner et al. 2008; Phillips and LeDoux 1992; Weiss et al. 1999).

Whilst the involvement of the hippocampus initially appears to be characteristic of a distinct mode of fear conditioning, evidence suggests it is actually the result of an increase in complexity of the conditioning (learning) task. By increasing the complexity of *delay conditioning*, animal brains become reliant on the hippocampal involvement (Beylin et al. 2001; Ivkovich and Stanton 2001). This demonstrates the requirement for greater neuronal processing (and thus neuroanatomical involvement) with increased complexity of cued conditioning.

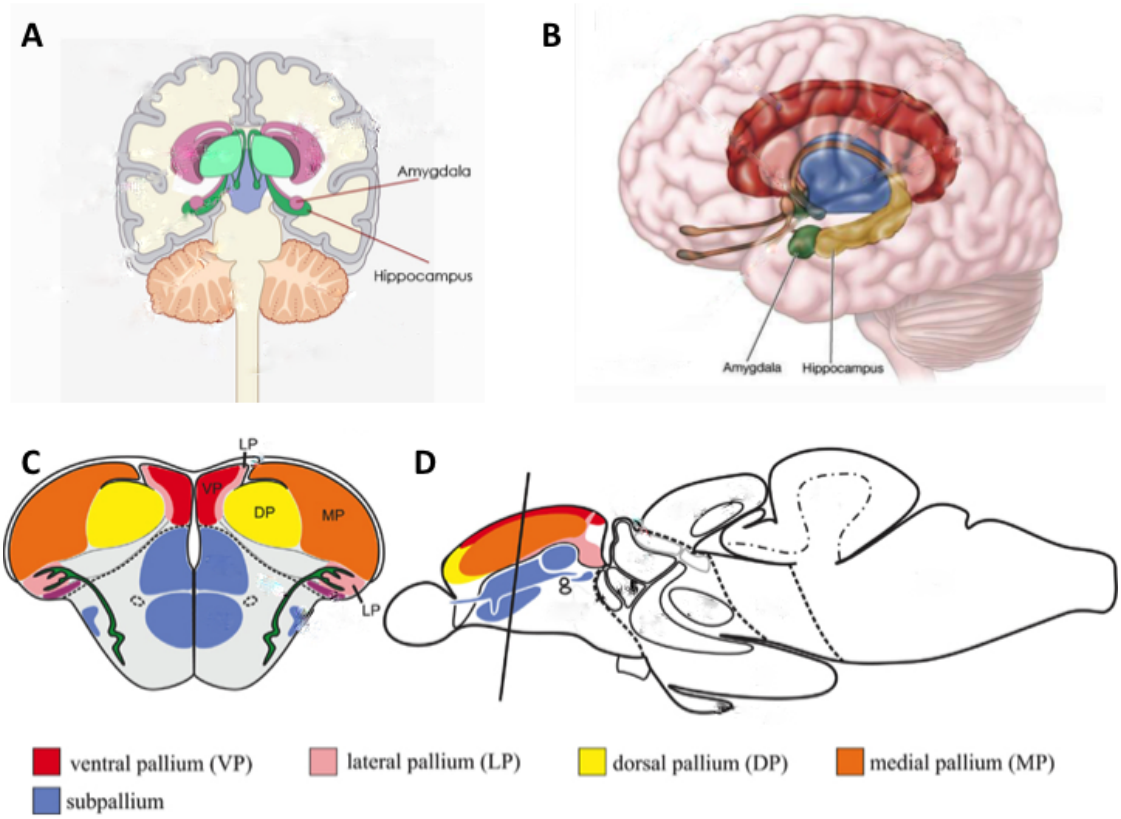

Figure 3.2: Conservation of limbic system structures associated with classical fear conditioning, in the human and zebrafish brain. Functions of the amygdala and hippocampus in of the human brain (coronal and sagittal sections; A & B, respectively) have been reported to be performed by the MP (/Dm) and LP (/Dl) (respectively) of the zebrafish brain (coronal and sagittal sections; C & D, respectively). (Images adapted from Buzzle.com® 2016; Mueller 2012; StudyBlue 2016)

3.1.3 Zebrafish neuroanatomy

Zebrafish do not possess the aforementioned neuroanatomical structures that govern fear conditioning in mammals (i.e. amygdalae and hippocampi). However, as with other non-mammalian organisms, various pallial regions in the ray-finned fishes (which include zebrafish and goldfish) have been associated with mammalian cortical and subcortical structures. Of relevance to fear conditioning, the medial (mP; termed Dm by Rupp *et al.*) and lateral (lP; termed Dl by Rupp *et al.*) pallial divisions (see Figure 3.2) have been structurally and topographically parallelled with the mammalian amygdalae and hippocampi, respectively (Braford Jr 1995; Ganz et al. 2014; Mueller et al. 2011; Northcutt 2006; Rupp et al. 1996).

These neuronal structures have also been functionally associated with their mammalian counterparts. Lesions speicific to the mP have been demonstrated to impair avoidance learning (Portavella et al. 2004b). This behaviour is associated with the mammalian amygdala (as elaborated on, above). Lesions specific to the lP have been demonstrated to impair spatial learning (Portavella and Vargas 2005; Vargas et al. 2006). This function has been associated with the hippocampus in mammalian and bird brains, and the pallial function is conserved in reptiles and fish (Morris et al. 1982; O'Keefe and Dostrovsky 1971; Olton et al. 1978a; Olton et al. 1978b; Rodrıguez et al. 2002; see Burgess et al. 2002).

Interestingly, this pattern of impairment in pre-conditioning mP and lP lesions (above) is mirrored when lesions are induced post-conditioning. Portavella and colleagues reported that - in goldfish - whilst mP lesions (induced following conditioning) affected the retention of both *delay* and *trace* fear conditioning, lP lesions only affected retention of *trace* conditioning (Portavella et al. 2004a). Thus, damage to these structures appears to affect retention (or recollection) of a conditioned fear response.

To parallel this impairment of retention in fish with mammalian findings, in a *delay fear conditioning* experiment in rodents, it was found that the frontal cortex (FC) is involved in early consolidation of CS retention, whilst the parietal cortex (PaC) is involved in both early and delayed consolidation (or possibly recollection) (Sacchetti et al. 2003). Thus, it is possible that the teleost mP fulfills some of the functions performed by the mammalian FC and PaC.

3.1.4 Classical fear conditioning in relation to clinical and experimental neurodegeneration

Evidence of impaired fear conditioning has been reported in several forms of neurodegeneration. Here, it is necessary to review this literature, as an indication of the potential research implications of an automated fear conditioning assay (when employed with zebrafish models of neurodegeneration).

Aluminium neurotoxicity

The subject of Al toxicity in the CNS has been explored in relation to AD neurodegeneration (see Bondy 2014; Bondy 2016; Doll 1993; Lidsky 2014). Whilst the possible role of Al as a causative factor in AD is still contentious, it is well accepted that Al does indeed induce neurotoxicity (e.g. Bondy 2014; Kumar and Gill 2014).

Al treatment has previously been demonstrated to inhibit *contextual fear conditioning* in rodents (Rizwan et al. 2016). However, as yet, there have been no studies assessing this phenomenon in zebrafish. Thus, investigating the effect of Al toxicity on fear conditioning (undertaken in Chapter 4) would indicate the efficacy of zebrafish as a replacement (or pre-assessment) for rodent models.

Of neuroanatomical relevance, Al has been demonstrated to accumulate in the amygdala, as well as various structures with efferent projections to the amygdala, including the entorhinal cortex, substantia nigra, and hippocampus (Walton 2009). Therefore, investigating the effects of Al toxicity on zebrafish fear conditioning would indicate the conservation of neurotoxic mechanisms specific to these structures. Additionally, this would provide a method of validating the assay (developed in the current chapter) as a screen for neurodegeneration-related impairment.

Alzheimer's-like diseases

Impaired fear conditioning has been reported clinically in AD patients using both *delay* and *trace* conditioning paradigms (e.g. Hamann et al. 2002; Hoefer et al. 2008; Woodruff-Pak and Papka 1996). Similarly, transgenic mice expressing mutated

hAPP show impaired fear conditioning, which is somewhat attenuated by treatment with a γ-secretase inhibitor (Barnes and Good 2005; Comery et al. 2005; Corcoran et al. 2002).

Interestingly, Woodruff-Pak and Papka reported that *delay fear conditioning* is in fact a more sensitive test in AD patients than *trace fear conditioning* (Woodruff-Pak and Papka 1996). Thus the development of this type of assay may prove more efficacious in relation to dementias of the Alzheimer's type. Further, due to the lack of hippocampal involvement in *delay fear conditioning*, it would provide a 'cleaner' assessment of amygdala (mP) functionality.

AD patients have been reported to present high levels of amygdala atrophy (Heun et al. 1997; Lehericy et al. 1994; Poulin et al. 2011). This has also been reported in AD patients with high CSF levels of Aβ_{42}, and in patients expressing the late-onset AD-related ApoE ϵ4 allele (Lehtovirta et al. 1995; Mattsson et al. 2014).

3.1.5 Aims

Classical aversion has previously been demonstrated in the zebrafish (Valente et al. 2012). Thus, the aim of this study is to replicate this finding, producing a scalable optimised assay, suitable for use in screening (i.e. genetic and pharmacological). This will be achieved by establishing the parameters which induce reproducible aversive learning, whilst assessing the ability of zebrafish to retain a conditioned response.

3.2 Methods

3.2.1 Equipment design and set-up

All specialist equipment was designed and constructed in collaboration with Zantiks Ltd. (Cambridge, UK), and set up as shown in Figure 3.3.

Individual assay tanks measured 11cm x 20cm x 10cm (D x L x H). Electrodes (stainless steel plates) were placed at adjacent ends of the tanks, to pass electric current along the 20cm lengths of the tanks. The conductance of the aquarium

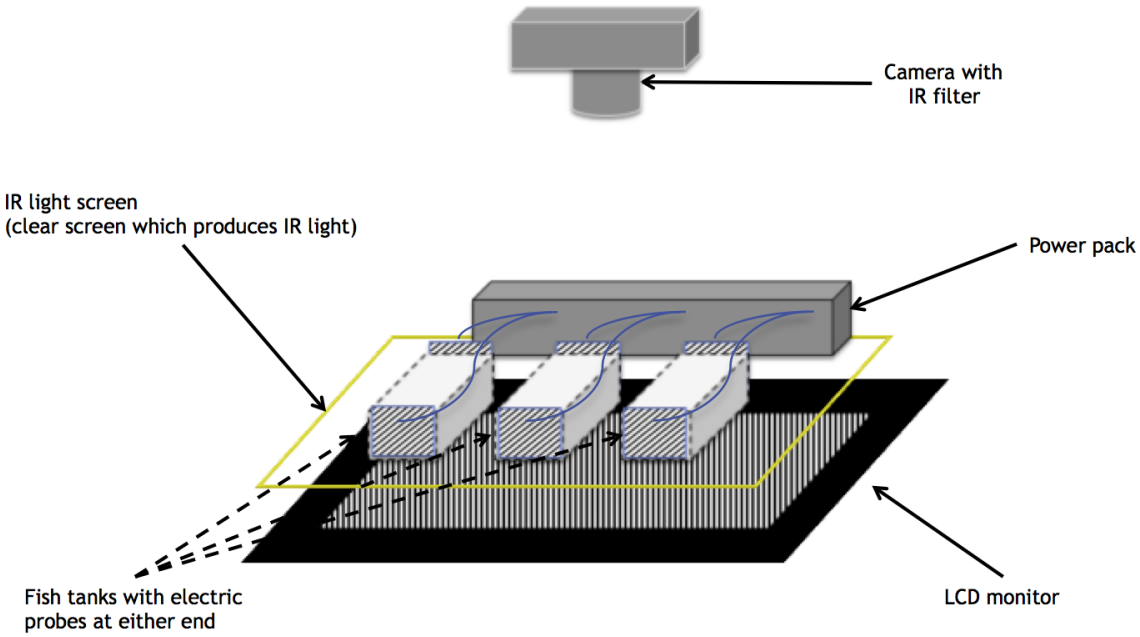

Figure 3.3: Aversion assay setup. A LCD monitor was placed flat (face-up) on a bench, with a transparent, infrared-emitting glass plate (Zantiks Ltd.) laid on top. Multiple tanks were then placed on top of the plate, with stainless steel probes at each end, connected to a bench-top power pack. An ethernet camera was hung above the equipment, with an infrared filter covering the lens.

water used in the assay was measured and adjusted to 0.4mS/cm. The use of an infrared-emitting transparent screen between the LCD monitor and tanks (in combination with the infrared filter over the camera lens) allowed the subjects to be detected and tracked over changing stimuli (presented on the LCD screen).

Stimuli used - presented in Figure 3.4 - were based on that adopted by Valente and colleagues (Valente et al. 2012). Stimuli were presented through the bottoms of the assay tanks. Both stimuli were presented during *baseline* and *probe* trials - one stimulus to each half of the tanks (Figure 3.4 A&B). The whole of the tanks were exposed to the CS and non-conditioned stimulus (non-CS) during conditioning (Figure 3.4 C&D).

Behaviour was monitored using EthoVision XT 9 software (Noldus), and extrapolated as *time spent in area* (for both CS and non-CS presenting areas) and *distance travelled* in 30-second time bins.

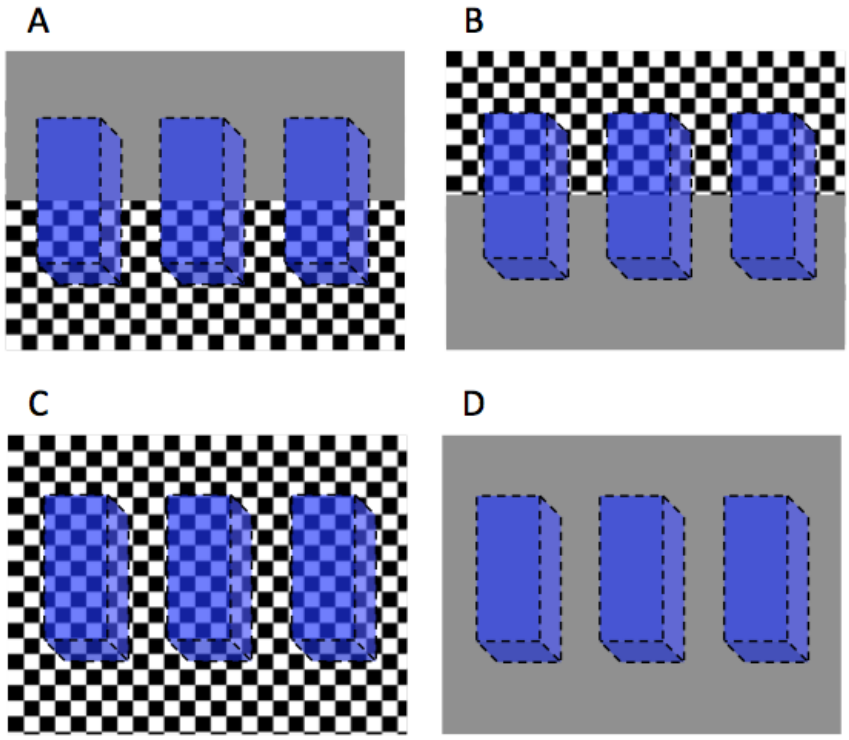

Figure 3.4: Stimuli used for aversive classical conditioning. During baseline and probe trials, both stimuli were presented - one to each side of the tanks (counterbalanced; A & B). Preferences were recorded using EthoVision XT software. During conditioning, CS and non-CS were presented to the whole of the tanks (C & D).

3.2.2 Assay design

The assay (including stimuli used) was designed based on a protocol developed by Valente and colleagues (Valente et al. 2012). This involved four stages, conducted in sequence, without any pause between them:

1. Habituation (30 minutes): Stimuli were presented via the LCD monitor, using a Microsoft PowerPoint slideshow. Both stimuli were presented at the same time - one to each half of the tank - by 'splitting' the slides. The side to which each stimulus was presented was alternated every 5 minutes, allowing subjects to habituate to the changing stimuli beneath the tanks. This presentation of both stimuli is in-keeping with habituation protocols adopted in human *fear conditioning* experiments (e.g. Hamann et al. 2002; Hoefer et al. 2008).

2. Baseline (30 minutes): The *baseline* trial was identical to the *habituation* trial, except that the time spent in each area (i.e. swimming above each stimulus) was recorded.

3. Conditioning (1.5 minutes): *Conditioning* involved the presentation of the CS for 1.5 seconds, at the end of which the US (a 9V DC electric shock lasting 80ms, unless stated otherwise) was delivered, the end of which coincided with the termination of CS presentation. The non-CS was then presented (immediately afterwards) for 8.5 seconds. This was repeated 9 times.

4. Probe (2 / 5 minutes): The *probe* trial involved both stimuli being presented with a 'split' slide, as in *habituation* and *baseline* trials. In the *voltage range-finding* and *voltage titration* experiments, the probe lasted 5 minutes, with stimuli presentation switching after the first 2 minutes (see Figures 3.5 and 3.6, below). Following the assessment of probe length (see Figure 3.7), a 2-minute probe (in which the stimuli did not switch sides) was utilised.

3.2.3 Subjects

All animals used in this study were wild-type (Tubingen) adult zebrafish, between three and six months post fertilisation. All animals were reared and housed as described in Section 2.1.

3.2.4 Data analysis

Data for *time spent in area* were then converted into CS-preference values (time spent in CS-presenting area / total time subject was tracked), for each time bin, using Micosoft® Excel® for Mac 2011.

Preference scores were then analysed using SPSS® Version 21 for Mac (IBM®). Data were fitted to a *linear mixed effects model*, using *preference for CS-presenting area* as the dependent variable, with *trial* (baseline or probe) as a fixed factor, *distance travelled* as covariate, and *subject ID number* as a random effect. The *residuals* of this model were plotted (SPSS *Legacy Boxplot*), and outliers removed at 1.5x interquartile range (IQR). The *linear mixed effects* model was then run again, without outlying data.

All post-hoc analyses were conducted using two-tailed t-tests, with Bonferroni correction applied where necessary.

3.3 Assay development results

3.3.1 Voltage titration

In order to establish an US that delivered significant aversion, the voltage of electric shock was titrated. Initially, a broad range-finding experiment was conducted to find a 'working range' (see Figure 3.5). This indicated that 7V and 9V (DC) present useful US in this assay.

Following the 'range-finding' experiment, the assay was repeated using the range that appeared to produce significant CS aversion (7V, 9V and 12V). The results of this *titration* experiment confirmed that 7V and 9V produced significant aversion of the CS in the *probe* trial (see Figure 3.6A).. The *effect sizes* (Cohen's *d*) and number of subjects required for power were estimated using G*Power software. These indicate that 9V produces the largest effect, with fewest subjects required for power (Figure 3.6 B & C, respectively).

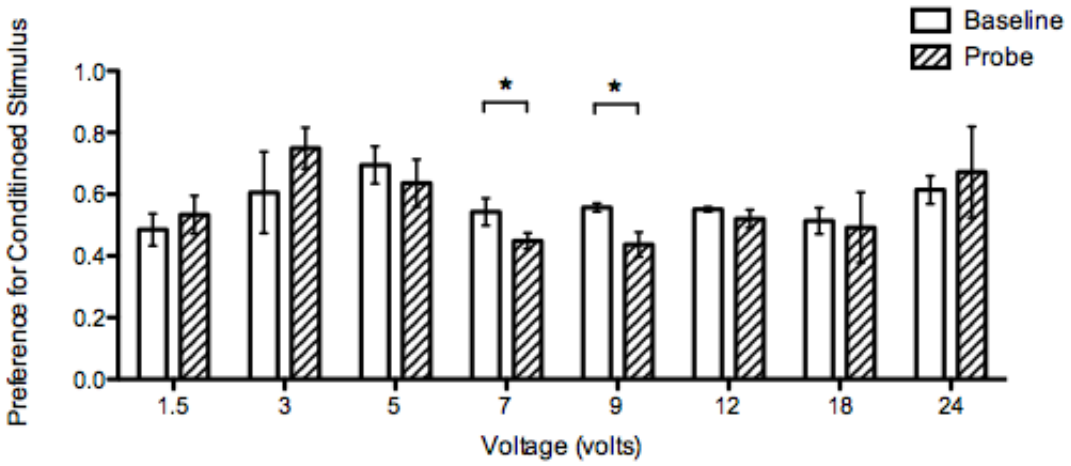

Figure 3.5: Voltage range-finding. In order to determine a useful voltage, an initial *voltage range-finding* experiment was conducted. This indicates that around 7V and 9V produced significant CS aversion (n=24). *, $p<0.05$; by two-tailed t-test. Data plotted as mean ± SEM.

3.3.2 Probe length / extinction

Whilst the optimal voltage to use in the US had been determined (9V), the small change in preference (approximately 0.5 in *baseline* trials vs approximately 0.4 in *probe* trials) may be problematic in sample groups with higher variance in basal preferences. Thus, the length of the *probe* trial was investigated to assess whether higher aversion for the CS was observed during the initial 2 minutes (i.e. before the stimuli presentation was switched). The results demonstrate that during the first two minutes of the *probe trial*, significantly greater aversion of the CS was observed than during the total 5-minute duration (see Figure 3.7). This effect was not related to any change in *distance travelled*, demonstrating that the fish travelled the same distances in each probe trial.

Subsequently, preference for the CS in the full (5-minute) probe was assessed against time (see Figure 3.8 A). This initially suggested that CS aversion extinguished over the course of the probe trial. However a significant difference was found between *Probe_1* (first 2 minutes) and *Probe_2* (last 3 minutes) (Figure 3.8 B). Subsequent assessment of extinction over time during *Probe_1* alone found no significant effect (Figure 3.8 C).

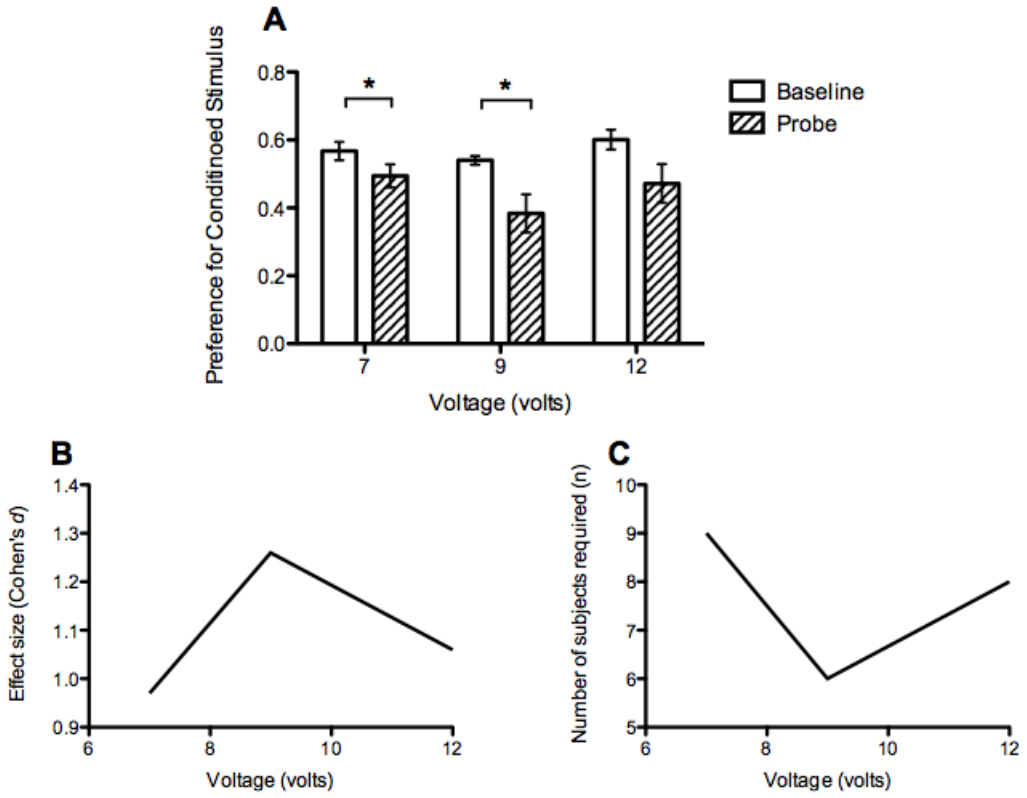

Figure 3.6: Voltage titration. Following the initial *range-finding* assessment, a *voltage titration* experiment was conducted. This confirmed that 7V and 9V produced significant US, as determined by CS aversion in the probe trial (A). Estimates of *effect sizes* and *n required for power* indicate that 9V produces the largest effect, with fewest subject required (B & C) (n=18). *, $p<0.05$; by two-tailed t-test. Data plotted as mean ± SEM.

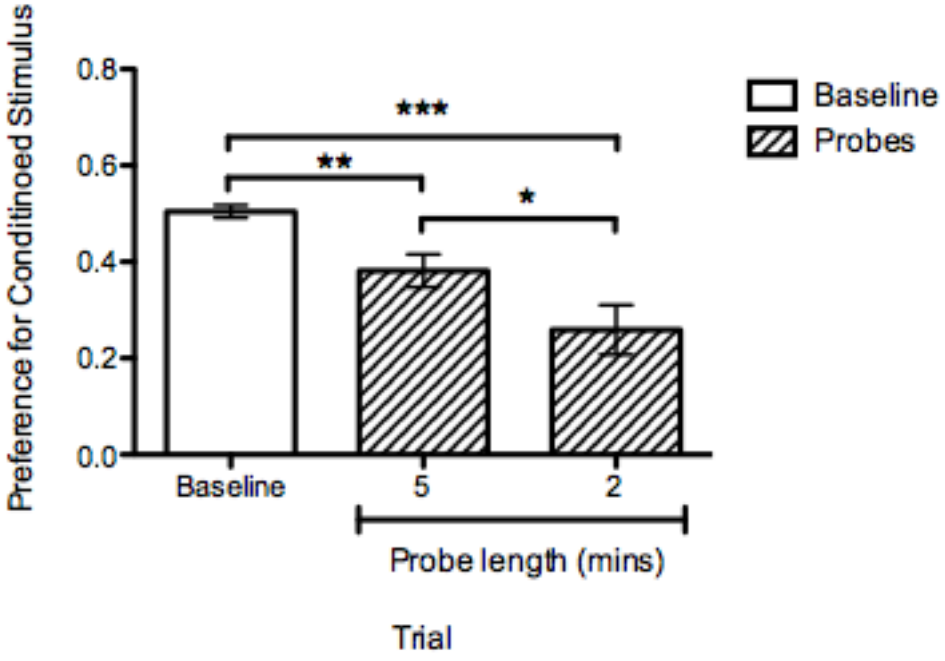

Figure 3.7: Probe length. During the first two minutes of the probe - prior to the 'switching' of the 'split' stimuli - greater CS aversion was observed than in the total (5-minute) probe (n=27). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; by two-tailed t-test. Data plotted as mean ± SEM.

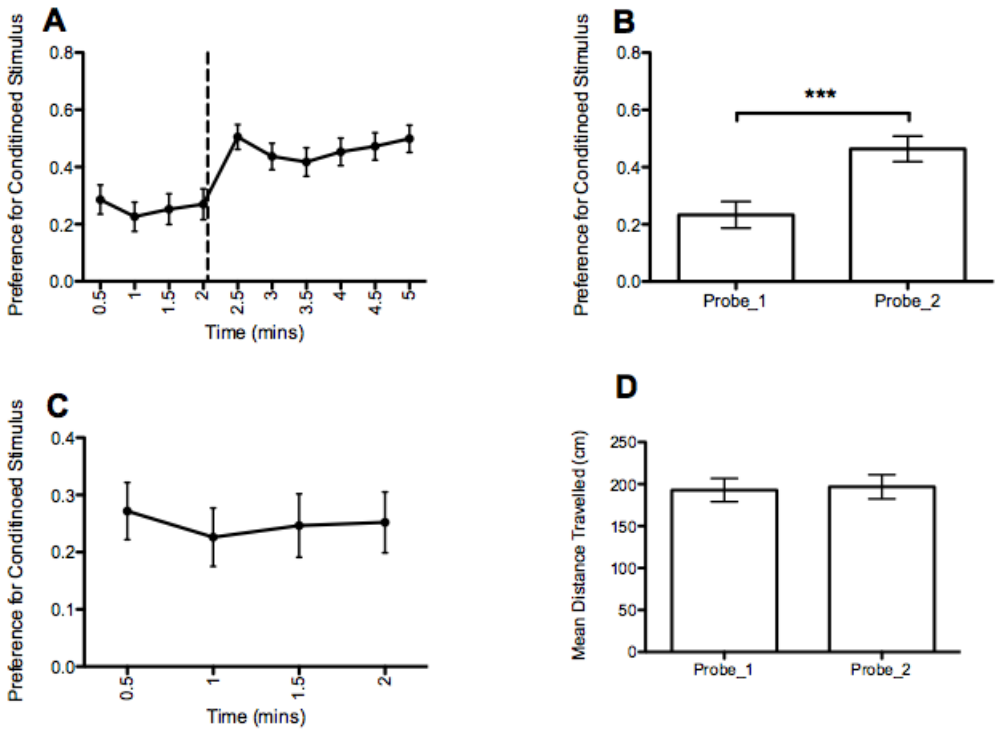

Figure 3.8: Extinction of conditioning. (A) Preference for the CS during the probe trial was plotted against time, with the point at which stimuli presentation was alternated indicated by the dashed vertical line. There was a significant effect for time - $F_{9,225.804}$=5.029, p<0.0005 - indicating that the conditioning was extinguishing during the probe. (B) A comparison between *Probe_1* (0-2 mins; prior to alternation of stimuli presentation) and *Probe_2* (2-5 mins; following alternation of stimuli presentation) demonstrated a significant difference between these two trials - $F_{1,30.587}$=8.338, p=0.007. (C) Analysis of *Probe_1* individually found no effect for time - $F_{3,70.239}$=0.327, p=0.806; (n=27). (D) No effect was found for distance between the two probes - $F_{1,32.07}$=0.11, p=0.742. Linear mixed effects model; ***, p<0.001; by post-hoc two-tailed t-test. Data plotted as mean ± SEM.

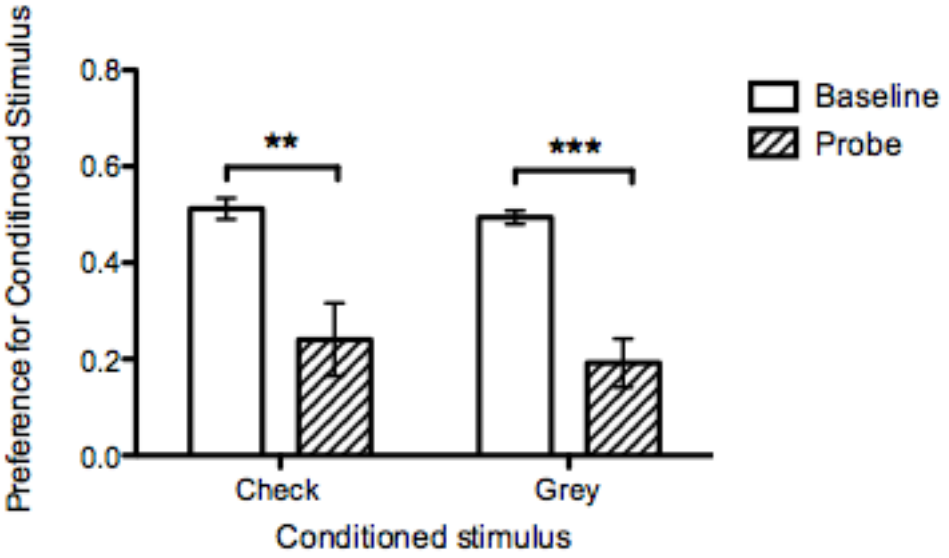

Figure 3.9: Stimuli comparison. In order to confirm that neither of the stimuli produced stronger aversive pairing, a comparison between the levels of aversion was assessed. No significant difference was found between the two CS - $F_{1,47}$=2.07, p=0.157; (n=27). *, p<0.05; **, p<0.01; ***, p<0.001; by two-tailed t-test. Data plotted as mean ± SEM.

3.3.3 Stimuli

Finally, in order to confirm that the CS used in the assay were unbiased, the level of aversion produced by pairing each stimulus was compared (see Figure 3.9). No significant difference was found between those subjects paired to the *check* stimulus and *grey* stimulus.

3.4 Discussion

The aim of this chapter was to develop a reproducible, scalable *fear conditioning* paradigm. This aim was achieved, with optimal aversion for the CS observed in the first two minutes following 9V US, with no stimulus bias. The results presented here demonstrate that zebrafish can be classically conditioned to avoid a CS in a reliable manner. This is in support of previous findings (i.e. Valente et al. 2012).

The assay developed here was a simple *delay fear conditioning* paradigm. This was chosen over a *trace* paradigm as it has been reported to be more specific to

amygdala functioning (see Beylin et al. 2001; Büchel et al. 1999; Clark and Squire 1998; Ivkovich and Stanton 2001; Marschner et al. 2008; Phillips and LeDoux 1992; Weiss et al. 1999). In light of findings that *delay* paradigms are more sensitive measure of AD cognitive decline than *trace* paradigms, implementing this assay using models of AD-related neurodegeneration appear the obvious choice for future validation of this assay (Woodruff-Pak and Papka 1996).

Initial voltage *range-finding* and *titration* experiments demonstrated that a 9-volt US (passed between electrodes along 20cm aquarium water; conductance=0.4mS/cm) produces the greatest aversion of CS in probe trials. This is in-keeping with the US used previously by Valente and colleagues (Valente et al. 2012).

Assessment of the length of probe found that greater CS-aversion was observed during the first 2-minutes of the probe, prior to stimuli switching sides. Initially, significant extinction was found over the duration of the total probe time. However ad-hoc analysis of the two separate fractions of the trial - *Probe_1* (0 - 2 mins); and *Probe_2* (2 - 5 minutes), with simuli switching between these two fractions - found a significant difference between these two probe trials. As this effect was not related to *distance* (with subjects moving approximately 200cm per 2-minute time bin in each fraction of the probe), it is evident that this extinction was not an artefact of 'freezing' with CS presentation. Thus, the alternation of stimuli appears to have induced extinction, with none occurring prior to this.

This suggests the possibility of retention over a longer period of time. From a purely academic point of view, this would be an interesting follow-up study; to assess the length of retention in comparison to other species. However as the purpose of this assay was for use in pharmaceutical screening, the robust, time-efficient nature of the two-minute probe trial (*Probe_1*) was deemed sufficient and no further development was undertaken at this point. (However further investigation was undertaken in the development of a *drug discrimination* assay; see Chapter 7).

No difference was found between subjects conditioned to either CS, demonstrating the unbiased nature of conditioning to these stimuli. In this regard, it should be mentioned that the two patterned stimuli were preferred to coloured stimuli as zebrafish have been shown to bias certain colours (Avdesh et al. 2010). Whilst it is possible to use unbiased colours, the possiblity of individual differences in colour preference could increase variability of baseline recordings. Thus, the patterned stimuli used here were based on those used previously (Valente et al.

2012).

With regard to data analysis, it was noticed that some zebrafish displayed freezing/darting behaviour following conditioning. This was observed to be irrespective of independent variable control (as reported previously; see Parker et al. 2012b). It is likely that this effect is due to variations in stress responding, following administration of the US. However, due to the stressful nature of the conditioning paradigm, these behaviours may be expected. In this regard, the exclusion of fish which display these erratic patterns of locomotion is likely to exclude true data points. However, this behaviour must be addressed in the statistical model to account for variance caused by this stress-related behaviour on conditioned learning. Thus, *Distance (cm)* was factored in to the *mixed effects model* as a covariate.

With practical relevance to translational applications, the development of this time-efficient, robust screening assay would facilitate disease research into disorders which affect the neuroanatomical structure(s) that govern this behaviour (i.e. the amygdala).

Due to the robust findings of impaired amygdala function and *delay fear conditioning* in AD patients and models, the current assay is likely to be useful in assessing genetic and toxicological factors in AD (and subsequently as a pharmaceutical screen).

In summary, the aim of developing a robust, automated assay of simple cognitive function has been achieved. However it is now necessary to validate the assay by utilising well-established models of neurodegeneration-related cognitive decline (/amygdala dysfunction).

Chapter 4

Aluminium Neurotoxicity

4.1 Introduction

Whilst the development of a robust aversive classical conditioning assay has been achieved (see Chapter 3), its utility as a screening tool for neurodegeneration-associated cognitive decline requires further validation. In order to achieve this, the consequences of pre-treatment with the neurotoxin Al was investigated. In this regard, assessing molecular dysregulations will indicate mechanisms of Al neurotoxicity, with a focus on genes related to AD. Additionally, this will provide an indication of the utility of zebrafish in these two areas of research (i.e. Al neurotoxicity and AD). Subsequently, behavioural assessment using the *classical fear conditioning* paradigm will indicate the cognitive consequences of Al toxicity, whilst additionally providing a validation of the assay in neurodegeneration-related behavioural research.

Al is an extremely abundant element in Earth's environment, and the second most abundant metal found in urban soils (Alekseenko and Alekseenko 2014). The World Health Organisation reported that human exposure from dietary consumption is between 2.5-13mg per day (Habs et al. 1997). Al is included in antiperspirants (amongst other cosmetic products), through which it has been associated with breast cancer (Darbre 2003; Darbre 2005). Additionally, it is a common ingredient in antacids, with GI absorbance increased by acidic dietary components (i.e. orange juice, citric acid) (Fairweather-Tait et al. 1994; Weberg and Berstad 1986).

4.1.1 Aluminium neurotoxicity

Al is well established as a neurotoxin which enters the CNS, exerting a multifaceted degenerative action on neurons:

Approximately 90% of Al in human uremic serum (pH 7.4) binds to *transferrin*, which is the only serum protein found to bind the metal (Cabezuelo et al. 1997). Transferrin's ability to transport molecules (including drug compounds) across the BBB suggests that it actively facilitates the otherwise passive diffusion of Al into the brain (Edwardson et al. 1992; Pardridge et al. 1991; Roskams and Connor 1990; Ulbrich et al. 2009). After crossing the BBB, Al accumulates in various neuroanatomical structures (many associated with AD; i.e. hippocampus, entorhinal cortex, temporal cortex), including the amygdala (which is central to fear conditioning, as discussed in Section 3.1.2; see Walton 2009; Ward et al. 2001).

Rodent studies have shown that, within minutes of (intravenous) Al exposure, the *brain extracellular fluid* : *blood* ratio reaches 0.2, which appears to be maintained thereafter (see Yokel 2002). The ionic species of Al in solution varies, depending pH, with high levels of $Al(OH)_3$ at pH 7 (Achak et al. 2008). $Al(OH)_3$ has been shown to be neurotoxic in rodents, and its inclusion in vaccines has been associated with increased neurological disorders (Petrik et al. 2007).

The mechanisms by which Al induces neuronal death have received notable attention. In contemplating this wealth of research, there appears to be two main cellular mechanisms of neurodegenerative signalling - mitochondrial dysfunction and ER stress (e.g. Savory et al. 2003; see Figure 4.1). However there still remains an ambiguity surrounding the relationships between these mechanisms.

Al has been found to interfere with the functioning of Na^+/Ca^{2+} exchangers, resulting in an increase in cytoplasmic Ca^{2+}, which subsequently accumulates in mitochondria (Szutowicz et al. 1998). Elevations in cellular Ca^{2+} have been demonstrated to result in increased generation of ROS by activation of the respiratory chain (Castilho et al. 1995; Hansson et al. 2008; see Starkov et al. 2004). This, in turn, leads to the secretion of cytochrome *c* from mitochondria, which forms a complex with apoptotic protease activating factor (APAF)-1 (in the cytoplasm) and subsequently triggers apoptosis via a caspase signalling cascade (Ghribi et al. 2001b; Savory et al. 2003).

Additionally, Al has been demonstrated to induce ER stress (e.g. Rizvi et al. 2014). Whilst the precise mechanism by which Al induces this is undetermined, it has been reported that Al causes downregulation of GRP78 (Rodella et al. 2008). Under 'normal' conditions, GRP78 is bound to the three unfolded protein response (UPR) proteins (PERK, IRE1, ATF6), inhibiting their action; when unfolded proteins build up inside the ER, GRP78 dissociates from the UPR proteins and binds these unfolded polypeptides (see Basha et al. 2012; Szegezdi et al. 2003). Thus, under-expression of GRP78 suggests a dysregulated increase in UPR signalling. In addition to the UPR proteins, GRP78 binds to caspase-7 and caspase-12, inhibiting their (apoptotic) consequenses (Momoi 2004; Reddy et al. 2003). Thus the down-regulation of GRP78 suggests an increase in the activities of these caspases. Aluminium has also been shown to increase expression of caspase-12, triggering an increased mode of apoptosis by its subsequent activation of caspase-9 (Morishima et al. 2002; Rizvi et al. 2014).

Al exposure has been shown to result in the translocation of both NF-κB and

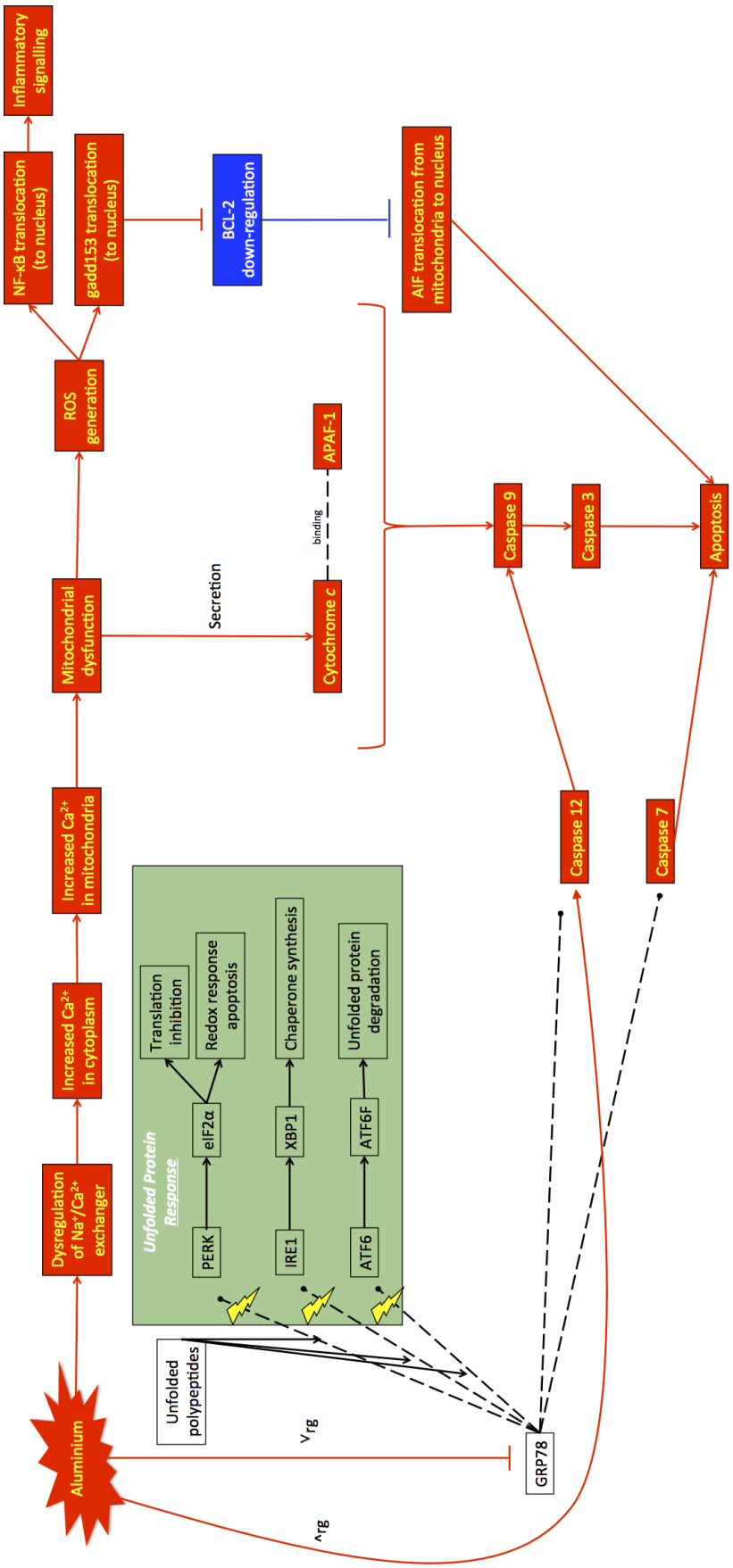

Figure 4.1: Aluminium exposure induces mitochondrial dysfunction and ER stress. A simplistic pathway of Al-induced cellular dysfunction in relation to molecular dysregulation. Al disrupts Na^{+}/Ca^{2+} exchanger functioning, resulting in an increase in cytoplasmic, and subsequently mitochondrial Ca^{2+}. This leads to the secretion of cytochrome *c*, which binds APAF-1 to initiate apoptotic caspase signalling. Additionally, the mitochondrial dysfunction leads to increased generation of reactive oxygen species (ROS), which causes the translocation of NF-κB (resulting in inflammation) and GADD153 (which inhibits the anti-apoptotic action of BCL-2). Al also down-regulates GRP78 (resulting in disregulated UPR signalling and increased activity of caspase 7 and caspase 9) and increases apoptotic signalling via upregulation of caspase 12. (Yellow text on red background, neurodegenerative effect; white text on blue background, neuroprotective effect; $\hat{}rg$, up-regulation; $\check{}rg$, down-regulation; yellow 'lightning symbol', dissociation of bound proteins.)

GADD153 transcription factors to the nucleus (Ghribi et al. 2001a). NF-κB has a complex role in inflammatory signalling, the extent of which is still uncertain (see Lawrence 2009). GADD153 has been shown to down-regulate the antiapoptotic BCL-2, sensitising cells to ER stress (Ghribi et al. 2001b; McCullough et al. 2001). BCL-2 exerts its antiapoptotic function by inhibiting the translocation of *apoptotic inducing factor* (AIF) from mitochondria to the nucleus (Daugas et al. 2000). Thus, the reported Al-induced mitochondrial clustering and translocation towards the nucleus suggest a facilitation of AIF's nuclear translocation and subsequent apoptotic function (Dewitt et al. 2006). However, as both NF-κB and GADD153 regulation is controlled by mitochondrial ROS, it is possible their consequential molecular dysregulations are actually secondary to mictochondrial dysfunction (Carrière et al. 2004; Gloire et al. 2006).

Thus, regardless of the specific molecular mechanisms which apparently facilitate it, there is confirmative evidence of Al-induced neurotoxic signalling.

4.1.2 Disputed role in Alzheimer's-like pathologies

As alluded to previously (Section 3.1.4), Al accumulates in neuroanatomical regions associated with AD neurodegeneration (e.g. Walton 2009). However, the potential role of aluminium in AD aetiology is somewhat controversial.

Whilst there is clearly a large genetic component to AD, some level of environmental interaction is clearly also involved. An early twin study revealed that genetics alone are an insufficient predictor of AD, with only around 40% concordance in both monozygotic and dizygotic twins (Nee et al. 1987). Subsequent twin studies have placed the concordance at 19-21% and 5-11% for monozygotic and dizygotic twins (respectively), with only 50% concordance in relation to the presence of the ApoE ϵ4 allele (Breitner et al. 1995; Räihä et al. 1996). Thus, whilst genotype clearly has a strong implication in these diseases, it is far from sufficient to holistically explain their pathogeneses.

Geographical studies have associated areas containing high concentrations of Al in drinking water, with increased instances of AD (e.g. Gauthier et al. 2000; Martyn et al. 1989; McLachlan et al. 1996). Similarly, Al exposure has been reported to induce phenotypes associated with AD pathology, in both clinical findings and laboratory animals.

However, the concept of Al as a causative or triggering factor in AD remains controversial. Dialysis encephalopathy results from insufficient removal of Al from the blood of dialysis patients, resulting in its accumulation in grey matter of the brain (Alfrey et al. 1976; McDermott et al. 1978). However, despite causing atrophy and cognitive impairments, the pathology of dialysis encephalopathy patients has been found to be distinct from that of AD patients (e.g. Shirabe et al. 2002). This disparity relates to the distinct molecular dysregulations between Al toxicity and AD, demonstrating that Al (at least independently) is insufficient to induce AD:

Early findings suggested that Al induces neither tau phosphorylation, nor its subsequent formation of paired helical filaments (PHF) (despite inducing aggregation) (Scott et al. 1993; Shea and Husain 1995). It has since been found that Al-induced NFT do contain hyperphosphorylated tau, with its expression found both in dialysis patients and in experimental animals (Harrington et al. 1994; Savory et al. 1995; Singer et al. 1996). However the inability of Al to induce PHF formation remains a difficulty for advocates of Al as a factor in AD aetiology.

Additionally, a lack of direct biophysical interaction between Al accumulation and Aβ plaques has been reported (Harrington et al. 1994; Landsberg et al. 1992). However, it may not be necessary for this neurotoxin to interact biophysically with amyloid. For example, an upstream dysregulation of either transcriptional control or molecular signalling may result in amyloid aggregation. Additionally, inhibition of proteases involved in the breakdown of amyloid peptides could induce much the same pathology. Thus a lack of direct interaction between Al and amyloid does not necessarily imply a lack of associated dysregulation.

Findings that Al in the brain increases with age demonstrates the accumulation of this neurotoxin (McDermott et al. 1979). However, no difference was found between the Al content of AD brains and age-matched controls (McDermott et al. 1979; Shore and Wyatt 1983). This suggests that, whilst Al may not be an independent causative factor in AD aetiology, it may be a facilitating factor in individuals with genetic vulnerability.

Whilst the concept of Al as a causative factor in AD remains controversial, it clearly disrupts signalling in pathways associated with AD. Thus, these molecular mechanisms affected by Al toxicity must be evaluated in relation to known AD signalling pathways, to assess its validity as a toxic model of neurodegeneration-related cognitive decline.

Molecular dysregulation

Tau is a MAP, facilitating tubulin's formation of microtubules and stabilising them (Weingarten et al. 1975; see Gong and Iqbal 2008). Tau is post-translationally modified; it is phosphorylated by GSK3β or Cdk5 (when in complex with its regulator) and dephosphorylated by phosphatases; notably PP2A (e.g. Sontag et al. 1996; see Stoothoff and Johnson 2005; Walton 2013). Hyperphosphorylation of tau has been associated with the formation of PHF in AD, resulting in NFTs (Bancher et al. 1989; Grundke-Iqbal et al. 1986; Morishima-Kawashima et al. 1995).

Some *amyloid cascade* hypotheses have pointed to the activation (phosphorylation) of GSK3β by Aβ (see Figure 4.2; Kremer et al. 2011; Pei et al. 2008; Terwel et al. 2008). However PP2A expression has been reported to be decreased in AD brains, suggesting a mechanisms of impaired dephosphorylation (rather than only hyperphosphorylation) (Vogelsberg-Ragaglia et al. 2001). In this regard, Al has been found to impair PP2A activity (Walton 2007; Yamamoto et al. 1990). Further, there is evidence that chronic Al exposure in humans results in AD-like brain pathologies, increased phosphorylated tau and cognitive decline (Harrington et al. 1994; Lu et al. 2014). Thus, whilst the effects of Al exposure on tau processing may not entirely resemble that of AD (i.e. a lack of evidence for increased GSK3β), it appears to affect a related pathway.

In addition, there are parallels between Al exposure and AD in the form of other molecular dysregulations. A preliminary study by Lukiw and colleagues found that $\sim$71% of genes highly dysregulated ($\geq$3-fold mRNA change) in AD are also affected by *in vitro* Al exposure (Lukiw et al. 2005). This includes the APP gene, which is highly associated with AD pathologies.

A protein up-regulation of APP has been reported *in vivo* in rodents treated with Al (e.g. Li et al. 2012). An extensive study by Wang and colleagues found that the expression of several genes commonly associated with AD are affected - at protein expression level - in Al-treated rats (Wang et al. 2014). The authors reported increases in APP, BACE1 and subunits of γ-secretase (i.e. PS1, NCT), in the hippocampi and cortices. Additionally, they reported decreases in some genes of the *a disintegrin and metalloproteinase* (ADAM) gene family in hippocampi (ADAM9, ADAM10, ADAM17) and cortices (ADAM10, ADAM17). These α-secretase enzymes cleave APP within the Aβ peptide region, inhibiting the

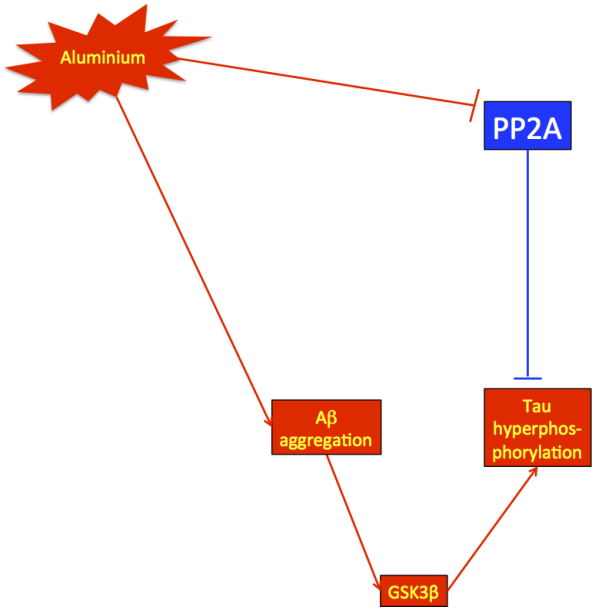

Figure 4.2: Effects of aluminium on the phosphorylation of tau. Tau is phosphorylated by GSK3β (a mechanism instigated by Aβ), and dephosphorylated by PP2A. Aluminium has been shown to decrease PP2A activity, suggesting that its facilitation of tau phosphorylation results from inhibition of dephosphorylation. (Yellow text on red background, neurodegenerative effect; white text on blue background, neuroprotective effect)

production of Aβ (non-amyloidogenic pathway). This increase in substrate (APP) and enzymes in the amyloidogenic pathway (BACE1 and γ-secretase), coupled with inhibition of the non-amyloidogenic enzymes (ADAM), resulted in increase cleavage of Aβ peptides (see Figure 4.3 for mechanisms of APP metabolism).

Interestingly, the authors only found an increase in cleavage of the highly-toxic Aβ_{42} peptide, not the relatively untoxic Aβ_{40} peptide (e.g. Wang et al. 2014; see Dahlgren et al. 2002). This suggests that Al affects the modulation of γ-secretase binding to / cleavage of APP, likely by affecting allosteric regulation, as reported to occur in other pharmacological interventions which affect the species of Aβ peptides produced (e.g. Shelton et al. 2009; Takeo et al. 2014).

In addition to (and likely resulting from) the up-regulation of innate AD-related genes, transgenic mice (expressing hAPP) exposed to high dietary Al have been reported to display increased expression of both Aβ_{40} and Aβ_{42} (i.e. increased transgene cleavage) (Praticò et al. 2002). This resulted in increased plaque formation, demonstrating that Al dysregulation affects cleavage of transgenic hAPP processing.

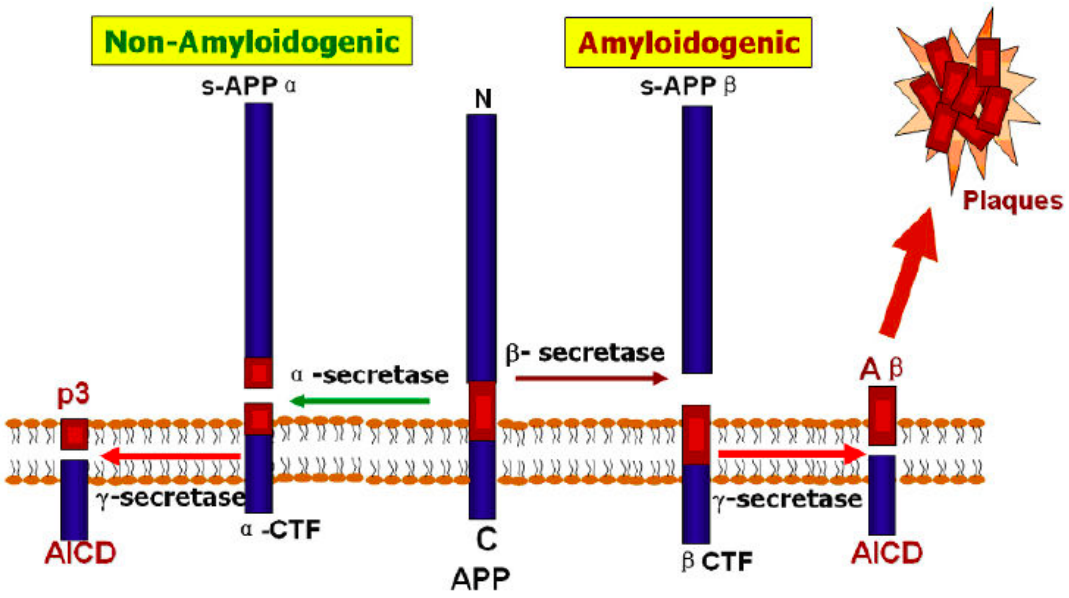

Figure 4.3: APP metabolism by secretase enzymes. In relation to AD pathologies, APP is generally regarded as being processed by one of two pathways. In the '*amyloidogenic pathway*', β-secretases (e.g. BACE1) cleave at a specific site (β site) of the protein, releasing a soluble fragment ($s\text{-}APP\beta$) from the N-teminal. The remaining transmembrane C-terminal fragment is subsequently cleaved by γ-secretase in one of several neighbouring sites, releasing an Aβ peptide between 39-42aa long. In the '*non-amyloidogenic pathway*', α-secretases (ADAM genes) cleave at a specific site (α site) of the APP protein, releasing a longer soluble fragment ($s\text{-}APP\alpha$) from the N-terminal. As the α site is located within the region of the Aβ peptide, this peptide is not released by the subsequent γ-secretase cleavage of the transmembrane C-terminal fragment. (Image from Zhang and Saunders 2009).

Whilst an Al-induced up-regulation of APP has been reported *in vivo* at a protein level, it is unclear whether this results from modulation of gene transcription. The only study to investigate the effects of Al on APP mRNA expression reported only preliminary data from a microarray study, which has yet to be replicated (Lukiw et al. 2005). Additionally, this study was conducted using primary culture, rather than *in vivo*. In this regard, there are clear disparities between the effects of Al *in vivo* and *in vitro*. For example, Al has been repeatedly demonstrated to induce cell death (apoptosis) *in vivo* (e.g. Prakash and Sudhandiran 2015). However an *in vitro* study found no effect of Al (up to 300μM) on cell survival in a neuroblastoma cell culture, in the absence of a synthetic Aβ peptide (Castorina et al. 2010). Further, as astrocytes are known to express APP, it is difficult to draw confirmative conclusions from *in vitro* studies in which neurons are cultured in the absence of glia. Thus, the effects of Al on the transcriptional regulation of APP *in vivo* are, as yet, unknown.

Similarly, the aforementioned finding of increased BACE1 protein expression is unconfirmed *in vivo* at a transcriptional level (Wang et al. 2014). One *in vitro* study reported a mRNA up-regulation of BACE1 following three hours of Al exposure, suggesting this may be a useful period of treatment (Castorina et al. 2010). However the effect was preceded (one hour following incubation) and succeeded (from 12 hours following incubation) by down-regulations. This delay may indicate that the reported up-regulation might not be in direct response to Al, but rather a result of an underlying primary dysregulation (i.e. APP up-regulation, Ca^{2+} dysregulation, mitochondrial dysfunction, ER stress), which results in an increase in BACE1 transcription. Thus, *in vivo* data is required to confirm the aforementioned *in vitro* study.

γ-secretase is a protease complex, formed of four subunits - presenilin (either PS1 or PS2), NCT, APH1, and PEN1 (see Newman et al. 2011; Wolfe 2008). Thus, the reported Al-induced up-regulation of PS1 and NCT has potential implications for the functionality of the γ-secretase complex (Wang et al. 2014).

However, γ-secretase has roles distinct from APP metabolism. For example, it has been demonstrated to cleave other transmembrane proteins (including Notch) and module Ca^{2+} signalling (e.g. De Strooper et al. 1999; Shideman et al. 2009). Interestingly, high expression of both presenilin subunits (PS1 and PS2) has been reported in mitochondria-associated membranes (MAM) (a membranous interface between mitochondria and ER), as has γ-secretase activity (Area-Gomez et al. 2009). Further, PS2 (but not PS1) has been demonstrated to facilitate mitochondrial Ca^{2+} influx by increasing MAM associations (Zampese et al. 2011a; Zampese et al. 2011b).

Thus, given the aforementioned Al-induced Ca^{2+} dysregulation in both mitochondria and ER (see Section 4.1.1), it seems likely that γ-secretase dysfunction plays a role in this.

Regardless of γ-secretase's role in Ca^{2+} signalling, it's high expression in MAMs suggests cleavage of APP in this cellular region. Indeed, high levels of Aβ peptide production (i.e. secretase cleavage of APP) has been reported in MAMs (Schreiner et al. 2015). In this light, the aforementioned extensive reporting of both mitochondrial dysfunction and ER stress (in both Al toxicity and AD) may be linked by processes of amyloid metabolism in the MAM. Further, this presents another molecular mechanism of cellular dysfunction linking Al toxicity and AD. However, given the likelihood of allosteric regulation, as well as the distinct functions of the individual subunits, assessment of gene expression will likely be an insufficient indication of γ-secretase functionality. Thus, expression of these genes will not be assessed in the present study.

The long-term purpose of the behavioural (*classical aversion*) assay - developed in Chapter 3 - is to identify neuroprotectant compounds and neuroprotective mechanisms. A strong candidate for investigation in this area is the function of PIN1:

PIN1 has been shown to inhibit aggregation of Aβ peptides, as well as hyperphosphorylation of tau, and GSK3β activity (Bulbarelli et al. 2009; Kimura et al. 2013; Lu et al. 1999; Ma et al. 2012; Pastorino et al. 2006). (Additionally, impaired PIN1 function has been reported in AD patient brains, although no genetic mutation was found to be associated with the disease (Maruszak et al. 2009; Sultana et al. 2006)).

There is evidence that Al induces a change to the secondary structure of PIN1, causing α-helices to change into β-sheets (Wang et al. 2013). In this regard, as the functionality of PIN1 was reported to be inhibited, it is possible that an up-regulation occurs to enhance the neuroprotective mechanism. Alternatively, it is equally likely that the neurotoxic action of Al is facilitated by a down-regulation of PIN1. Thus, the regulation of this gene is of interest for both these modes of neurodegeneration.

Thus, in light of the objective to identify neuroprotective mechanisms, the assessment of PIN1 activity (following Al exposure) would provide an indication of potential disruption to this pathway. The signalling pathway proposed for

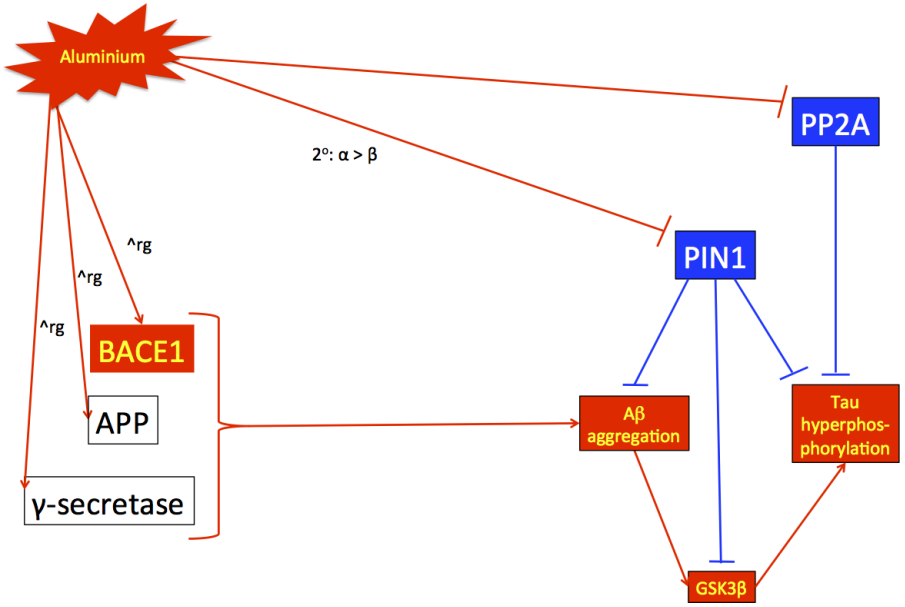

Figure 4.4: Aluminium dysregulates AD-related genes and disrupts neuroprotective signalling. Aluminium exposure increases the protein expression levels of both APP and BACE1. It has also been demonstrated to affect the secondary structure of PIN1, converting α-helices into β-sheets. This, in turn, disrupts the neuroprotective consequences of PIN1 signalling (i.e. inhibition of Aβ aggregation and dephosphorylation of tau). (Yellow text on red background, neurodegenerative effect; white text on blue background, neuroprotective effect; *^rg*, up-regulation; 2^o $\alpha > \beta$, protein secondary structure change from α helix to β sheet)

investigation (see Figure 4.4) involves two genes commonly associated with the amyloidogenic pathway of AD - namely APP and BACE1 (zebrafish *appb* and *bace1*). qPCR will be conducted to assess whether previous reports of Al-induced protein up-regulations of these genes are transcriptionally modulated. It is therefore hypothesised that mRNA expression of these two genes will be increased following Al exposure. Additionally, the effect of Al on PIN1 transcription will be assessed, as an indication of whether this neuroprotective pathway is affected. This may manifest as a protective up-regulation (in response to APP and BACE1 expression), or as a degenerative down-regulation (possibly as a direct result of Al exposure). Thus, a two-tailed hypothesis is proposed - that PIN1 expression will be affected by Al. Together, these data are intended to provide evidence of Al-induced molecular dysregulation, and an insight into an affect on a neuroprotective mechanism.

Neuroanatomical accumulation and behavioural phenotypes

Al has been reported to accumulate in various regions - both in humans and experimental animals - with highest levels detected in the hippocampus (McDermott et al. 1979; Yuan et al. 2012). Interestingly, the regions which accumulated Al in rats were also found to contain higher products of lipid peroxidation, supporting links between Al exposure and oxidative stress (Yuan et al. 2012). Mice exposed to Al have demonstrated decreased spatial memory function, measured by perfomance in a water maze (Kaneko et al. 2006). With regard to human exposure, ex-workers who had been exposed to Al dust (at an Al melting plant) over the course of their careers were found to perform poorly in a range of cognitive tests (compared to controls) ten years after retirement (Polizzi et al. 2002).

As the amygdala has been found to accumulate Al, it seems likely that toxicity would result in impaired performance in the *classical aversion assay* (Developed in Chapter 3) (Walton 2009). Indeed, it has also been reported that Al-treated mice display impaired fear conditioning (Hashmi et al. 2015). Interestingly, this was found to be ameliorated by non-steroidal anti-inflammatory drugs (NSAID), which are also believed to protect against AD progression in arthritic patients, as well as rodent models of AD (e.g. Heneka et al. 2005; Lim et al. 2000; Lim et al. 2001; Myllykangas-Luosujärvi and Isomäki 1994; Rogers et al. 1993).

4.1.3 Aims

The initial aims of this investigation are to establish whether Al toxicity induces a transcriptionally-regulated change in expression of zebrafish *appb*, *bace1* and *pin1*. This will test the hypothesis that Al exposure affects the expression of genes associated with neurodegeneration. Additionally, an attempt will be made to qualitatively assess whether this results in neuronal apoptosis within the zebrafish amygdala-like structure - the Dm (mP). Finally, cognition (in relation to Dm functionality) will be assessed using the *classical aversion assay*, as developed in Chapter 3. This will test the hypothesis that Al toxicity induces impairment to *classical fear conditioning*.

4.2 Methods

4.2.1 Subjects

Wild-type (TU) adult zebrafish were bred and reared in our facility, as described previously (e.g. Parker et al. 2012b). Fish were housed in groups of 30, in ∼28°C water, on a 14h:10h light:dark cycle. Fish were transferred to the room where behavioural experimentation was conducted at least 24 hours prior to experimentation. All experiments were conducted in accordance with the Animals (Scientific Procedures) Act, 1986, under license by the Home Office (UK).

4.2.2 Aluminium exposure

Zebrafish were individually exposed to *aluminium chloride* ($AlCl_3$) for 3 hours, at doses stated. This length of time has previously been demonstrated to be sufficient to cause a mRNA up-regulation of both *appb* and *bace1* in zebrafish (Nik et al. 2012).

4.2.3 qPCR

Zebrafish were sacrificed by decapitation, brain tissue dissected out and frozen immediately on dry ice. Total RNA was extracted, DNAse-treated and reverse transcribed, as described in Sections 2.2 and 2.4.

Standard curves were loaded on each plate from PCR samples of known *fragment concentrations*, as determined using Avogadro's constant. *Fragment numbers* in test samples were determined by the qPCR machine (BioRad CFX Connect) from the Cq values, based on the standard curves.

Regarding post-hoc testing; for appb and bace1 expression analyses, one-tailed t-tests or one-sided Dunnett's tests were conducted (as indicated). This is due to the previous evidence demonstrating an $AlCl_3$-induced up-regulation for these genes at the protein level; (e.g. Wang et al. 2014). Thus, should down-regulations be detected, this would be regarded as an feedback from high protein expression

(i.e. homeostatic dysregulation caused by inhibition of proteolytic breakdown of the relevant protein). For pin1 expression, two-tailed t-tests or two-sided Dunnett's tests were conducted (as indicated). This is because of the lack of previous evidence indicating a possible direction of dysregulated expression.

4.2.4 Fluoro-Jade® B staining

Zebrafish were treated with 100μM $AlCl_3$ for three hours, then allowed to recover for one hour. This 'recovery period' was intended to replicate the *habituation* (30 minutes) and *baseline* (30 minutes) periods of the *classical aversion assay*, during which they were not exposed to Al. Fish were then sacrificed by decapitation, brain tissue was dissected out, and frozed immediately in OCT compound, on dry ice.

Brains were cut at -20°C, in 10μm sections. Slides were then stored at -20°C, until processing.

Fluoro-Jade® B was purchased from Millipore, and slides stained as described in Section 2.25.

4.2.5 Classical aversion assay

The *classical aversion assay* was conducted as developed and described in Chapter 3, using 2-minute probes.

Subjects were treated in aquarium water containing $AlCl_3$ (at the doses stated) for three hours. Subjects were then netted into assay tanks (which did not contain $AlCl_3$), and assayed immediately.

As discussed in Section 3.4, *Distance travelled* was factored into the *mixed effects* statistical model as a covariate, to account for any variance caused by differences in locomotion.

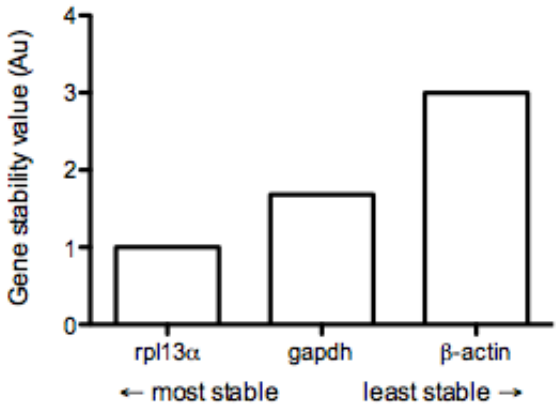

Figure 4.5: Stability of housekeeping genes. qPCR data were entered into the online *RefFinder* tool. The results demonstrate that rpl13α was the most stably expressed gene.

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4.3 Results

4.3.1 Molecular dysregulation

qPCR was undertaken to assess transcriptional regulation of gene (appb, bace1, and pin1) expression, following acute aluminium exposure. Zebrafish were exposed to $AlCl_3$ for three hours, as this exposure time has previously been found to be sufficient for Al-induced transcriptional dysregulation (Castorina et al. 2010). If not sacrificed immediately, fish were placed in a recovery tank for the period of time specified.

Determination of stable house-keeping gene

qPCR amplification was conducted on three housekeeping genes - β-actin, gapdh, rpl13α (in tandem with experimental genes).

Gene stability was calculated using the '*RefFinder*' online tool (Lab 2016). This tool integrates multiple algorithms which assess gene stability, assigning a *stability value* based on these outputs (Xie et al. 2012). The results demonstrate that rpl13α was most stably expressed in the experimental conditions (see Figure 4.5). Thus, experimental genes were normalised to rpl13α in subsequent $AlCl_3$ experiments.

Effects of acute $AlCl_3$ exposure (100μM) on gene expression

The expression levels of appb, bace1 and pin1 were assessed following acute exposure to $AlCl_3$ (100μM). Results (see Figure 4.6) found no transcriptional up-regulation of appb or bace1 ($p = 0.33$ & 0.098, respectively; by one-tailed independent t-tests). Whilst the up-regulation of pin1 failed to reach significance, the effect may be viewed as marginally significant (p=0.06; by two-tailed independent t-test). In light of this, power analysis was conducted (using G*Power version 3.1 software) for pin1 data. This estimated that 24 subjects would be required in total (12 per group), indicating that the study may have been under-powered.

Prolonged effects of $AlCl_3$ (100μM) on gene expression

The prolonged effects of $AlCl_3$ exposure on gene expression were determined at 30 minutes and 60 minutes post exposure (to coincide with initiation of baseline and conditioning in the classical aversion behavioural assay). Results are presented in Figure 4.7. No effect was found for appb expression (p=0.849). A significant up-regulation was found for bace1 expression (p=0.006) at 60 minutes post exposure. The ANOVA model for pin1 expression failed to reach significance (p=0.06); however a significant up-regulation was detected post-hoc at 30 minutes post exposure, which had attenuated by 60 minutes.

Dose-dependent effects of $AlCl_3$ on gene expression at one hour following treatment

In order to confirm the effects of $AlCl_3$ on gene expression at one-hour post exposure, dose-response effects were assessed by simple linear regression (see Figure 4.8). The up-regulation of appb failed to reach significance (p=0.052). A significant up-regulation was found for bace1 expression (p=0.041). No effect was found for pin1 expression (p=0.305).

Dose-dependent effect of $AlCl_3$ on dapk1 expression

Whilst no effect was found for *pin1* expression in the previous experiments, it was subsequently hypothesised (ad-hoc) that *dapk1* expression would be affected. This

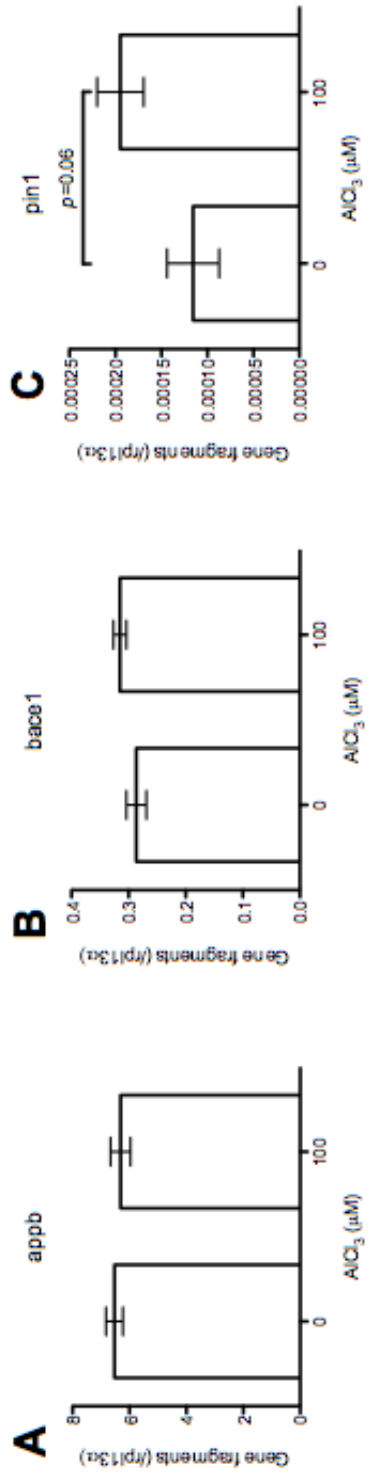

Figure 4.6: Effects of $AlCl_3$ treatment on gene expression. qPCR expression analyses (‘*fragment number*’) were calculated and normalised to rpl13α expression. No significant effect was found for appb (p=0.33) or bace1 (p=0.098) expression (by one-tailed t-test). The increase in pin1 expression failed to reach significance (p=0.06; by two-tailed t-test) (n=12). Data plotted as mean ± SEM.

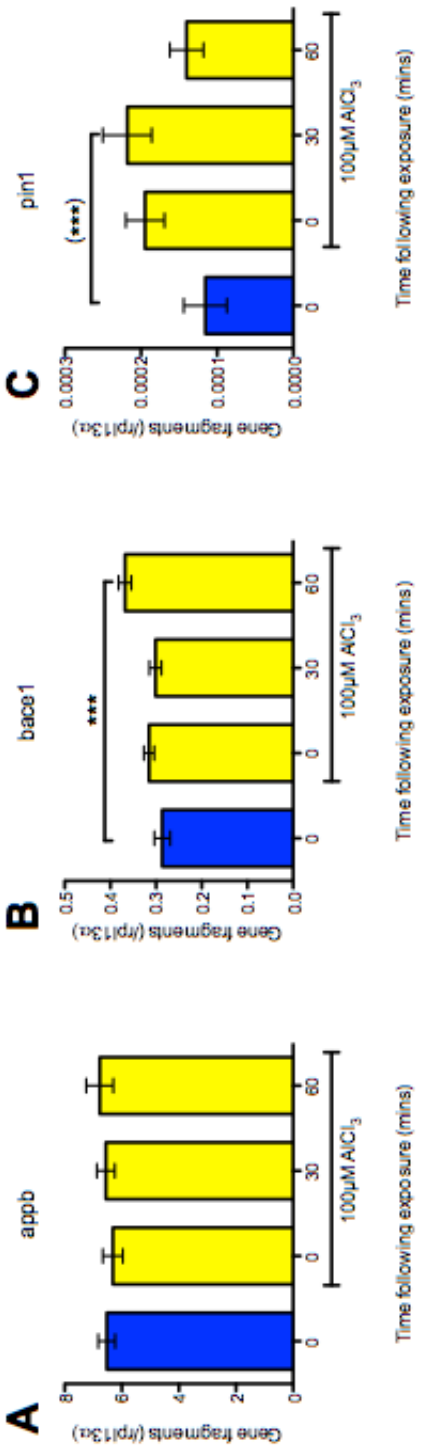

Figure 4.7: Prolonged effects of aluminium exposure on gene regulation. Animals were exposed to $AlCl_3$ (100μM) or saline for three hours, then placed in a 'recovery tank' and sacrificed at the times specified. No effect was found for appb expression ($F_{3,20}$=0.266, p=0.849). A significant effect was found for bace1 expression ($F_{3,19}$=5.595, p=0.006). The pin1 up-regulation failed to reach significance ($F_{3,19}$=2.925, p=0.06), although a significant up-regulation was found post-hoc at 30-minutes following exposure (n=24). ***, p<0.001; by one-way ANOVA, then one-sided (*appb* and *bace1*) or two-sided (*pin1*) Dunnett's test. Data plotted as mean ± SEM.

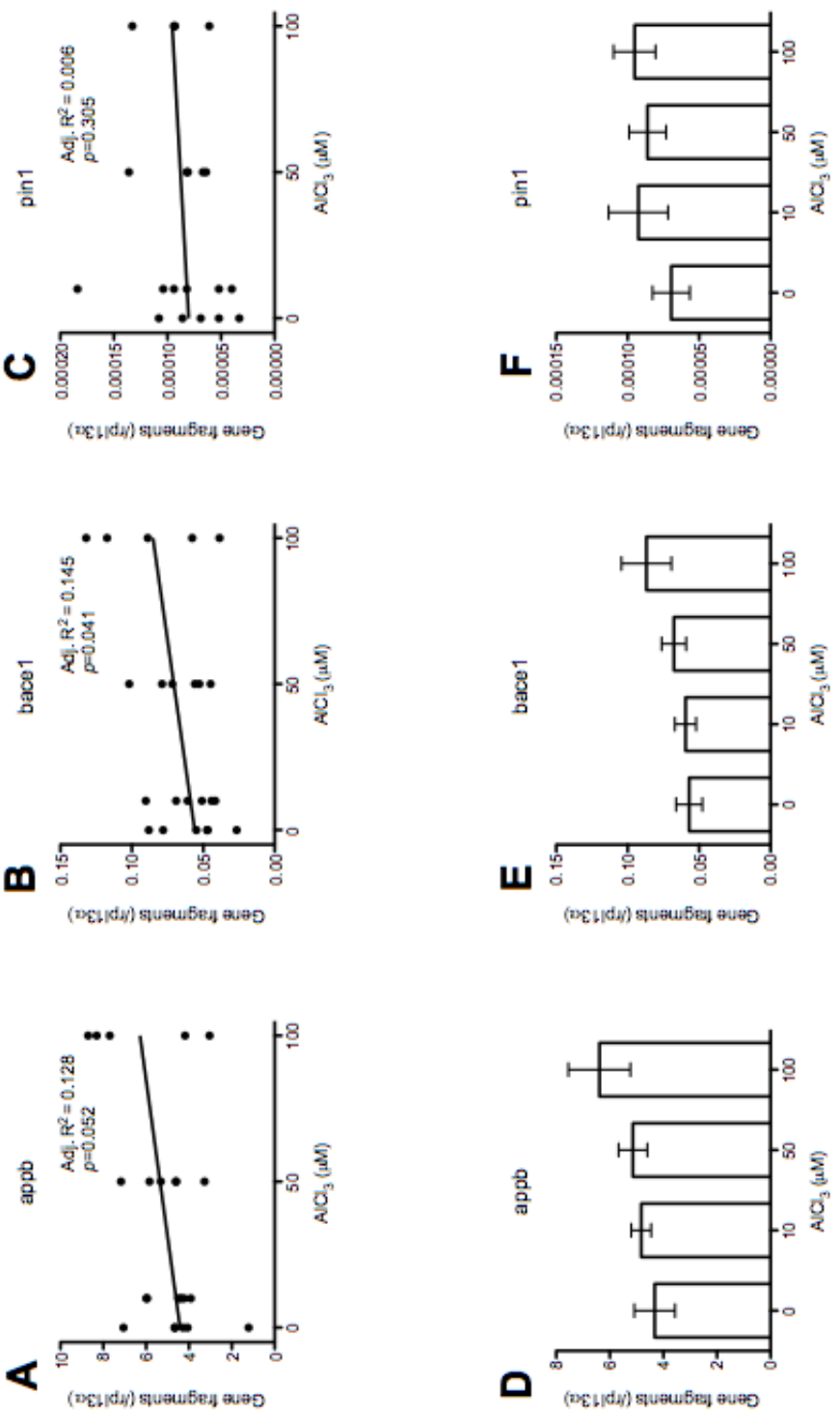

Figure 4.8: Dose-dependent effects of $AlCl_3$ on gene expression at one-hour following exposure. (A & D) The up-regulation of appb failed to reach significance ($F_{1,22}$=4.237, p=0.052). (B & E) A significant up-regulation of bace1 was found ($F_{1,22}$=4.745, p=0.041). (C & F) No effect was found for pin1 expression ($F_{1,19}$=1.114, p=0.305) (n=23). By simple linear regression. Data plotted as raw values (A-C) and mean ± SEM (D-F).

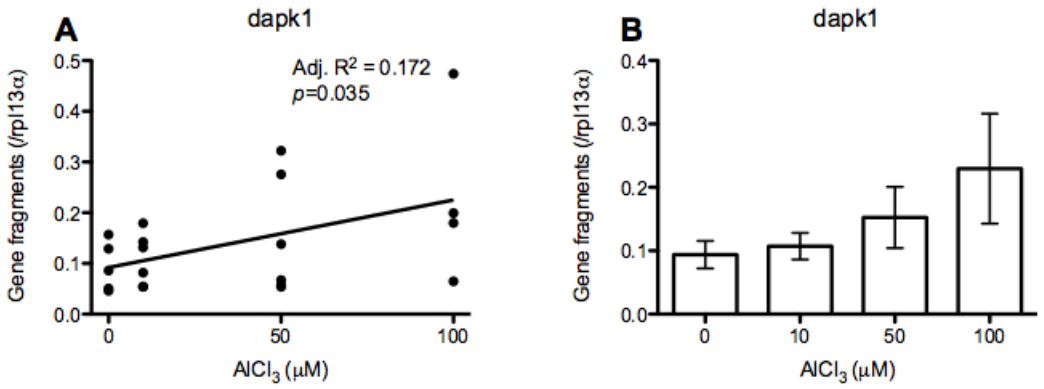

Figure 4.9: Dose-dependent effect of $AlCl_3$ on dapk1 expression at one hour post exposure. A significant up-regulation was found ($F_{1,20}$=5.162, p=0.035). By simple linear regression. Data plotted as raw values (A) and mean ± SEM (B).

is due to findings that *dapk1* inhibits *pin1*, inhibiting its neuroprotective functions. Thus, in light of the failure to trigger *pin1*'s neuroprotective signalling, it was hypothesised that Al may induce neurodegenerative signalling via *pin1* inhibition. In this regard, preliminary data suggests a significant up-regulation of *dapk1* (p=0.035)

4.3.2 Neurodegeneration

In order to qualitatively associate $AlCl_3$ toxicity with neurodegeneration, Fluoro-Jade® B staining was conducted on telencephalic sections (which include the relative pallial divisions; see Figure 3.2) of the zebrafish brain. Zebrafish were treated with 100μM $AlCl_3$ for three hours, then placed in a recovery tank for one hour, before being sacrificed.

Results are presented in Figure 4.10 (larger images in Section 9.1). Some level of degeneration was observed in the ventral pallium (Vp) and Dc in both groups (control and treated). The Dm showed no detectable Fluoro-Jade® B staining in the control subjects. In $AlCl_3$-treated brains, increased staining was observed in the Dm. However, due to the inconsistent quality of the staining, these data are regarded as preliminary and by no means confirmative.

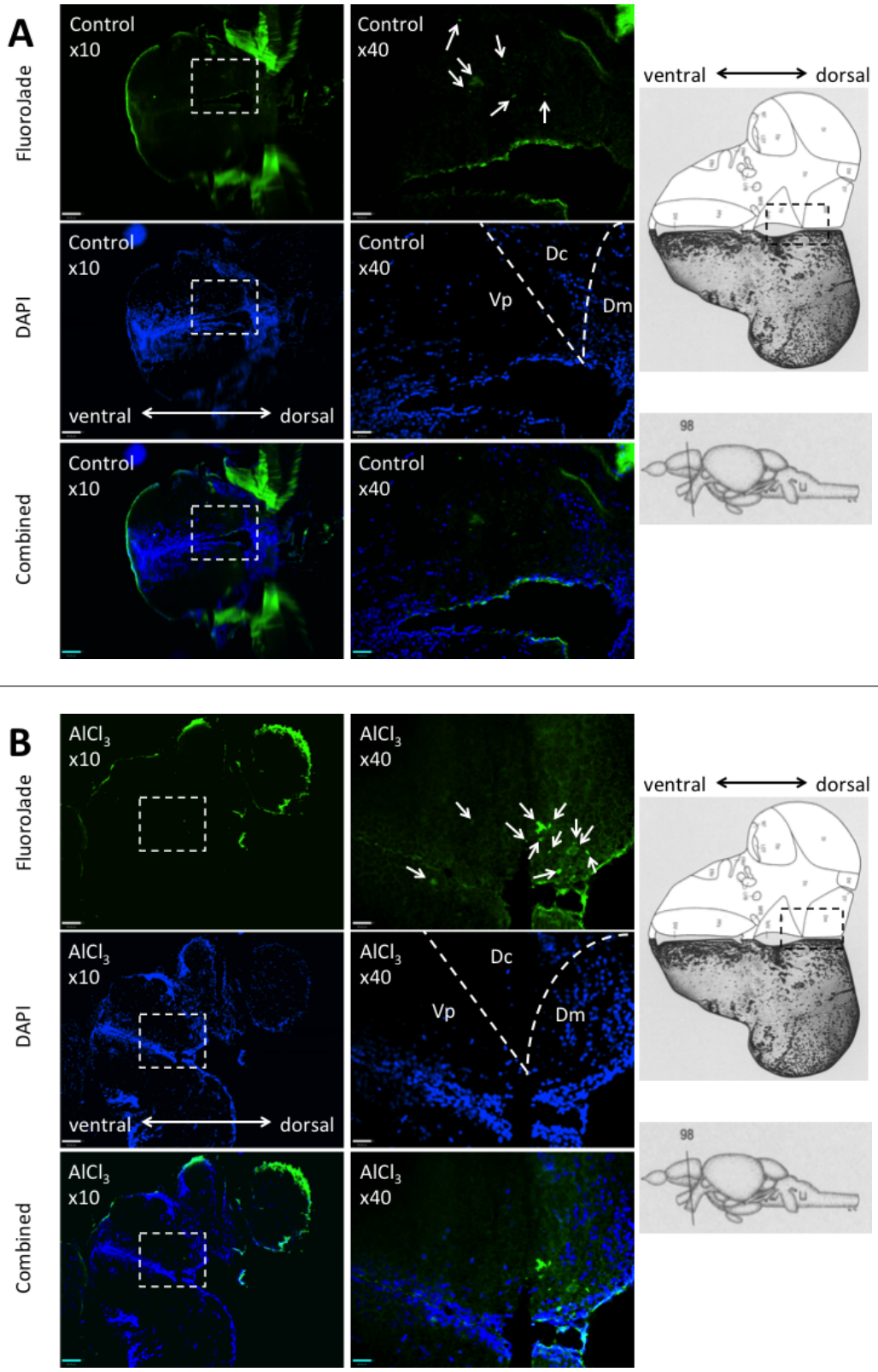

Figure 4.10: Neurodegeneration following $AlCl_3$ treatment. FluoroJade® B staining was conducted on 10μm sections of zebrafish telencephalon. Increased staining is apparent in the $AlCl_3$-treated brains (B) compared to saline-treated brains (A). (Larger images are included in Appendices; Section 9.1.) Approximate positions of brain sections are indicated on the right-hand side (both A & B), with focal region indicated in the coronal (top) graphics (images from Wulliman et al. 2012).

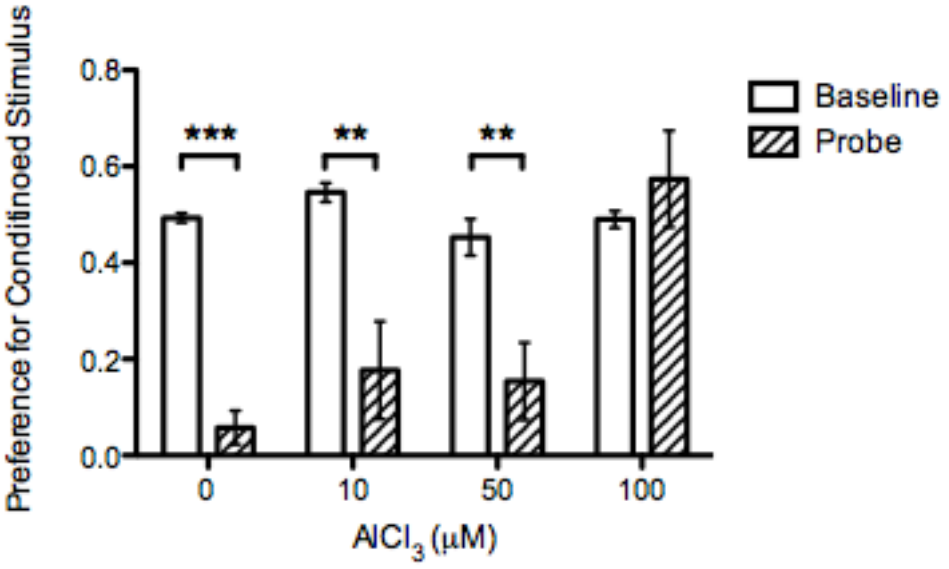

Figure 4.11: $AlCl_3$ dose-response curve. Analysis by *linear mixed effects model* found significant effects for '*$AlCl_3$ concentration*' ($F_{3,23.379}$=6.673, p=0.002) and '*trial*' (baseline vs. probe) ($F_{1,28.622}$=24.329, p<0.0005). A significant interaction ($AlCl_3$ concentration * trial) was also found ($F_{3,23.623}$=5.3, p=0.006). Post-hoc analyses found that, at low concentrations of $AlCl_3$ ($\leq$50μM), significant aversion of the CS was observed in the probe trial. However this effect was not seen at the highest dose (100μM) (n=29). **, p<0.01; ***, p<0.001; by two-tailed t-test with Bonferroni correction applied. Data plotted as mean $\pm$ SEM.

4.3.3 Dose-response curve for $AlCl_3$ on classical aversion

In order to assess whether $AlCl_3$ toxicity induced a behavioural phenotype, the *classical aversion assay* (developed in Chapter 3) was employed. The results demonstrate a dose-dependent effect for $AlCl_3$ (see Figure 4.11). Thus, $AlCl_3$ significantly impaired performance ('*learning*') in this behavioural measure of Dm (amygdala) function.

4.4 Discussion

The aims of this chapter were to assess whether Al toxicity in zebrafish induces a transcriptionally-regulated dysregulation of genes associated with AD pathology (*appb* and *bace1*) as well as up-stream genes which affect their function (*pin1* and *dapk1*); and whether these resulted in an impairment to *classical fear conditioning*.

The initial results demonstrate that acute exposure to $AlCl_3$ has no immediate effect on *appb* or *bace1* expression. The up-regulation of *pin1* failed to reach significance; however post-hoc power analysis estimates suggested that this may have been underpowered. Thus it seems plausible that a significant up-regulation would be found with increased power. Should this be detected in future replications, it would appear to suggest that Al may actually be a valid therapy for AD-related neurodegeneration, as the neuroprotective gene (*pin1*) is up-regulated in the absence of any effect on the neurodegenerative (amyloidogenic) genes (*appb* and *bace1*). However, given the extensive reporting that Al accumulates in the brain, the effects immediately following exposure may be less relevant than the prolonged effects (e.g. Alfrey et al. 1976; McDermott et al. 1978; Walton 2009; Ward et al. 2001).

As the *classical aversion assay* involves a 30-minute habituation period, followed by a 30-minute baseline period (prior to conditioning), gene expression was assessed after 30- and 60-minutes recovery. No effect was found for *appb* at any of the time points following exposure. However, *bace1* expression was found to significantly increase at 60-minutes following exposure. Thus, whilst there is no effect on the amyloidogenic substrate (*appb*), the key enzyme in cleaving the amyloidogenic pathway (*bace1*) was found to be up-regulated. Logically, this would be sufficient to induce an increase in Aβ peptide cleavage, although this was not tested in the present study. Additionally, whilst the inferential statistical model (ANOVA) failed to find a significant effect for *pin1* expression, post-hoc analyses indicate that a significant up-regulation may occur at 30 minutes following treatment, which attenuates by 60 minutes.

In relation to the *classical aversion assay*, the time when conditioning starts (60 minutes following treatment) coincides with an increase in expression of *bace1*, whilst a possible up-regulation of *pin1* appears to have attenuated. This suggests that, at the onset of conditioning, there is an up-regulation of neurodegenerative (amyloidogenic) signalling, in the absence of *pin1*-facilitated neuroprotective signalling. However, the functional effects implied by these data are unconfirmed without assessment of enzyme activity. Whilst this level biochemical analysis is beyond the scope of the current thesis, it would form an important future investigation.

At face value, the lack of effect on *appb* appears to contradict previous studies which have reported an up-regulation of protein expression (e.g. Li et al. 2012; Wang et al. 2014). However, Al has been found to inhibit the proteolytic

breakdown of Aβ peptides (Sakamoto et al. 2006). Thus, despite no transcriptional up-regulation, it is highly plausible that there is still an increase in *appb* protein, as a substrate for the up-regulated *bace1* enzyme. Thus, future research in which protein expression is compared to mRNA expression would elucidate the nature of the regulatory mechanisms which govern this Al-induced molecular dysregulation (amyloidogenesis). In this regard, an attempt was made to assess APP protein expression via western blot. However the antibody used failed to consistently probe bands of the same size, and was thus deemed unreliable to include in the results presented here (see Appendices, Section 9.2 for examples of antibody inconsistency).

As these data indicate a pattern of gene expression potentially leading to increased Aβ production during conditioning (in the behavioural assay), an investigation of the dose-response effects (of $AlCl_3$) was undertaken. These data indicate that increased concentration of $AlCl_3$ significantly increased expression of *bace1* at one hour following exposure. Whilst earlier findings (in the current study) failed to find an effect for *appb* mRNA expression, the dose-response curve indicates that a subtle affect may be present; although, again, this marginally failed to reach significance (p=0.052). Indications that any potential up-regulation of *pin1* is attenuated by 60 minutes post exposure are supported in the dose-response investigation, in which no effect was found.

Whilst these data support the hypothesis that neurodegeneration-related signalling mechanisms are facilitated by Al exposure, the complex post-translational interactions between these genes still make it difficult to predict apoptosis. Thus, the expression of a final gene - *dapk1* - was assessed.

dapk1 has been found to inhibit *pin1* (by phosphorylation), thus inhibiting the neuroprotective consequences of *pin1* signalling (Figure 4.12; Lee et al. 2011). Additionally, Ca^{2+} activates CaN which, in turn, activates dapk1 (by dephosphorylation) to induce apoptosis (see Nair et al. 2013). Thus, previous findings of Al-induced increases in cellular Ca^{2+} suggest an increase in *dapk1* activity (Szutowicz et al. 1998). Presently, *dapk1* was found to be significantly up-regulated at one-hour post $AlCl_3$ exposure. Therefore any neuroprotective function of any residual increase in *pin1* protein expression is likely to be inhibited (to some extent) by the increase in *dapk1* expression.

Whilst not quantitative, the findings of neurodegeneration (by Fluoro-Jade® B staining) support the utility of Al as a positive control in neurodegeneration studies.

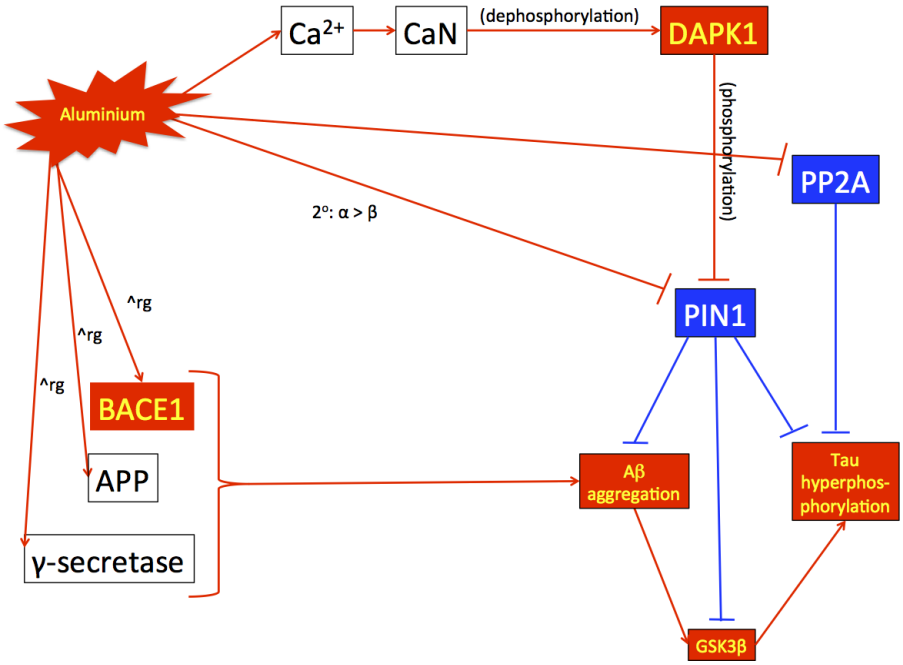

Figure 4.12: Effects of *dapk1* signalling on *pin1* neuroprotective signalling. *dapk1* phosphorylates *pin1* to inactivate it. This would inhibit the neuroprotective signalling of *pin1* (inhibition of Aβ aggregation and dephosphorylation of tau). *dapk1* is activated by CaN (via dephosphorylation), which itself is activated by Ca^{2+}, found to accumulate in cells following Al treatment.

Further, the increased neurodegeneration was detected in the Dm. This area has been reported to be analogous to the tetrapod mP, and the mammalian amygdala (see Ganz et al. 2014). Therefore neurodegeneration was specifically detected in the neuroanatomical area associated with *classical fear conditioning*; an area where Al has previously been found to accumulate in rodents (Walton 2009).

Finally, behavioural impairment was detected in the *classical fear conditioning* assay. This demonstrates that the molecular dysregulation and / or neurodegeneration in the zebrafish brain results in a quantifiable functional impairment, supporting the use of Al-induced neurotoxicity as this model. Thus, the utility of this behavioural assay as a screen in neurodegeneration research is validated.

To summarise, the effects of aluminium on gene transcription support the hypothesis that $AlCl_3$ induces neurodegenerative signalling, in relation to a pathway involved in AD. This is further supported by the qualitative finding of increased neurodegeneration in the Dm, detected by Fluoro-Jade® B staining. The subsequent finding of impaired *fear conditioning* allows the acceptance of the hypothesis that acute $AlCl_3$ exposure results in impaired cognition. This validates

the utility of zebrafish as model for neurodegeneration-related cognitive decline, demonstrating a conservation of mechanisms found in mammals. Additionally, the behavioural findings validate the utility of the *fear conditioning assay* as a screening tool in neurodegeneration research.

Chapter 5

Genetic Models of Neurodegeneration

5.1 Introduction

Previous chapters of this thesis have suggested that *classical fear conditioning* in zebrafish may be used to detect behavioural changes in neurotoxin-induced neurodegeneration (see Chapters 3 and 4). The current chapter will further validate this assay by assessing a transgenic zebrafish line of relevance to a neurodegenerative disease.

As a tool for genetic research, zebrafish have been used extensively in gene manipulation studies. The focus of this chapter will be the genetic basis for AD; however it must be noted that investigations into several other neurodegenerative diseases (as well as non-degenerative psychiatric disorders) have utilised zebrafish as a genetic model.

5.1.1 Alzheimer's-like pathologies

The concept that '*Alzheimer's disease*' is a group of distinct diseases (albeit with similar pathologies) was argued in the *General Introduction* of this thesis (see Section 1.1.2). Here, a more in-depth assessment of the various mutations which may result in AD pathologies is presented.

APP mutations

The most commonly researched gene in AD research is the APP. As discussed previously, its sequential cleavage by BACE1 and γ-secretase results in Aβ peptides; the 42-amino acid species of which is associated with amyloid plaque formation (Selkoe 1999). Thus, amyloidogenesis-centred animal models have focussed on affecting the secretases' binding / cleavage of APP, or else Aβ peptide aggregation.

The majority of APP mutations have been found around the binding sites of secretase enzymes. Wiley and colleagues categorised those proximal to the β- and α- sites (BACE1 and α-secretase binding sites, respectively) as *Class I*, and those proximal to the γ site (γ-secretase binding site) as *Class II* (see Figure 5.1; Wiley et al. 2005). The current thesis chapter adopts this same classification.

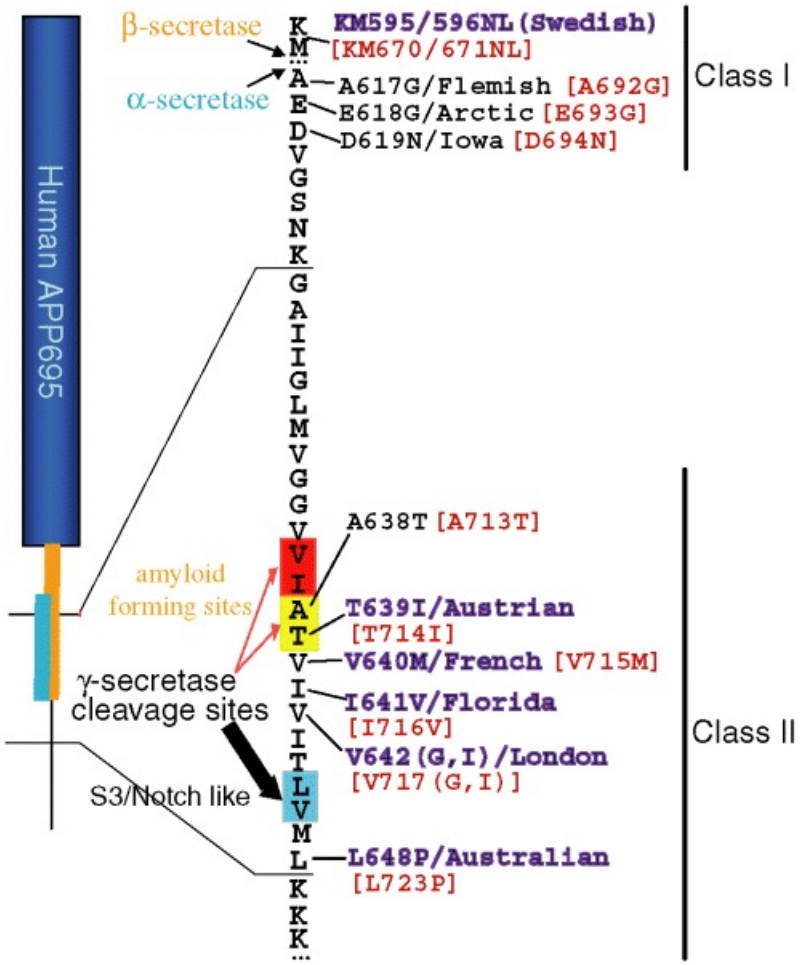

Figure 5.1: Classification of APP mutations. Mutations located around the β- and α-cleavage sites are regarded as *Class I* mutations. The are the *Swedish* (double), *Flemish*, *Arctic*, and *Iowa* mutations. Mutations located around the γ-cleavage site are regarded as *Class II* mutations. These are the *Austrian*, *French*, *Florida*, *London*, and *Australian* mutations. (Image from Wiley et al. 2005).

Class I - Mutations currently known are the *Swedish*, *Flemish*, *Arctic*, and *Iowa* mutations. Additionally, *Dutch* and *Italian* mutations are at the same site as the *Arctic* mutation. Despite their close proximity, the mechanisms by which these mutations disrupt Aβ are distinct.

For example, the *Swedish* double-mutation has been shown to increase production of Aβ peptides by cleavage in vesicles (formed in the Golgi apparatus, implicating a mechanism of extracellular secretion) (Haass et al. 1995). Behaviourally, this has been shown to result in a range of cognitive impairments, including spatial learning and fear conditioning deficits, in transgenic rodent models (Hanna et al. 2012; Lalonde et al. 2002).

The *Flemish* mutation - despite being a *Class I* mutation - actually increases γ-secretase activity. It is located within the '*APP substrate inhibitory domain*', which is bound by γ-secretase and normally inhibits its activity; however the *Flemish* mutation impairs this inhibitory mechanism (Tian et al. 2010).

Aβ peptides containing the *Arctic* mutation have an increased tendency to form protofibrils (Nilsberth et al. 2001). Interestingly, this mutation has been shown to alter the location of APP - from cell membrane (a primary site of ADAM10 activity) to intracellular - resulting in lower availability to α-secretase activity (Gutwein et al. 2003; Sahlin et al. 2007). The *Iowa* and *Dutch* mutations have been shown to facilitate fibril formation of the cleaved Aβ peptide (Van Nostrand et al. 2001). Additionally, the *Arctic*, *Flemish*, *Dutch*, and *Italian* mutations have all been associated with reduced proteolytic breakdown (Tsubuki et al. 2003). Thus, the location of these mutations - all of which are in the region of α-cleavage site - indicates that this region plays a role in Aβ degradation.

Class II - Mutations proximal to the γ-cleavage site are the *Austrian*, *French*, *Florida*, *London*, and *Australian* mutations. Interestingly, despite their pathogenic effects, many of these mutations have been found to decrease γ-secretase activity (Wiley et al. 2005). However, all of these mutations have been shown to increase production of Aβ_{42} (i.e. cleavage at the 42$^{\text{nd}}$ residue of the Aβ peptide) (Hock and Lamb 2001). In this regard, the *Austrian* mutation has been reported to affect the length of Aβ peptide, resulting in greater Aβ_{42} and fewer Aβ_{40} peptides cleaved (Kumar-Singh et al. 2000).

Unclassed - In addition to these neurodegeneration-associated mutations, one mutation has been found to protect against AD development / progression and Aβ

plaque formation, as well as 'normal' age-related cognitive decline (Callaway 2012; Jonsson et al. 2012). This '*Icelandic*' mutation is located within the region of *Class I* mutations, however it has been found to inhibit APP interaction with BACE1 (Das et al. 2016).

The above mutations at the secretase-cleavage sites in APP have been one approach adopted by researchers generating transgenic models of AD. However others have investigated mutations in the secretase genes themselves.

Secretase mutations

Whilst the concept of mutations to the substrate (APP) has been extensively explored, there have also been numerous investigations into mutations of the metabolising enzymes - the secretases.

Regarding the α-secretases, mutations in ADAM10 have been identified in LOAD patients, which reduced α-secretase activity and increased Aβ peptides *in vitro* (Kim et al. 2009).

Although BACE1 mutations have been demonstrated to affect APP metabolism, BACE1 deletion has been shown to attenuate Aβ deposition and memory deficits in transgenic mice expression mutated hAPP (Laird et al. 2005). However these mice displayed synaptic dysfunction in the hippocampi, as well as hippocampal-related cognitive and emotional deficits. Thus, in light of the interconnectivity between the hippocampi and amygdalae, it is likely that the deletion of BACE1 would impact fear conditioning.

The clinical evidence of BACE1 mutations is even more unclear. A population study of Swedish patients found that no BACE1 SNP was associated with markers of dysregulated amyloid metabolism or BACE1 activity (Sjölander et al. 2010). However, in a Chinese population study, two polymorphisms in the BACE1 promotor were associated with increased risk of LOAD (Wang and Jia 2010). Interestingly, a BACE1 SNP has been associated with PD, suggesting an association between the dysregulated pathways in these two neurodegenerative diseases (Lange et al. 2014).

Regarding the γ-secretase subunits, mutations to both PS1 and PS2 (the proteolytic units) have been found in genealogical studies (Bruni 1998; Levy-Lahad et al. 1995; Rogaev et al. 1995; Scheuner et al. 1996). Further, these mutations

have been demonstrated to increase cleavage of the Aβ_{42} peptide *in vitro* and *in vivo* (Citron et al. 1997; Duff et al. 1996; Holcomb et al. 1998; Jankowsky et al. 2004). However, different PS1 mutations have been shown to have differential affects on the resultant production of Aβ_{40} and Aβ_{42} (e.g. Bentahir et al. 2006).

ApoE ϵ4 and late-onset Alzheimer's diseases

ApoE is the principal transporter of cholesterol in the CNS, facilitating multiple metabolic and signalling processes (Elshourbagy et al. 1985; Pfrieger 2003). Whilst neuronally-produced cholesterol is sufficient for the development of neurons, the formation of synapses requires additional ApoE-cholesterol, produced by astrocytes (Mauch et al. 2001). This reduces the high metabolic demands (required for cholesterol synthesis) on neurons, by allocating this to glia (see Shobab et al. 2005).

Both neurons and astrocytes regulate cholesterol synthesis via a homeostatic feedback mechanism, which requires the ApoE-facilitated endocytosis of cholesterol (Leduc et al. 2010; Shobab et al. 2005). Excess cholesterol is converted (into 24-hydroxy-cholesterol) by neurons and excreted across the BBB, thus retaining CNS homeostatic regulation (Björkhem et al. 1997; Lund et al. 2003; see Vance et al. 2005).

However, human ApoE is polymorphic, with three known alleles - ϵ2, ϵ3 and ϵ4. Expression of the ϵ4 allele has been extensively associated with LOAD, in a gene dose-dependent manner (e.g. Corder et al. 1993; Strittmatter et al. 1993). In this regard, it has been demonstrated that all alleles of ApoE are involved in Aβ clearance from the brain (Holtzman et al. 1999). However ϵ2 and ϵ3 expression facilitate greatest clearance, whilst the relatively insufficient ϵ4 clearance results in highly diffuse plaque formation (Castellano et al. 2011). ϵ3 has been demonstrated to bind Aβ_{40} peptides with around 20-times the affinity of ϵ4 (LaDu et al. 1994). Additionally, all alleles have been demonstrated to protect against hydrogen peroxide-induced oxidative stress, with ϵ2 providing highest protection and ϵ4 providing lowest protection (Miyata and Smith 1996).

Interestingly, knocking out the endogenous apoE gene has been shown to reduce Aβ deposition and plaque formation (in transgenic hAPP mice), which is reinstated by expression of the human ϵ3 and ϵ4 alleles (Holtzman et al. 2000). This indicates the role of ApoE in generic Aβ transport, whether extracellular

deposition or clearance.

The $\epsilon 4$ allele has also been associated with a range of degeneration-associated functions (e.g. tau phosphorylation, impaired neurite outgrowth, down-regulation of androgen receptor), which are reversed by $\epsilon 3$ (Mahley et al. 2006). Thus, it appears that many neurodegenerative effects of $\epsilon 4$ may result from impaired neuroprotective functions, otherwise performed by the other alleles. In addition, the $\epsilon 3$ and $\epsilon 4$ alleles have been demonstrated to differentially modulate the neuroanatomical transcription of PIN1 (signalling discussed in Section 4.1.2), suggesting another mechanism by which it can affect neuroprotective signalling (Lattanzio et al. 2014).

However, in light of the complex nature of transgenic ApoE allele expression, particularly with regard to the complication of endogenous gene expression, manipulation of this gene was viewed as too intricate to provide a robust validation of the *classical aversion assay*.

It is extremely important to note that, whilst these mutations all appear to result in similar pathological phenotypes (with the obvious exception of the '*Unclassed' APP mutation*), the biochemical and cellular mechanisms by which these dysregulations manifest are vastly distinct. Thus, by regarding them all under the umbrella term '*Alzheimer's disease*', the distinct causes and mechanisms of these dysfunctions are somewhat disregarded. For this reason, in the current article, the term '*Alzheimer's-like diseases*' has been used.

Zebrafish models of Alzheimer's-like diseases

In assessing the amyloidogenic aspects of AD, it is necessary for the model organism to possess orthologues of the APP gene, as well as the secretase genes which cleave it (or else transgenically express these genes). In this regard, zebrafish possess two APP paralogues - *appa* and *appb* (Guo et al. 2012; Musa et al. 2001; Xi et al. 2011). They also possess orthologues of the BACE1 and presenilin genes (which code for β- and γ- secretases, respectively) (Groth et al. 2002; Leimer et al. 1999; Nik et al. 2012; Xi et al. 2011).

A potential obstacle to investigating amyloidogenesis in laboratory animals is the ability of their endogenous Aβ peptides to form plaques. Whilst some non-primate mammals have been demonstrated to possess a plaque-forming APP

gene (e.g. polar bears, dogs, cats), these do not include many commonly-used laboratory animals, such a rodents (e.g Cummings et al. 1996; Tekirian et al. 1996). Therefore, the necessity to *knock-in* hAPP is common to both rodents and zebrafish, suggesting that the AD-related molecular mechanisms may be more similar between these species than with humans.

The popularity of amyloid cascade hypotheses has lead to production of many mouse models transgenically expressing hAPP (as discussed above). However zebrafish research into AD has paid more attention to tauopathies (Bai et al. 2007; Paquet et al. 2009; Tomasiewicz et al. 2002). Whilst these models have revealed NFT resembling AD pathology, the lack of amyloid dysregulation questions the efficacy of these lines as models specific to AD.

However, attempt has been made to investigate the processing of hAPP in zebrafish. Joshi and colleagues demonstrated that under-expression of *appb* (by morpholino injection) results in a morphological phenotype in embryos (Joshi et al. 2009). The authors reported that the phenotype was rescued by unmutated hAPP expression, but not $hAPP_{SWE}$ expression. This demonstrates a conservation of function between the hAPP and zebrafish *appb* genes, which is affected by the AD-related Swedish mutation. A subsequent study found that the phenotype is not induced by under-expression of *appa*, suggeting *appb* functioning to be of greater importance in early zebrafish development (Song and Pimplikar 2012). Whilst this developmental phenotype cannot be viewed as a model for an age-related degenerative disease, it may present a method of morphologically assessing amyloid processing; however a greater understanding of the functional conservation between hAPP and *appb* is necessary. Regardless, the phenotype rescue of *appb* by hAPP demonstrates some level of conservation, justifying the utility of zebrafish for the transgenic study of hAPP.

In this regard, a zebrafish line which transgenically expresses $hAPP_{LON/SWE}$ (expressed under the pan-neuronal HuC promoter sequence) has been obtained via a collaboration with the Gothilf laboratory (Tel Aviv University). A transgenic mouse line expressing $hAPP_{LON/SWE}$ has been reported to display a range of cognitive deficits, including *fear conditioning* (Faizi et al. 2012). Thus, it is hypothesised here that zebrafish expressing this mutant transgene will also display impaired *fear conditioning*. Additionally, should no behavioural phenotype be observed, the potential for Al to increase *bace1* expression (as reported in Chapter 4) may facilitate amyloidogenic processing and result in a detectable phenotype.

Alternative hypotheses of molecular dysregulation

Whilst amyloid cascade hypotheses have dominated much of biomedical research into AD, amyloid-centred therapies have tended to fail in clinical development (Mangialasche et al. 2010). It is argued in this thesis that AD-related amyloidogenesis results from an array of molecular dysregulations producing similar pathologies (grouped as AD). Thus, the failure of many clinical trials to genotype patients means that patients will be administered therapies for similar cognitive impairments which result from distinct molecular dysregulations.

Thus, whilst mutations to APP and the secretases are clearly affective in some AD pathologies, it is highly likely that dysregulated APP metabolism can result from up-stream dysregulations. In this regard, the consequences of DAPK1-PIN1 signalling present an interesting candidate for investigation.

As discussed previously, PIN1 signalling has neuroprotective consequences in AD research by inhibiting both Aβ aggregation and tau phosphorylation (Bulbarelli et al. 2009; Kimura et al. 2013; Lu et al. 1999; Ma et al. 2012; Pastorino et al. 2006; see Section 4.1.2). Whilst this suggests that mutations which inhibit PIN1 may be a causal factor in AD, no PIN1 mutation has been associated with the diseases (Maruszak et al. 2009; Sultana et al. 2006). However, PIN1 is deactivated by DAPK1 (see Figure 4.12).

An over-activity or over-expression of *dapk1* leads to increased phosphorylation of PIN1, decreasing its neuroprotective activity (Bialik and Kimchi 2011; Lee et al. 2011). Interestingly, H_2O_2-induced oxidative stress has been found to result in DAPK1 activation, suggesting a possible down-stream facilitation of neurodegeneration in AD instances where DAPK1 is not primarily affected (Fan et al. 2014). Of relevance to AD, intronic DAPK1 mutations have been associated with LOAD, suggesting a possible effect from mis-expression, rather than dysfunction (Li et al. 2006). Additionally, increased DAPK1 expression has been reported in the hippocampi of AD patients, however no difference was found in cortical expression (Hainsworth et al. 2010; Kim et al. 2014).

Behavioural deficits

As mentioned previously, the transgenic expression of $hAPP_{LON/SWE}$ in mice resulted in impaired *fear conditioning* (amongst other cognitive deficits) (Faizi et al. 2012). It should be noted that the authors reported an effect in *contextual*, but not *cued* fear conditioning. However, the *cued* assay used was a *trace fear conditioning* paradigm, which involves hippocampal activity in addition to the amygdala (see Section 3.1.2). Thus, hippocampal involvement may mask an amygdala-centred effect, or *vice versa*.

In this regard, in a double-transgenic mouse model (hAPP and PS1), the impairment to fear conditioning was associated with neither increased neurodegeneration, nor amyloid plaque formation; but rather morphological changes in dendritic spines in the lateral amygdala (Knafo et al. 2009). In the same mouse model, dendritic dysfunction has been associated with close proximity to amyloid plaques in cortical and hippocampal sections (Grutzendler et al. 2007). Dendritic dysfunction has also been associated with the single transgenic expression of mutated hAPP (Spires et al. 2005). Similar dendritic abnormalities have been found in brains of AD patients (Grutzendler et al. 2007; see Knobloch and Mansuy 2008)

Thus, it is hypothesised in the present study that transgenic expression of $hAPP_{LON/SWE}$ impairs *delay fear conditioning*.

5.1.2 Mechanisms of transgenic expression in zebrafish

Regarding mechanisms of transgenic expression, HuC promoter has been utilised to drive transgene expression in zebrafish (e.g. Lyons et al. 2003; Sato et al. 2006; St John and Key 2012). HuC is expressed 'pan-neuronally', including at early stages of emryonic development (Kim et al. 1996; Lyons et al. 2003). Thus, by fusing a transgene down-stream to the HuC promotor sequence, transgenic expression can be driven across CNS neurons. This strategy was adopted by the Gothilf lab, in their generation of the Tg(HuC:$hAPP_{LON/SWE}$) line (unpublished).

Alternatively (or additionally), the GAL4/UAS system can be utilised to drive transgene expression. GAL4 is a *transcription activator* found in yeast, which binds UAS to initiate transcription (Ornitz et al. 1991; Scheer and Campos-Ortega 1999). Several transgenic lines have been developed which express GAL4 under the control

of tissue-specific promoter sequences (e.g. HuC and s1101t are neuron-specific) (Dell et al. 2013; Kim et al. 1996; Lyons et al. 2003; Schoonheim et al. 2010). Thus, the subsequent introduction of a transgene - cloned down-stream of UAS - results in tissue-specific expression. Additionally, by replacing the GAL4 C-terminal activation domain with a viral activation domain (VP16, from herpes simplex) the activation of UAS is increased (Croston et al. 1992; Sadowski et al. 1988).

In this regard, the Et(e1b:GAL4-vp16)s1101t (developed by Baier lab, Max Planck Institute) expresses GAL4 specifically in CNS neurons (e.g. Dell et al. 2013; Schoonheim et al. 2010). This provides an efficacious tool for generating new CNS-expressing trangenic lines. Thus, by cloning a gene down-stream of UAS, its expression can be driven by the neuronally-expressed GAL4.

With regard to expression vectors, plasmids containing Tol2 transposable elements may be utilised. When co-injected with *transposase* mRNA, this system facilitates insertion into genomic DNA (Suster et al. 2009). The pBr-Tol2-UAS-*MCS*-mCherry (where *MCS* = *multiple cloning site*) - developed by Masa Tada (UCL), presents a tool which would facilitate this genetic insertion.

5.1.3 Aims

The aim of this research is to assess the effects of AD-related transgene expression on cognition (i.e. performance in the *fear conditioning assay*), in zebrafish. This will be conducted using the Tg(HuC:hAPP$_{\text{LON/SWE}}$) line. Additionally, new gene manipulation lines will be developed, as tools for future research. These will utilise the transgenic expression of the human Aβ_{40} peptide, as well as an over-expression of the zebrafish *dapk1* gene. Whilst these assessment of these new lines is beyond the time-frame allowance of this thesis, their development will be important for understanding the molecular pathways affected by the relative molecular dysregulation.

5.2 Methods

5.2.1 Subjects

Tg(HuC:hAPP) fish were a kind gift from Yoav Gothilf (Tel Aviv University). This line transgenically express hAPP, with expression driven by the pan-neuronal HuC gene. A green fluorescent marker (expressed in the pancreas) indicates the presence of the transgene.

The imported hAPP-expressing fish were heterozygous; these were incrossed to produce siblings of mixed genotypes (25% homozygous, 50% heterozygous, 25% wild-type). These were then identified as carriers or non-carriers of the transgene (by fluorescent microscopy). The non-carrier (non-fluorescent) offspring were isolated, raised and inbred to produce the wild-type strain (termed "hAPP-WT" in this thesis). The carrier (fluorescent) offspring were raised; these were then outcrossed to identify them as homozygous (100% fluorescent offspring) or heterozygous (50% fluorescent offspring) via Mendelian genetics. The homozygous fish were isolated, raised and inbred to produce the homozygous strain (termed "$\text{hAPP}_{\text{LON/SWE}}$" in this thesis).

In order to develop new transgenic lines, a line which expresses GAL4 throughout the CNS was utilised. These fish - Et(e1b:GAL4-vp16)s1101t - were a kind gift from Tom Hawkins (UCL).

5.2.2 RT-PCR

Zebrafish were sacrificed by decapitation and brain tissue dissected out. They were then frozen immediately on dry ice and stored at -80°C until processing.

RNA was extracted, DNAse-treated and reverse transcribed, as described in Sections 2.2 & 2.4. The resultant cDNA was used to PCR-amplify hAPP and HuC genes, .

PCR reactions were conducted using the following primers:

Gene	Primer sequence	Fragment size	Annealing temperature
hAPP	Forward: TTGATGTGACTGAAGGGAAG Reverse: CCAATGATTGCACCTTTGTT	1,186 bp	45^{o}C
HuC	Forward: TTAGCTTTGTCACTGATGAAACACATA Reverse: AAGGAAATCTTCGACCCACG	1,000 bp	48^{o}C

In order to increase PCR product yeild, BSA and DMSO were added to the reaction.

Reaction mix:

Thermopol Buffer	2.5μl
dNTPs (10mM)	0.5μl
ddH_2O	16.87μl
Taq DNA polymerase	0.13μl
BSA (10mg/mL)	0.25μl
DMSO (100%)	1.25μl
Forward primer (10μM)	0.5μl
Reverse primer (10μM)	0.5μl
cDNA	2.5μl
Total	**25μl**

In order to increase specificity of the primers, '*touch-down*' PCR was utilised.

Thermocycles:

Initial denaturation	95^{o}C	30 seconds	
Denature	95^{o}C	30 seconds	x10 cycles
Anneal	60°C - 50°C*	1 minute	
Extension	68^{o}C	1.5 minutes	
Denature	95^{o}C	30 seconds	x35 cycles
Anneal	45°C (hAPP) / 48°C (HuC)	1 minute	
Extension	68^{o}C	1.5 minutes	
Final extension	68^{o}C	5 minutes	
Hold	4^{o}C	∞	

* Annealing temperature reduced by 1°C each cycle

5.2.3 Fluoro-Jade® B staining

Zebrafish were treated with 100μM $AlCl_3$ for three hours, then allowed to recover for one hour. They were then sacrificed by decapitation, brain tissue dissected out, and frozed immediately in OCT compound, on dry ice.

Brains were cut at -20°C, in 10μm sections. Slides were then stored at -20°C, until processing.

Fluoro-Jade® B was purchased from Millipore, and slides stained as described in Section 2.25.

5.2.4 Classical aversion assay

Zebrafish were moved to the behaviour room at least 24 hours prior to experimentation. The *classical aversion assay* was conducted as described and developed in Chapter 3, using 2-minute probes.

As discussed in Section 3.4, *Distance travelled* was factored into the *mixed effects* statistical model as a covariate, to account for any variance caused by differences in locomotion.

5.2.5 qPCR

Zebrafish were sacrificed by decapitation and brain tissue dissected out. They were then frozen immediately on dry ice and stored at -80°C until processing.

RNA was extracted, DNAse-treated and reverse transcribed, as described in Sections 2.2 & 2.4.

Standard curves were loaded on each plate from PCR samples of known *fragment concentrations*, as determined using Avogadro's constant. *Fragment numbers* in test samples were determined by the qPCR machine (BioRad CFX Connect) from the Cq values, based on the standard curves.

qPCR was conducted using primer sequences listed in Section 2.17, Table 2.2.

5.2.6 Cloning new transgenic zebrafish lines

Human Aβ_{40} was transgenically expressed in zebrafish, whilst the endogenous zebrafish *dapk1* gene was over-expressed.

Human Aβ_{40} sequences were a kind gift from Damian Crowther (University of Cambridge). Zebrafish *dapk1* gene was generated by PCR amplification from cDNA (generated as described above), using *dapk1*-specific forward (TTACAATCCTCCCATTTCCTGCAA) and reverse (ACACAATAGGCCCGATTCCC) primers.

Reaction mix:

Buffer HF	5μl
dNTPs (10mM)	0.5μl
ddH_2O	14.25μl
Phusion DNA polymerase	0.25μl
Forward primer (10μM)	1.25μl
Reverse primer (10μM)	1.25μl
cDNA	2.5μl
Total	**25μl**

Thermocycles:

Initial denaturation	98^{o}C	1.5 mins	
Denature	98^{o}C	10 seconds	x35 cycles
Anneal & extension	72^{o}C	3.5 minutes	
Final extension	72^{o}C	10 minutes	
Hold	4^{o}C	∞	

Both genes were cloned into pGEM®-T Easy (Promega) (as described in Section 2.16), transformed (as described in Section 2.9) and MiniPrep'ed (Section 2.12). They were subsequently digested from the vector using EcoRI-HF (New England Biolabs) in the following reaction:

CutSmart®Buffer	5μl
ddH_2O	3μl
EcoRI-HF	2μl
Vector DNA	40μl
Total	**50μl**

The digested DNA was run on a 1% agarose gel, and the correct sized bands excised and extracted, as per Section 2.11. The extracted genes were then cloned into pCS2+ at the EcoRI site, as described in Section 2.26.

Following ligation into pCS2+ the genes were sequenced to confirm insertions in the correct orientations. The genes were then PCR-amplified from the plasmid, using SP6 promoter (forward primer) (ATTTAGGTGACACTATAG) and M13 Reverse primer (GGTCATAGCTGTTTCCTG), in the following reaction:

Reaction mix:

Buffer HF	10μl
dNTPs (10mM)	1μl
ddH_2O	28.5μl
Phusion DNA polymerase	0.5μl
SP6 (F) primer (10μM)	2.5μl
M13 reverse primer (10μM)	2.5μl
cDNA	5μl
Total	**50μl**

Thermocycles:

Initial denaturation	98^{o}C	30 secs	
Denature	98^{o}C	10 seconds	x35 cycles
Anneal	43^{o}C	30 secs	
Extension	72^{o}C	30 secs	
Final extension	72^{o}C	10 minutes	
Hold	4^{o}C	∞	

The PCR products were purified as described in Section 2.14 and digested with NotI-HF (New England Biolabs) in the following reaction:

CutSmart®Buffer	5μl
ddH_2O	3μl
NotI-HF	2μl
Vector DNA	40μl
Total	**50μl**

The gene - flanked by a blunt end (5‘) and NotI site (3‘) - was then cloned into the expression vector - pBr-Tol2-UAS-*MCS*-mCherry (a kind gift from Masa Tada, UCL), at these sites, in the following reaction:

Ligation Buffer	3μl
Vector	(50ng)
Insert	(3x fragment number of vector)
T4 DNA Ligase	1μl
ddH_2O	(to 30μl)
Total	**30μl**

The resultant vector DNA was used to transform competent cells (see Section 2.9), MaxiPreped (see Section 2.13) and ethanol precipitated (see Section 2.15).

Purified plasmids (0.02ng) was then co-injected with *transposase* mRNA (0.02ng) (see Section 2.28) into Et(e1b:GAL4-vp16)s1101t embryos, at the one-cell-stage (see Section 2.29 for injection volume calculations).

Gene carriers were identified by fluorescent protein expression (mCherry-FP) at $\geq$2-DPF (see Section 2.30).

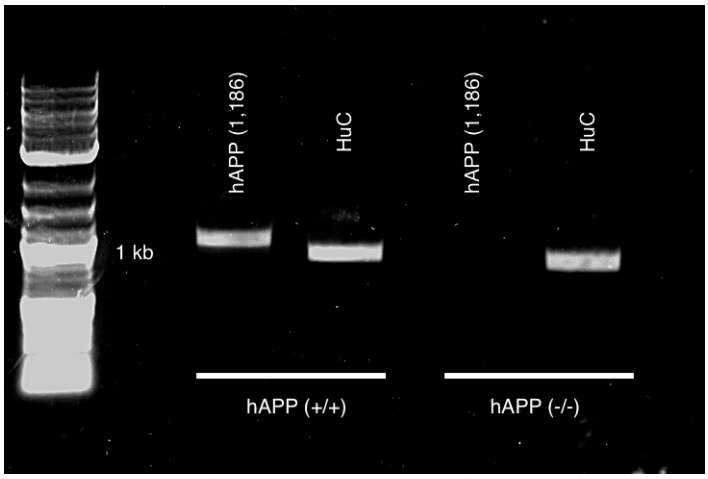

Figure 5.2: Detection of hAPP expression in transgenic fish. cDNA from Tg(HuC:hAPP;ins:eGFP) (homozygous vs wild-type) fish was amplified by PCR, using primers specific for hAPP and HuC. The hAPP gene fragment was only detected in the homozygous fish, demonstrating it's transgenic expression. The relatively similar expression of HuC (semi-quantitative) serves as a control.

5.3 Results

5.3.1 Assessing expression of hAPP$_{LON/SWE}$ transgene

In order to confirm the transgenic expression of hAPP, reverse-transcription PCR (RT-PCR) was conducted on cDNA from the brains of Tg(HuC:hAPP;ins:eGFP) fish. Results (see Figure 5.2) demonstrate that the hAPP gene was expressed in the homozygous fish, with no expression detected in the wild-type cousins. The relatively similar levels of HuC expression (semi-quantifiable by the strength of the bands) demonstrate that there were no problems in sample processing (i.e. RNA extraction, reverse-transcription, PCR), and that similar amount of cDNA were used in the PCR.

5.3.2 Effects of hAPP transgenic expression on *classical aversion* learning

In order to determine whether transgenic hAPP expression impaired amygdala-related cognitive functioning, the fish were assessed in the *classical aversion* assay (developed in Chapter 3).

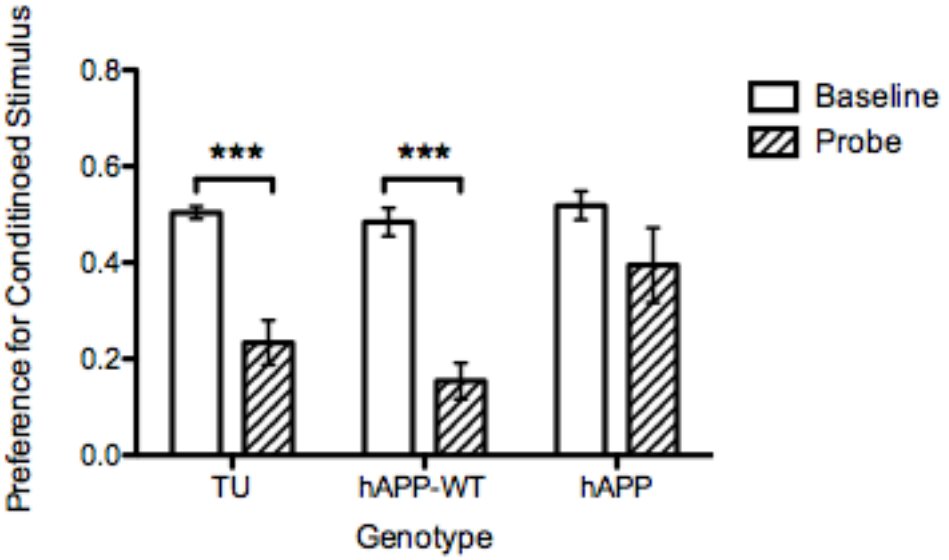

Figure 5.3: Performance of 6.5-MPF transgenic hAPP zebrafish in *classical aversion* learning. Transgenic hAPP-expressing fish were assayed using a *classical aversion* paradigm. Both wild-type lines, but not the hAPP-expressing line, significantly avoided the CS in the probe trials. Significant effects were found for genotype ($F(2,78.271)=5.278$, $p=0.007$) and trial ('baseline' vs 'probe') ($F(1,78.353)=58.114$, $p<0.0005$). A significant *genotype*trial* interaction was detected ($F(2,78.009)=4.661$, $p=0.012$; (n=81). Linear mixed effects model; ***, $p<0.001$; by two-tailed t-test with Bonferroni correction applied. Data plotted as mean ± SEM.

6.5 months post fertilisation

The effect of transgenic hAPP expression on *classical aversion learning* was initially assessed in adult zebrafish at 6.5-months post fertilisation (MPF). The results (see Figure 5.3) demonstrate that, whilst the two wild-type strains significantly avoided the CS in the probe trial, no significant aversion was observed in the transgenic strain.

3.5 months post fertilisation

Following confirmation that *classical aversion* learning is impaired in the transgenic hAPP line, a pilot study was conducted to assess whether this impairment is also observable in young adult fish (at 3-MPF). Results of this pilot indicated that no impairment was present, with all genotypes significantly avoiding the CS in the probe trials (see Figure 5.4). No inferential statistics were conducted on these data, owing to the low number of subjects used in the pilot (n=11).

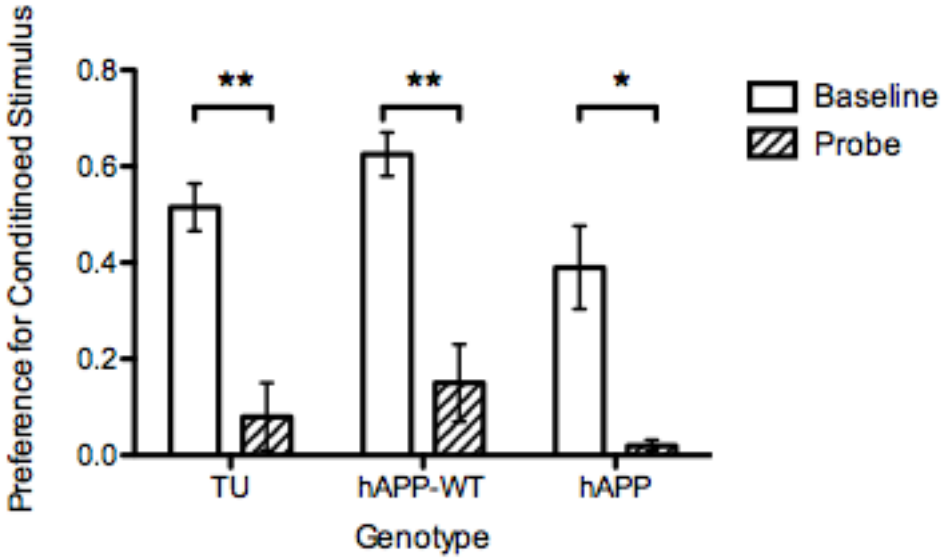

Figure 5.4: Pilot - performance of 3.5-MPF transgenic hAPP zebrafish in *classical aversion* learning. Transgenic hAPP-expressing fish were assayed using a *classical aversion* paradigm. All three genotypes significantly avoided the CS in the probe trials (n=11). *, $p<0.05$; **, $p<0.01$; by two-tailed t-test with Bonferroni correction applied. Data plotted as mean ± SEM.

Age-related effect

The above data indicates that impaired cognition in the hAPP genotype may present an age-related effect. In order to investigate this, zebrafish were assessed at three different ages - 3-MPF, 6.5-MPF, and 14.5-MPF. The results are presented in Figure 5.5.

At 3-MPF, all three genotypes significantly avoided the CS in the probe trials. However from 6.5-MPF, the transgenic hAPP line failed to display this behaviour. No significant main effect was found for *age*; however significant interactions were found for *genotype*age* and *genotype*trial*age*.

5.3.3 *pin1* expression

qPCR was conducted to assess whether *pin1* expression was affected by the transgenic expression of hAPP. Results are presented in Figure 5.6. No effect was detected in either young (3-MPF) or old (14.5-MPF) fish.

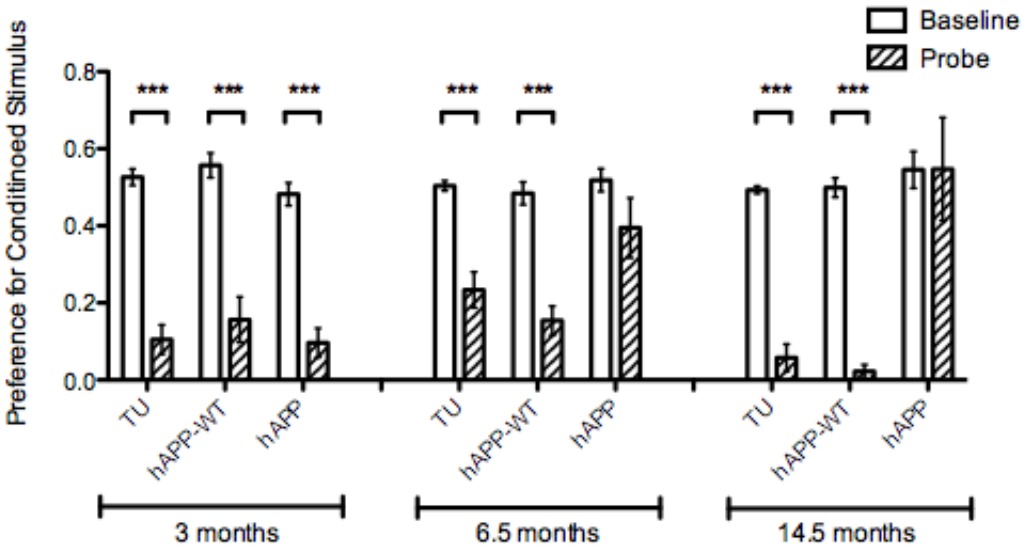

Figure 5.5: Transgenic hAPP expression induces an age-related effect on cognition. Zebrafish - both wild-type and transgenically expressing hAPP - were assessed for their performance in the *classical aversion assay* at three ages. At 3-MPF, all three genotypes significantly avoided the CS in the probe trials. However in older fish (6.5-MPF and 14.5-MPF) hAPP expression inhibited learning. Significant effects were found for *genotype* ($F(2,150.368)=8.113$, $p<0.0005$) and *trial* ($F(1,148.041)=159.398$, $p<0.0005$). No significant effect was found for *age* ($F(2,149.576)=2.375$, $p=0.096$). However significant interactions were found for *genotype*age* ($F(4,149.525)=4.675$, $p=0.001$) and *genotype*trial*age* ($F(4,147.190)=2.503$), $p=0.045$. Pairwise comparison of the genotypes found that hAPP was significantly different from TU ($p=0.002$) and hAPP-WT ($p<0.0005$). No significant difference was found between TU and hAPP-WT ($p=0.824$) (n=155). Linear mixed effects model; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; by two-tailed t-test with Bonferroni correction applied. Data plotted as mean ± SEM.

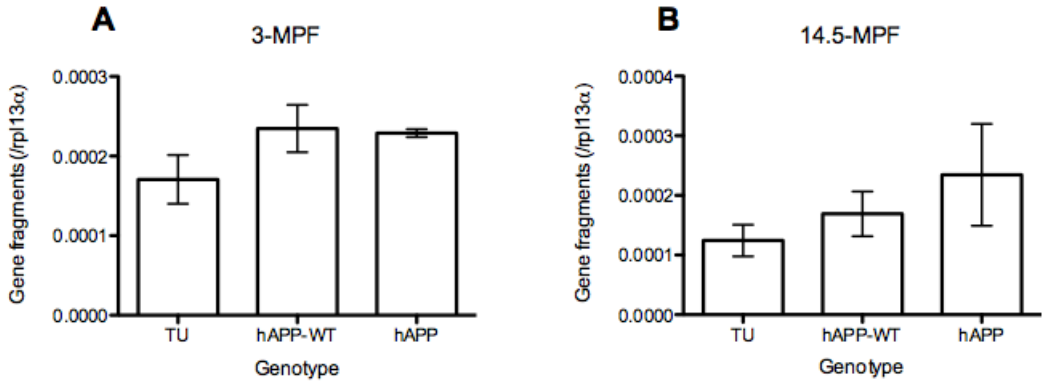

Figure 5.6: Effect of transgenic hAPP expression on *pin1* expression. The expression of *pin1* was assessed by qPCR in two wild-type lines (TU and hAPP-WT) against transgenic hAPP fish, at 3.5-MPF (A) and 14.5-MPF (B). No significant difference was detected at either age - 3.5-MPF ($F(2,10)=1.618$, $p=0.246$); and 14.5-MPF ($F(2,10)=0.993$, $p=0.404$) (n=15). By one-way ANOVA. Data plotted as mean ± SEM.

5.3.4 Neurodegeneration

In order to assess whether transgenic expression of $hAPP_{LON/SWE}$ causes neurodegeneration, Fluoro-Jade® B staining was conducted on telencephalic sections of zebrafish brains.

Results are presented in Figure 5.7 (larger images are presented in the Appendix, see Section 9.3). An increase in fluorescent staining indicates increased neurodegeneration in the brains of transgenically-expressing $hAPP_{LON/SWE}$ fish. However, due to the inconsistent quality of the staining, these data are regarded as preliminary and by no means confirmative.

5.3.5 Developing new transgenic lines of AD-related neurodegeneration

Whilst a phenotype was detected in the Tg(HuC:hAPP) line, one of the issue in AD-related translational neurobiology has been a lack of effective models (discussed in the *Introduction*; see Section 5.1.1). Additionally, given the range of molecular dysregulations which result in Alzheimer's-like phenotypes, it is apparent that a range of mutant models is required to investigate the different diseases of AD (discussed in *General Introduction*; see Section 1.1.2). Thus, new gene manipulation lines were generated. Whilst the assessment

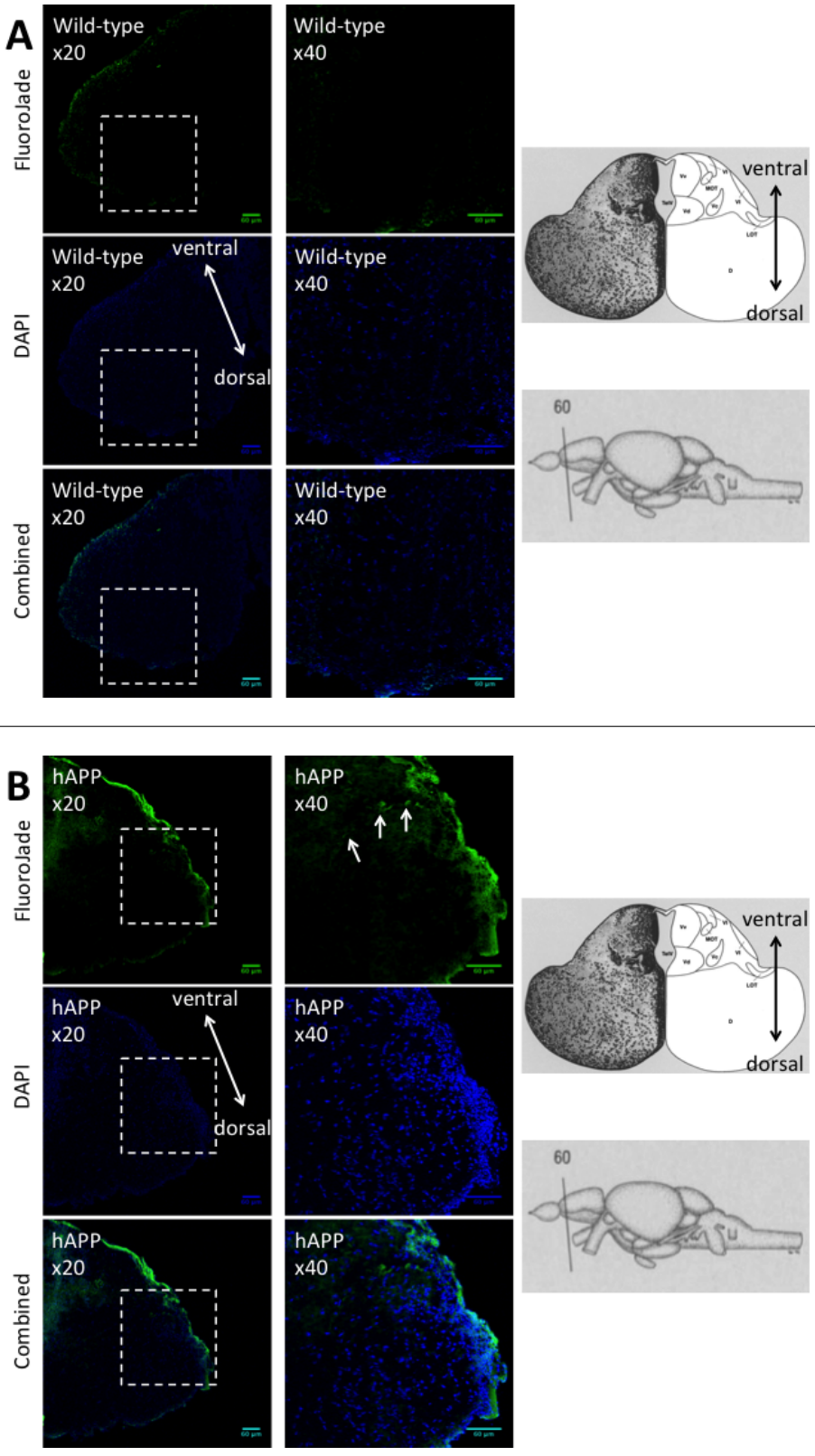

Figure 5.7: Neurodegeneration in transgenic $\mathbf{hAPP_{LON/SWE}}$ line. FluoroJade® B staining was conducted on 10μm sections of zebrafish telencephalon. Increased staining is apparent in brains in transgenic $hAPP_{LON/SWE}$-expressing brain (B) compared to wild-type brains (A). (Larger images are included in Appendices; Section 9.3). Approximate positions of brain sections are indicated on the right-hand side (both A & B) (images from Wulliman et al. 2012).

of these lines is not within the time-frame allowed for this thesis, they may provide a useful tool for future research in this area. Two genes were chosen for manipulation - $A\beta_{40}$ and *dapk1*.

Embryos were injected at the one-cell stage (into the cell), then reared as per standard protocols. At 2-DPF they were checked for fluorescent protein expression, as a marker of transgene expression. Images of un-injected and injected embryos are presented in Figure 5.8. These demonstrate that expression of the transgenes were successfully driven by the Et(e1b:GAL4)s1101t line.

5.4 Discussion

Assessment of the transgenic $\text{hAPP}_{\text{LON/SWE}}$ zebrafish line found expression of the transgene via RT-PCR. This confirms that the full-length protein is transcribed in the adult fish.

With regard to a behavioural phenotype, 6.5-MPF fish displayed impaired cognition in the *classical aversion assay*. However, no effect was found at 3.5-MPF. In light of this, an age-effect was investigated. The results of this analysis found that impairment to *classical aversion* is present in old ($\geq$6.5-MPF), but not young (3-MPF) fish. This is in-keeping with rodent studies, which have also found age-related cognitive impairments resulting from the transgenic expression of mutated hAPP (Chen et al. 1998; Kelly et al. 2003; Van Dam et al. 2003).

No significant effect was found for *pin1* expression, indicating that homeostatic regulation of amyloid toxicity does not feedback to this neuroprotection pathway. However, this assessment of mRNA expression is not indicative of the functional activity of *pin1*. Thus, further investigation is necessary to elucidate whether APP affects the phosphorylation of *pin1*.

Additionally, an increase in neurodegeneration is apparent in $\text{hAPP}_{\text{LON/SWE}}$ fish. However, there is evidence that neurodegeneration is not causative in cognitive deficits of transgenic rodents (Stein and Johnson 2002). Therefore, future assessment of the transgenic zebrafish line may wish to investigate the effect of hAPP on dendritic spines - a morphological dysfunction previously found in hAPP-expressing mice - in the transgenic fish, at a younger age (Irizarry et al. 1997; Stein and Johnson 2002).

In development of new genetic models of AD, two over-expression zebrafish lines have been generated. The **hA**β (trangenic over-expression) is not expected to induce

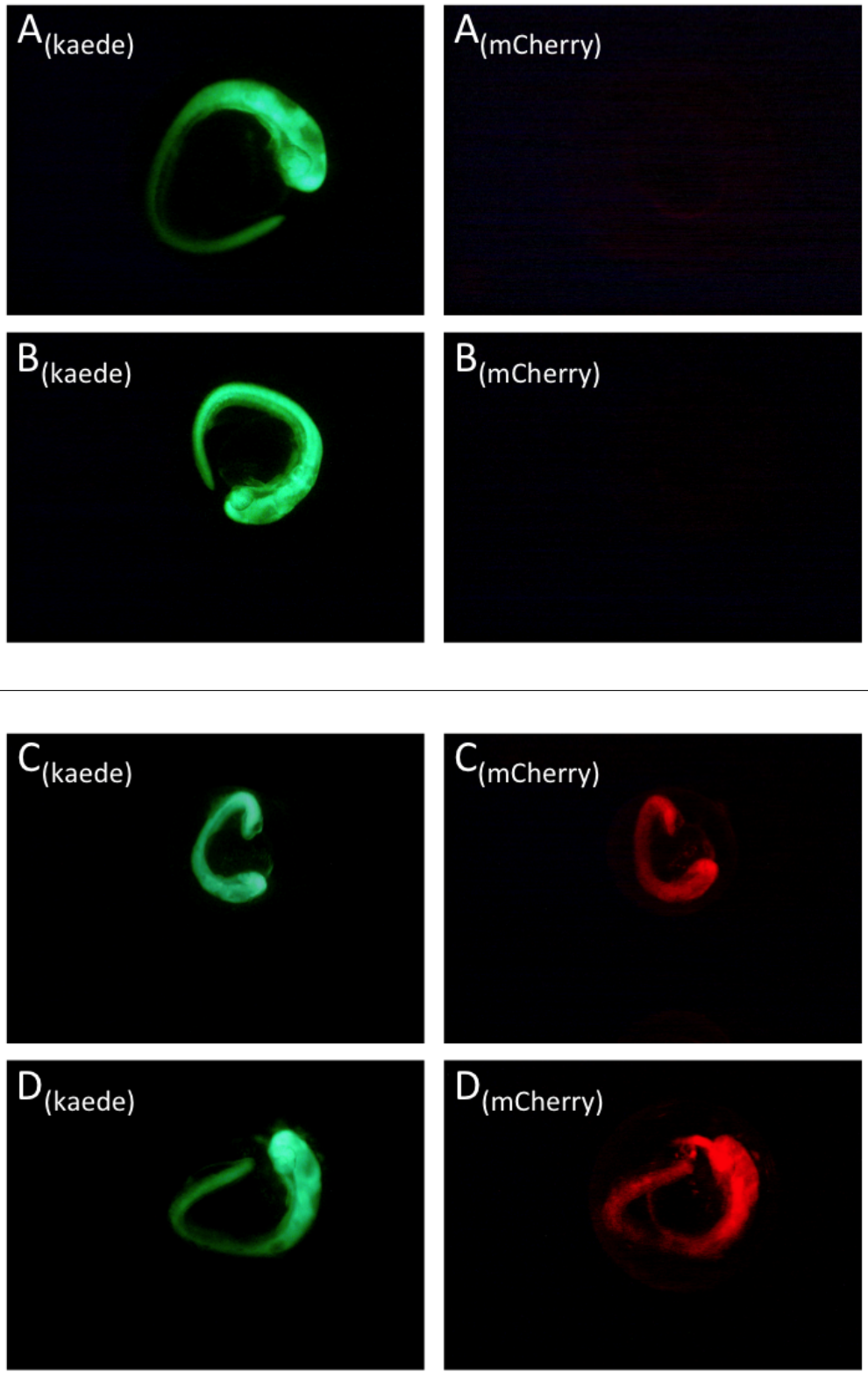

Figure 5.8: Expression of transgenes in embryos following injection. Zebrafish embryos were injected with expression plasmid and *transposase* mRNA (0.02ng of each in Danieau Buffer) at the one-cell stage. Un-injected controls show *kaede* expression, but no mCherry-FP (A & B). Injected embryos show expression of both *kaede* and mCherry-FP (as a marker of transgene expression) (C & D).

neurotoxicity or significant effects on cognition. However it may provide insight into the effects of pharmacological interventions which aim to modulate γ-secretase activity (i.e. which reduce Aβ_{42} cleavage and induce higher Aβ_{40} cleavage). The ***dapk1*** (endogenous over-expression) is an entirely new tool for investigation, as no stable gene-manipulation lines have been developed in any experimental organism. However, previous research into aberrant DAPK1-PIN1 signalling consequences - both *in vitro* and *in vivo* - indicate that a *dapk1* over-expression (as developed here) would result in neurodegeneration in tandem with amyloidogenesis and tau hyperphosphorylation (e.g. Bialik and Kimchi 2011; Bulbarelli et al. 2009; Kim et al. 2014; Lee et al. 2011; Wu et al. 2011a). Thus, whilst the analysis of these lines is beyond the time allowance of the current thesis, there is sufficient knowledge of these genes to formulate hypotheses in future studies.

Finally, as the central theme of this thesis is a facilitation of translational neurobiology, neuroprotective compounds must be regarded. In this regard, two extremely common drugs have repeatedly been associated with anti-apoptotic function in the CNS - nicotine and caffeine.

Nicotine has been shown to protect against Aβ toxicity in primary neuronal culture (e.g. Kihara et al. 1997; Liu and Zhao 2004). Several nicotinic receptors have been associated with facilitating this signalling, including $\alpha4\beta2$, $\alpha7$ (Kihara et al. 1998; Shaw et al. 2002). This is particularly interesting in light of the extensive reporting that cholinergic pathways are particularly vulnerable in early stages of AD progression (e.g. Collerton 1986; Coyle et al. 1983; Francis et al. 1999; Terry and Buccafusco 2003). Additionally, a periodontal study found that smokers express higher levels of PIN1 (in gingival biopsies), suggesting that it may affect the DAPK1-PIN1 pathway investigated in the current study (Cho et al. 2015).

Caffeine has been reported to exert neuroprotective properties - both *in vitro* and *in vivo*, as well as reducing Aβ in hAPP$_{\mathrm{SWE}}$-expressing mice (Arendash et al. 2006; Arendash et al. 2009; Rosso et al. 2007; Zamani et al. 1997). This mode of neuroprotection has been associated with adenosine A_{2a} receptor activity, which apparently facilitates this mechanism (Dall'lgna et al. 2003; Dall'Igna et al. 2007; Rosso et al. 2007; Ribeiro et al. 2002).

Whilst these two compounds exert neuroprotection, they have both been extensively investigated in light of their addictive properties (e.g. Benowitz 2010; Griffiths and Mumford 1996; Pohler 2010; Stolerman and Jarvis 1995; Swanson et al. 1994). Additionally, findings that caffeine and nicotine are often co-self-administered indicates that they may facilitate each others reinforcing mechanisms (e.g. Swanson et al. 1994). This suggests that the neuroprotective properties of these two compounds may facilitate one-another. Thus, there appears to be an association between AD-related

neuroprotective mechanisms and addictive mechanisms. In this light, it is highly necessary to screen neuroprotective compounds for reinforcing properties - a key stage of drug development which also incurs high ethical and economic costs.

Chapter 6

Assessing the Utility of Zebrafish Conditioned Place Preference as a Model for Abuse Potential

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6.1 Introduction

As mentioned earlier in this thesis, uncovering neuroprotective signalling mechanisms - and discovering pharmacological interventions which target these mechanisms - is a key goal in neurodegeneration research. However, the fact that these compounds target the CNS presents many problems in relation to adverse effects on brain functioning. Notable amongst these problems is the potential reinforcing properties of compounds; this is a particular concern in AD treatment, as the cholinergic system - damaged early in AD (nAChR-α7 has been reported to directly interact with Aβ peptides; see Wang et al. 2000b) and the target of most current therapies - is heavily involved in reward signalling pathways (discussed in Section 6.1.1, below). This chapter will begin by reviewing common 'drugs of abuse' with reported neuroprotective properties, describing the mechanisms by which they are understood to act. With regard to their abuse potential, these drugs will then be assessed for their ability to induce drug-seeking behaviour in zebrafish (via a CPP paradigm). These results will be discussed in relation to findings from studies of human subjective experience, assessing the potential utility of zebrafish CPP to model human drug reward and abuse potential.

6.1.1 Reward signalling

The mesocorticolimbic system is often regarded as the '*reward pathway*' of the mammalian brain. This involves DAergic projections emanating from the VTA, ascending to the NAcc (mesolimbic) and medial pre-frontal cortex (mPFC) (mesocortical), as well as descending projections to the DRN (Figure 6.1a; see Ikemoto 2010; Peyron et al. 1995). In addition to DAergic, GLUergic neurons - identified by *in situ* expression of VGluT2 mRNA - have been found in these pathways (e.g. Yamaguchi et al. 2011).

Similarly, serotonin signalling has been heavily implicated in reward signalling, with various 5-HT receptor subtypes expressed throughout midbrain, cortical and limbic structures (see Hayes and Greenshaw 2011). Midbrain projection from the raphe nuclei (i.e. the dorsal and median nuclei; DRN and MRN, respectively) - extend to the forebrain, VTA and NAcc (see Hu 2016; Mylecharane 1995). The DRN sends efferents to the NAcc, mPFC and VTA; the MRN projects to regions of the forbrain distinct to those with DRN afferents (see Figure 6.1b; De Deurwaerdère et al. 1998; Van Bockstaele et al. 1993; Vertes 1991; Vertes et al. 1999).

GABA signalling functions as the principal inhibitory mechanism of the adult brain (Davies 2003). GABA-Rs in the VTA (both $GABA_A$ and $GABA_B$) have been implicated in modulating DA release in the NAcc, whilst $GABA_A$ has been shown to interface between

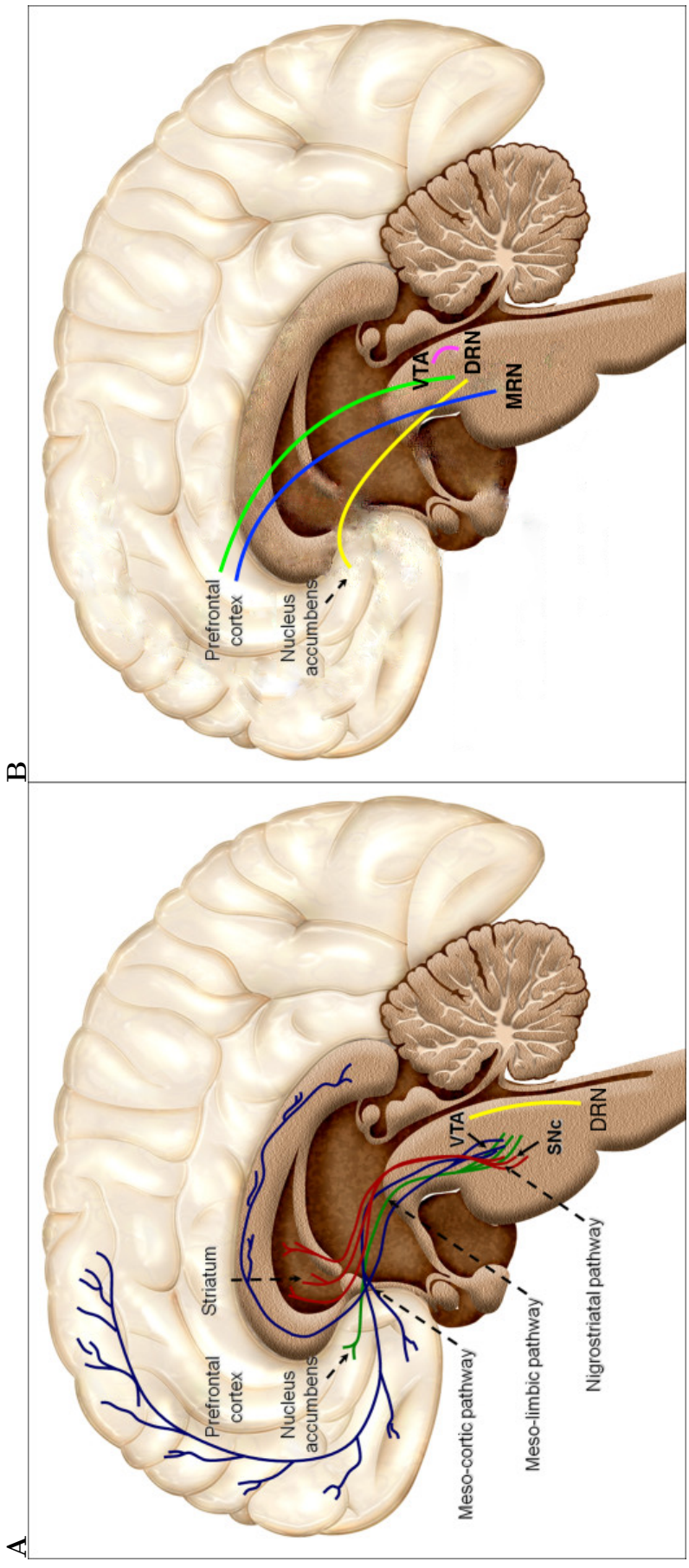

Figure 6.1: Pathways involved in reward signalling. (A) The mesocorticolimbic system is made up of two sets of DAergic projections, originating in the VTA; the mesolimbic system (green) projects to the NAcc, whilst the mesocortical pathway (purple) projects to the PFC. DAergic projections from the VTA have also been found to extend to the DRN (yellow). Additionally, the nigrostriatal pathway (red) extends from the substantia nigra (SNc) to the striatum. (B) Serotonergic pathways project from the DRN to the VTA (purple), NAcc (yellow) and PFC (green); and from the MRN to the PFC (blue). (Images adapted from Arias-Carrión et al. 2010)

DAergic and non-DAergic mechanisms in the VTA, modulating signalling between them (Laviolette and Van Der Kooy 2001; Suaud-Chagny et al. 1992; Xi and Stein 1998). Interestingly, GABAergic neurons have also been found to project from the VTA to the PFC, whilst GABA (but not DA) is reportedly involved in signalling from the NAcc to the PFC, facilitating communication between the two key structures of reward signalling (Carr and Sesack 2000a; Carr and Sesack 2000b). Thus, whilst not fully elucidated, the importance of this neurotransmitter in regulating mesocorticolimbic reward signalling is evident.

Like GABA-Rs, opioid receptors are inhibitory. They are widely expressed throughout brain structures, including the VTA, NAcc, FC, DRN, and MRN (see Ding et al. 1996; Le Merrer et al. 2009; Xia and Haddad 1991). Opioid receptors in the DRN are reported to modulate local serotonin release, indicating a mechanism of opioid reward pharmacology (Tao and Auerbach 2002). However DA receptors appear to facilitate opiate reward signalling, as D_2-null mice do not display opiate-induced CPP (Maldonado et al. 1997). This highlights the importance of DA in reward signalling - even where the principal target of a compound is not a DA-R - as well as the complex interactions between neurotransmitter systems required to facilitate this mechanism.

NMDA-R have also been reported throughout the reward circuitry, including the VTA, NAcc, PFC, DRN and MRN (see Covington III et al. 2008; De Kock et al. 2006; Petralia et al. 1994; Rodriguez et al. 2000; Takita et al. 1997; Tao and Auerbach 1996). NMDA signalling in the VTA has been found to increase DA excretion in the NAcc (Suaud-Chagny et al. 1992). Further, NMDA-R activity in the DRN and MRN has been shown to trigger 5-HT release locally, whilst DRN activity also increases its secretion in the NAcc (De Kock et al. 2006; Tao and Auerbach 1996).

The mesocortical pathway has also been shown to receive activating (or at least modulating) signals from cholinergic inputs to VTA DAergic neurons (Omelchenko and Sesack 2006). Thus, cholinergic signalling appears to play a role in triggering reward signalling, likely in combination with other neurotransmitter mechanisms.

The fact that NMDA and cholinergic mechanisms affect (or even instigate) reward signalling is of particular relevance to the current thesis, as all drugs currently approved to treat AD symptoms target cholinergic or NMDA signalling. Three drugs (donepezil, rivastigmine and galantamine) inhibit the breakdown of ACh by AChE, prolonging its action (Arias et al. 2005; Nordberg and Svensson 1998). The fourth drug (memantine) antagonises NMDA receptors, inhibiting glutamate over-activity and subsequent excitotoxicity (Erdö and Schäfer 1991; Reisberg et al. 2003; Volbracht et al. 2006).

In this light, it must be mentioned that AChE inhibition (pre-treatment, by donepezil

- the most commonly prescribed AD drug) has been reported to actually inhibit nicotine-induced reinforcement (measured by SA) in rodents (Kimmey et al. 2014). However, in light of the variegated natures of cholinergic receptor complexes and their signalling mechanisms, it is evident that other compounds affecting this neurotransmitter have the inverse effect. For example, application of a muscarinic agonist has been shown to increase both DA and 5-HT in the VTA, as well as increasing DA in the NAcc and FC (Gronier et al. 2000). Indeed (although not a commonly held hypothesis), it has been proposed that, whilst both types of AChR's are involved in triggering VTA-driven reward signalling, mAChR's play a greater role than nAChR's (Yeomans and Baptista 1997). Thus, whilst clearly heavily involved in the modulation of reward signalling, the specific functions of muscarinic and nicotinic receptors (in their various receptor isoforms) is still not entirely elucidated.

Additionally, several reinforcing compounds which do not target cholinergic pathways have been found to exert neuroprotective properties (discussed below in Section 6.1.2). Thus, the necessity to screen CNS-acting neuroprotective drugs for their *abuse potential* (reinforcing effects) is extremely important to the safety pharmacology stages of drug development (particularly in relation to AD).

6.1.2 Neuroprotective drugs with reinforcing properties

Stimulants

Nicotine has a well characterised pharmacology. It affects cognitive functions and emotional state via activation of nAChRs in the DAergic neurons of the mesolimbic system and related structures, leading to activation of cells within this pathway (D'souza and Markou 2011). Several receptor subtypes have been associated with facilitating this mesolimbic activation, including $\alpha 4$, $\alpha 6$, $\alpha 7$ (Besson et al. 2012; Sanjakdar et al. 2015; Tapper et al. 2004). With regard to rewarding behaviour, nicotine has been extensively reported to cause drug-seeking responses, inducing SA in humans and rodents, and CPP in both rodents and zebrafish (Donny et al. 1995; Fudala et al. 1985; Kedikian et al. 2013; Kily et al. 2008; Perkins 1999b; Rose and Corrigall 1997; Vastola et al. 2002; Walters et al. 2006).

Nicotine has been demonstrated to trigger neuroprotective signalling against amyloid toxicity, which is also mediated by nAChRs (requiring the $\alpha 7$ subunit), resulting in decreased Aβ aggregation (Arias et al. 2005; Jonnala and Buccafusco 2001; Liu et al. 2007a; Shimohama 2009). In light of this, findings from a rodent study that nicotine CPP is mediated by the $\beta 2$, but not the $\alpha 7$ subunit (determined by both antagonism

and genetic deletion), indicate that the rewarding and neuroprotective consequences of nicotine pharmacology occur by distinct signalling mechanisms (Walters et al. 2006). However, findings that the SA-aquired nicotine reinforcement actually requires the $\alpha 7$ subunit demonstrate the complexity of nicotine reward signalling, suggesting that $\alpha 7$ is necessary for the active (SA), but not passive (CPP) aquisition of reward. Additionally, nicotine has also been shown to increase PIN1 expression, potentially triggering the downstream neuroprotective properties of this protein (Cho et al. 2015).

Caffeine is an antagonist of the adenosine receptors - A_1, A_{2A}, A_{2B}, and A_3. In the CNS, the adenosine receptors are modulatory, with A_1 facilitating inhibition and A_{2A} facilitating excitation. Thus, the A_{2A} receptor mediates caffeine's affect on arousal (Huang et al. 2005). These two receptors exert counterbalancing tonal homeostatic functions - antagonism of A_1 has been shown to facilitate LTP; a function inhibited by A_{2A} antagonism (Costenla et al. 2010; Ribeiro and Sebastiao 2010).

The rewarding effects of caffeine result from an increase in DA secretion in the NAcc, a phenomenon likely mediated by antagonism of the A_1 receptor (Solinas et al. 2002). Its reinforcing properties are well established, inducing SA in both humans and rodents, as well as CPP in rodent paradigms (e.g. Bedingfield et al. 1998; Hughes et al. 1993; Liguori and Hughes 1997; Nehlig 1999).

In relation to neuroprotection, caffeine has been shown to rescue 'normal' age-related cognitive decline in rodents (Costa et al. 2008). It increases neuronal survival by decreasing caspase 3 activity in a rodent model of PD; although evidence shows that high concentrations lead to increased caspase 3-induced apoptosis (Kang et al. 2002; Nakaso et al. 2008). Additionally, AD patients have been found to intake significantly less caffeine than age-matched control subjects, although no causality is discernible from those data (Maia and De Mendonça 2002; Ribeiro and Sebastiao 2010). In this regard, antagonism of the A_{2A} receptor subtype has been associated with neuroprotection in several models of neurodegenerative disorders (including AD), highlighting the potential role that excitotoxicity may play in these diseases (Gomes et al. 2011).

Alcohol

The neuropharmacology of *ethanol* reward is relatively 'dirty', exerting effects on several receptor targets within the mesolimbic and mesocortical pathways. A commonly researched mechanism is EtOH's modulation of both $GABA_A$ and $GABA_B$ receptors (Davies 2003; Grobin et al. 1998; Koob et al. 1998; Liang et al. 2006). It has been shown to increase $GABA_A$-regulated ionic currents, with a similar effect on another major inhibitory receptor - glycine (Aguayo 1990; Celentano et al. 1988; Davies 2003; Lynch

2004; MIHIC 1999; Nestoros 1980). In light of GABA's modulation of DA signalling (discussed above), EtOH's activation of these receptors has an implicit role in reward signalling. Further, findings that glycine signalling modulates GABA signalling from the NAcc to the VTA (feedback from DAergic signalling in the other direction) demonstrate the importance of glycine in reward mechanisms (see Harvey and Yee 2013).

In addition to these indirect increases in DA, EtOH has been found to directly stimulate DAergic neurons in structures of the mesocorticolimbic pathways, including the VTA, NAcc and mPFC (Brodie et al. 1990; Brodie et al. 1999; Ding et al. 2011; Gessa et al. 1985; Weiss et al. 1993; Yoshimoto et al. 1992). Interestingly, EtOH's direct action on the NAcc has also been shown to feed in to the VTA where it activates nAChRs, which subsequently feed back to increase NAcc DA (Ericson et al. 2003; Ericson et al. 2008).

Despite the aforementioned increase in pre-frontal DA, EtOH has been demonstrated to decrease signalling in the PFC (Tu et al. 2007). This effect is due to its inhibition of ionic currents at NMDA-Rs (Davies 2003; Hoffman et al. 1989; Hoffman et al. 1990; Lovinger et al. 1989; Nagy 2008; Weitlauf and Woodward 2008). To add to this array of pharmacological consequences, EtOH also increases 5-HT in the NAcc, as well as inhibitting its clearance from the brain (Daws et al. 2006; Yoshimoto et al. 1992).

Regarding drug-seeking behaviours, EtOH has been extensively reported to induce SA and CPP in rodent studies (e.g. Bozarth 1990; Cunningham and Noble 1992; Cunningham et al. 2000; Rassnick et al. 1992; Spanagel et al. 1995). It has also been demonstrated to induce CPP in zebrafish, supporting the current investigation into the range of drugs which induce CPP in this model organism (Collier et al. 2014; Kily et al. 2008).

The detrimental consequences on the CNS of excessive, chronic EtOH consumption has received much research attention (e.g. Crews et al. 2004; Gazdzinski et al. 2005; Lieber 1991; Morris et al. 2010b). Further, there is evidence that EtOH's effects on NMDA and $GABA_A$ receptors causes apoptosis in the developing brain (e.g. Ikonomidou et al. 2000). However findings that (moderate levels of) EtOH are neuroprotective has been reported in relation to several manifestations of CNS atrophy. For example, EtOH's activations of GABA and HIF1α signalling have been associated with neuroprotective signalling in rodent models of ischaemia-reperfusion and ischaemia brain injuries (Qi et al. 2009; Wang et al. 2012). Moderate EtOH pre-exposure has been associated with decreased risk of AD and other dementias by reducing toxicity from pro-neuroinflammatory proteins, including Aβ (Brust 2010; Collins et al. 2010). Interestingly the protection against Aβ has been associated with an increase in NMDA-R activity; an effect inhibited by memantine (the NMDA-R antagonist currently prescribed to treat AD!) (Mitchell et al. 2009).

Cannabinoids

Of the many cannabinoids currently identified, Δ^9-tetrahydrocannabinol (THC) is the most commonly researched compound, largely due to its psychoactivity. It is a partial agonist of both CB_1 and CB_2, with CB_1 activity found to produce the psychoactive consequences of THC exposure (Pertwee 1988; Pertwee 2008; Svíženská et al. 2008). CB_1 also appears to facilitate the reinforcing effects of THC, by inducing DA release in the VTA and PFC (Diana et al. 1998; French 1997). Non-human primates (NHP) have been demonstrated to self-administer THC, but evidence indicates that rodents do not (Justinova et al. 2003; Wakeford et al. 2016). The zebrafish Dc is small relative to the rodent PFC, suggesting that THC would not be reinforcing in this model (see Section 6.1.4 for conservation of these structures). However, should the principle site of THC reward be the mammalian VTA, it is possible that its action on the zebrafish PTN may achieve this signalling. Thus it is necessary to directly investigate the presence of THC-induced reward signalling in the zebrafish brain.

CB_1 (activated by the endocannabinoid CB_1 agonist, 2-Arachidonoyl glycerol) triggers neuroprotective consequences via inhibition of NF-κB (Panikashvili et al. 2001; Panikashvili et al. 2005). CB_1 activity has been shown to reduce glutamate-induced excitotoxicity (Shen and Thayer 1998). In relation to age-related neurodegeneration, AD patients present decreased neuronal expression of CB_1 (Ramírez et al. 2005). Both CB_1 and CB_2 are expressed in amyloid plaques, and co-localise with molecular markers of (degenerative) microglial activation; microglial activation was found to be attenuated by agonists of both cannabinoid receptors (Ramírez et al. 2005). THC has been shown to inhibit AChE (the same function performed by three of the four currently-prescribed AD treatments), reducing its potential to facilitate aggregation of Aβ (Eubanks et al. 2006).

THC has also been found to protect neurons in an *in vitro* model of PD, via CB_1-independent signalling (Carroll et al. 2012). It attenuates MS-associates neuroinflammation, neurodegeneration, spasticity and tremor (Baker et al. 2000; Pertwee 2002; Pryce et al. 2003; Ungerleider et al. 1988). CB_2 is reportedly up-regulated in a rodent model of ALS ($SOD1^{G93A}$), and THC treatment has been found to delay the onset of symptoms and prolong longevity in this model (Moreno-Martet et al. 2014; Raman et al. 2004).

Additionally, other (non-psychoactive) cannabinoids have been identified, which may prove more socially acceptable as treatments for neurodegeneration. For example, cannabidiol (CBD) has been found to reduce Aβ toxicity, whilst reducing a range of neurodegenerative consequences including ROS production, caspase3 expression and Ca^{2+} dysregulation (Iuvone et al. 2004). The fact that CBD is an antagonist of both CB_1 and CB_2 demonstrates the complexity of cannabinoid signalling and its potential as

a target of neuroprotective pharmacological intervention (Pertwee 2008).

Anaesthetics

Anaesthetics present a class of drug to which many people are exposed at some point their lifetimes. Whilst exposure to *general anaesthetics* is somewhat specific to surgical procedures, *local anaesthetics* are commonly used in dental treatment practices. However the abuse potential of both anaesthetic subcategories has been reported.

Ketamine is commonly regarded as a general anaesthetic, although local anaesthetic properties have been reported (Dowdy et al. 1973; Durrani et al. 1989). It is a non-competitive NMDA-R antagonist (i.e. it reduces the maximal activity of NMDA responses). The inhibition of this excitatory receptor results in a range of acute, reversible cognitive impairments related to PFC functioning, including memory and attentional deficits, psychological dissociation and psychosis (in a manner paralleling certain characteristics of schizophrenia) (Malhotra et al. 1996; Moghaddam et al. 1997; Verma and Moghaddam 1996).

Additionally, ketamine has been found to directly increase DA secretion in the PFC (Verma and Moghaddam 1996). Interestingly, following repeated exposure to ketamine, despite increases in basal DA, the acute increase in DA is attenuated (Lindefors et al. 1997). However the authors reported an increase in acute serotonin activity in the mPFC. Similarly, it has been reported that ketamine increases 5-HT signalling in the DRN (Tso et al. 2004). These enhancements of serotonergic mechanisms are associated with the antidepressant and anxiolytic effects of ketamine (Gigliucci et al. 2013).

Ketamine also (weakly) agonises both μ- and κ-opioid receptors (Hirota et al. 1999). Its stronger affinity for μ-opioid receptros may further explain the reinforicing properties of ketamine, as this induces NAcc DA release (which is countered by κ-opioid receptors, as discussed below in Section 6.1.2; see Hirota and Lambert 1996; Hustveit et al. 1995; Spanagel et al. 1992). In rodent studies it has been found to be self-administered, as well as inducing CPP (De Luca and Badiani 2011; Du et al. 2017)

Ketamine's antagonism of NMDA-Rs has also been found to protect CNS neurons in rodent models of ischaemia and epileptic seizures (Church et al. 1988; Fujikawa 1995). In this regard, it is worth noting that other NMDA-R antagonists (e.g. the AD drug memantine and the PD drug amantadine) have also been reported to be protective against various CNS insults, including hypoxia, excitotoxicity and ischaemia (Danysz and Parsons 2003; Kornhuber et al. 1993; Miguel-Hidalgo et al. 2002; Weller et al. 1993). Additionally, the positive effects of ketamine on the cardiovascular system has been proposed to improve

blood-flow to the brain (Himmelseher and Durieux 2005).

PCP is also a NMDA-R antagnost, however its pharmacology is much 'cleaner' than that of ketamine. Thus, the above-mentioned consequences of ketamine's antagonism of NMDA-Rs is true of PCP, including subsequent modulation of DAergic and serotonergic pathways, as well as the induction of psychosis (see Kapur and Seeman 2002). In rodents it is self-administered, and produces CPP (Marglin et al. 1989; Marquis et al. 1989).

PCP has been found to exert some neuroprotective effect on cerebral ischaemia, via inhibition of creatine kinase release, an enzyme over-active in PD patients (Lu et al. 1992; Takubo et al. 2003). Interestingly, under-expression of creatine kinase has been associated with both AD and HD pathologies, exemplifying the distinct molecular dysregulations which occur in different neurodegenerative conditions (Aksenov et al. 1997; Kim et al. 2010).

Procaine is a local anaesthetic which has been widely utilised by dental practitioners in peripheral tissue, due to its inhibition of Na^+ channels (Wagner and Ulbricht 1976).

Within the CNS, procaine modulates DA secretion in a similar manner to cocaine (Hernandez et al. 1991). Additionally, it has also been shown to inhibit NMDA currents *in vitro*, apparently acting on the CNS in a manner similar to ketamine and PCP (Nishizawa et al. 2002). It induces SA in NHPs, however there is no evidence that it is reinforcing in rodent models (Beardsley and Balster 1993; Ford and Balster 1977; Gong et al. 1996; Woolverton and Balster 1979).

Procaine has also been found to protect neurons against Aβ toxicity, a function possibly facilitated by its inhibition of glutamate excitotoxicity and mitochondrial dysfunction (Lecanu et al. 2005). It may also protect against traumatic neuronal injury via inhibition of HSP-70 (Tunc et al. 2002).

Antidepressants

Bupropion (weakly) inhibits re-uptake of norepinephrine and DA, and has been shown to increase DA in the NAcc (Ascher et al. 1995). It induces rodent SA and CPP, and is abused by humans (e.g. Baribeau and Araki 2013; Ortmann 1985; Reeves and Ladner 2013; Tella et al. 1997).

Bupropion also appears to exert neuroprotection against excitotoxicity in a manner relevant to epileptic seizures, which the authors linked to its inhibition of glutamate excitotoxicity (Lin et al. 2011; Lin et al. 2013). Further, it has been reported that

bupropion induces an up-regulation of the neuroprotective SOD1, a possible mechanism in this protective effect (Li et al. 2000). It has also been found to protect against glaucoma, a function linked to its "anti-TNF" properties (Stein et al. 2014).

Opiates

Opiates agonise the opioid receptors - δ, κ, μ, ζ, and nociceptive receptor. μ-opioid receptors have been demonstrated to positively modulate DAergic secretion in the NAcc, whilst κ-opioid receptors have the inverse effect (Spanagel et al. 1992). The neuroprotective properties of opiates are largely believed to be facilitated by δ receptors . However antagonism of μ-opioid receptors has also been found to induce neuroprotection, suggesting that this receptor plays a role similar to the balance between adenosine receptors A_1 and A_{2A} in a counterbalanced modulation of both these phenomena (see Section 6.1.2; Liao et al. 2003).

Morphine acts by stimulating μ-opiate receptors, resulting in increased DA secretion in the NAcc (Di Chiara and Imperato 1988). Rodent studies have demonstrated that microinjection into the VTA increases DA in the NAcc, as well as inducing CPP (Leone et al. 1991; Mucha and Herz 1985; Phillips and LePiane 1980). It is self-administered by rodents, and has also been shown to induce CPP in zebrafish (Glick et al. 1991; Lau et al. 2006).

Morphine has been found to modulate ubiquitination in a neuroprotective manner, via the production of NO (Rambhia et al. 2005). It appears to offer some protection against Aβ neurotoxicity via NO, as well as downstream modulation of caspases -2 and -3 (possibly by CaMKII) (Lin et al. 2004; Pak et al. 2005). Pre-exposure has also been shown to protect against ischaemia (Zhao et al. 2006).

Fentanyl is also a μ-opioid agonist, although with greater affinity than morphine. It is self-administered by both NHPs and rodents, and it induces rodent and zebrafish CPP (Broadbear et al. 2004; Morgan et al. 2002; Mucha and Herz 1985; Nishida et al. 1989; Stewart et al. 2015).

Kuzak and colleagues criticised the lack of adequate fentanyl use in an emergency medical procedure as, despite clear indications of neuroprotective benefits, few patients are administered this drug (Kuzak et al. 2006). Fentanyl has also be found to protect against ischaemic damage in a rodent model (Wang 2006).

6.1.3 Neuroprotective drugs without reinforcing properties

Additionally, two neuroprotective drugs were included in the screen as negative controls for reward signalling.

Atomoxetine is a treatment for attention deficit hyperactivity disorder (ADHD), a condition largely associated with PFC dysfunction (Arnsten 2006). It inhibits re-uptake of DA, 5-HT and norepinephrine, thereby prolonging their signalling in the PFC (Bymaster et al. 2002; Ding et al. 2014). It increases cortical cholinergic signalling, which is associated with an increase in cognitive function (Tzavara et al. 2006). However, atomoxetine is not considered to have significant abuse liability in humans (Jasinski et al. 2008). It does not affect DA in the NAcc and does not induce CPP in rodents (Bymaster et al. 2002; Peña et al. 2011; Swanson et al. 2006).

Atomoxetine has been demonstrated to improve functional recovery in rodent traumatic brain injury (TBI) and spinal cord injury (SCI) (Hou et al. 2016; Reid and Hamm 2008). It has been shown to be neuroprotective in rodent ischaemia (Park et al. 2015). Additionally, atomoxetine has been reported efficacious in restoring some level of *executive function* in PD patients, although not proposed to be actually neuroprotective in this disease (Marsh et al. 2009).

Naloxone is an inverse agonist of μ-opioid receptors, counteracting the effects of opiates (Sawynok et al. 1979). It thus does not induce reward signalling. However naloxone protects against microglial production of ROS - a detrimental consequence of Aβ signalling - and has shown efficacy as a treatment for ischaemia in a rodent model (Chang et al. 2000; Liao et al. 2003; Liu et al. 2002). It also appears to protect DAergic neurons from microglia and ROS, possibly having positive implications for PD treatment (Liu et al. 2000).

6.1.4 Conservation of reward in zebrafish

The parallels between mammalian and teleost reward signalling have been alluded to above. However the extent of conservation between these species is still unclear. Indeed, there is currently a lack of comprehensive insight into the range of pharmacological agents which induce rewarding behaviour in zebrafish. However the conservation of certain key neuroanatomical structures and behavioural responses is supported in the literature (see Figure 6.2).

Reward signalling

Many of the aforementioned regions involved in reward signalling (see Section 6.1.1, above) are structures specific to the mammalian brain (with the exception of raphe nuclei, which are conserved in zebrafish; see Turner et al. 2016; Yokogawa et al. 2012). However zebrafish have similar structures, argued to be orthologous to those of the mammalian brain. For example, the PTN has been reported to functionally represent the mammalian VTA (Klee et al. 2012; Parker et al. 2013b; Rink and Wullimann 2001). Expression patterns in the zebrafish Vd have been paralleled with the NAcc (Rink and Wullimann 2001). Similarly, the Dc has been paralleled with various cortical regions, including some basic functions performed by the mammalian PFC (Mueller et al. 2011; Parker et al. 2013b). These zebrafish neuroanatomical structures are represented graphically in Figure 6.2.

6.1.5 Safety pharmacology

Safety pharmacology is a stage of drug development, concerned with assessing and predicting possible adverse effects of novel compounds. One of the key elements of this is assessing the abuse potential of these compounds.

Abuse potential is the likelihood that a substance will be misused (abused), due to reinforcing pharmacological properties. All drugs approved for distribution in the UK, Europe, and the US are required to be assessed for their abuse potential in order for physicians to make more informed decisions when prescribing medication.

6.1.6 Reward-seeking behaviours

Safety pharmacology relies heavily on the observation of reward-related behaviours. These have been developed into screening assays in the pharmaceutical industry. Current industrial protocols utilise rodents for this purpose, assessing abuse potential via assays of classic reward-seeking behaviours, including SA and CPP (as well as DD, discussed in Chapter 7) (Panlilio and Goldberg 2007; Tzschentke 2007).

Self administration

SA involves the *ad libitum* administration of a drug, under the control of the subject itself. Often the drug dose is decreased following an initial aquision of SA (at a higher dose).

Figure 6.2: Neuroanatomical structures hypothesised to be orthologous with mammalian reward signalling structures. The PTN (A, in yellow), Vd (B, in red) and Dc (B, in green) have been paralleled (to some extent) with the mammalian VTA, NAcc and PFC (respectively). Locations of coronal sections are shown saggitally in (C-D). (Image adapted from Wulliman et al. 2012.)

The number of administration responses, or total drug consumption, is then measured as an index of reinforcement (e.g. Collins et al. 1983; Palmatier et al. 2006).

The development of a zebrafish SA assay would clearly be efficacious to industrial safety pharmacology, however there is difficulty in controlling administration of the drug. For example, the continuous locomotor activity of zebrafish poses problems for the insertion and stable maintenance of a transcranial cannula. It is possible to administer BBB-penetrating compounds via aquarium water (a method commonly utilised in zebrafish CPP paradigms); however controlling the acute termination of drug exposure (as *discrete trials*) presents a problem. Netting the fish induces acute stress, which would likely overshadow drug reinforcement. It may be possible to develop a '*wash chamber*' to treat fish and subsequently remove drug via a rapid change of chamber water. However, again the stress of this process will likely interfere with reward signalling.

Another possible method is to infuse food with a drug. However this presents its own set of problems. For example, many drugs of abuse dampen appetite (e.g. cocaine, amphetamine, nicotine). Additionally, it is almost impossible to reliably calculate the concentration of drug consumption as this would depend on the volume of food dispensed, the consistency of this volume between administrations, and the quantity of food actually consumed by the fish. Further, the administration is likely to be restricted by the maximal quantity of food the fish can consume. This would pose further problems for genetic studies in which mutations affect growth (i.e. size) and appetite, as well as likely presenting sex differences due to the relatively smaller size of the male zebrafish.

Conditioned Place Preference

Despite SA paradigms having higher face and predictive validity in relation to drug-seeking behaviours, the relative simplicity of CPP has lead to its wide-spread utilisation (in rodents) as a measure of reward signalling (Bardo and Bevins 2000; Tzschentke 1998).

Essentially, CPP is a pavlovian association between an environmental context (i.e. the CS) and the rewarding properties of a pharmacological agent (i.e. the US). The animal's preference - following conditioning - when given the choice of CS and a neutral stimulus (non-CS) is indicative of the rewarding properties of the US (drug). Typically, the specifics of this assay vary around the following procedural steps (e.g. Kily et al. 2008; Parker et al. 2016):

1. Measurement of baseline preferences when given the choice of two distinct stimuli

2. Multiple conditioning sessions to the least-preferred (CS) of the two stimuli, paired by exposure to a rewarding drug (US)
3. Probe assessment of '*conditioned preference*' for CS vs non-CS.

Several drugs abused in human society have been found to induce CPP in zebrafish. These include amphetamine, cocaine, ethanol, morphine, and nicotine (Darland and Dowling 2001; Kily et al. 2008; Lau et al. 2006; Ninkovic and Bally-Cuif 2006).

6.1.7 Aims

The aims of this chapter are to:

1. Validate a zebrafish CPP paradigm using a positive control (fentanyl)
2. Assess the ability of a range of drugs (with neuroprotective properties) to induce CPP in zebrafish

6.2 Methods

6.2.1 Subjects

Wild-type Tubingen zebrafish (*Danio rerio*) were bred in-house, or else aquired from a commercial supplier (Wades Tropical Import Ltd., UK) at 4 week post fertilisation. All fish were raised in the fish facility at Queen Mary, University of London, according to standard protocols. Based on power analyses conducted previously on studies using nicotine and EtOH, 20 fish were used at each drug concentration. CPP analyses were conducted at 3-4 months post fertilisation. All experiments were conducted in accordance with the Animals (Scientific Procedures) Act, 1986, under ethical guidelines by Queen Mary Animal Care and Use Committee, and under license by the Home Office (UK).

6.2.2 CPP

CPP experiments were conducted as previously described (e.g. Parker et al. 2013a; Parker et al. 2016). All experimental procedures were conducted in 20cm x 15cm x 30cm (WxHxL)

plastic assay tanks (see Figure 6.3).

One week prior to habituation, fish were singly housed. They were then exposed to two separate ***conditioning*** sessions (one per day, on two consecutive days). Each conditioning session involved placing the fish in the assay tank, giving it freedom to explore the entire tank (i.e. both stimulus-presenting '*zones*'), for 20 minutes each.

Baseline preferences were measured on the day following the second habituation session. This was achieved by allowing the fish to explore the entire tank (as with the habituation sessions), for 10 minutes. Video recordings (to calculate basal and probe preferences for stimuli) were taken, and fish were tracked live using EthoVision XT 9 software (Noldus), with a ceiling-mounted camera suspended above the assay tanks. The 'arenas' were drawn to envelope the tanks, and divided into two 'zones' (corresponding to the two stimuli presented in the tanks; see Figure 6.3). The proportion of '*time spent in zone*' was calculated from these data, as a direct index of stimulus preference. Fish which displayed basal proportional preferences greater than 0.75 for either of the stimuli were excluded from the rest of the study. This ranged between 5% and 40% fish per drug conditioning (i.e. across all doses).

Conditioning was conducted on three consecutive days. Fish with basal preferences below the exclusion threshold were conditioned to associate the non-preferred of the two stimuli with drug administration. Initially, fish were placed in the tank - with a plastic divider separating the two stimuli-presenting areas - on the side presenting the preferred stimulus (for that particular fish). They were allowed to explore just this stimulus-presenting area (non-CS) for 20 minutes, in the absence of drug. The barrier was then removed and fish encouraged to relocate to the non-preferred stimulus-presenting side, and the barrier replace (restricting the fish to exposure to this stimulus). Immediately following this relocation the drug (or vehicle) was administered by addition to the tank water from 50mL stock (at 50x treatment concentration). Fish were then allowed to explore this stimulus-presenting area (CS) in the presence of the drug (US).

Probe preferences were calculated in the same manner as baseline preferences, except that only the latter five minutes of the 10-minute trial was analysed.

6.2.3 Locomotion

Drugs which failed to induce CPP (both those which had been hypothesised to induce it, and the negative controls), were assessed for their effect on locomotor activity. Effect on

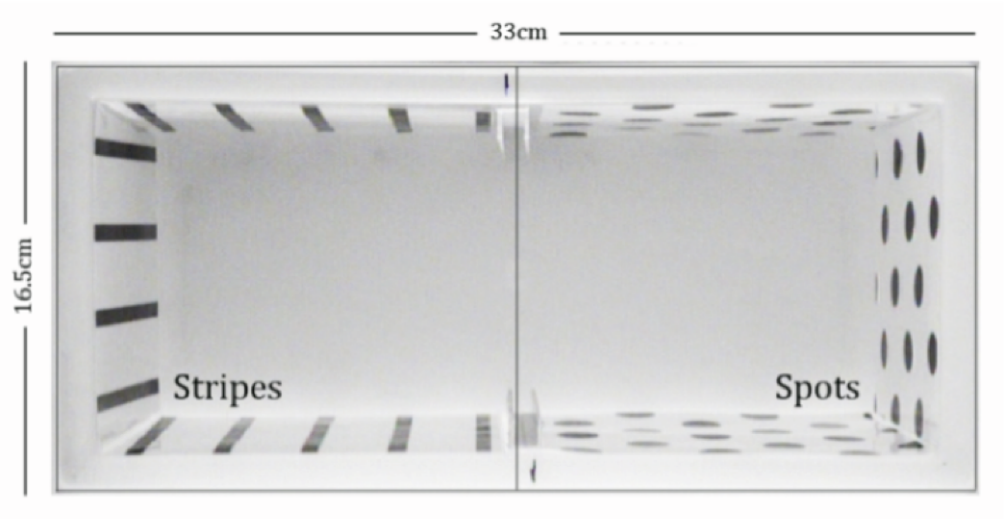

Figure 6.3: CPP assay tank. All stages of CPP experimentation were conducted in opaque rectangular tanks (20cm x 15cm x 30cm; WxHxL) containing 2.5L aquarium water. Basal and post-conditioning (probe) preferences for each stimulus was assessed by giving free access to the entire tank. During conditioning sessions, a divider was inserted in the centre of the tank (indicated here by a vertical line through centre of tank), restricting access to one of the two stimulus-presenting arenas.

locomotion was used as a proxy measure, to indicate whether or not the drug might cross the BBB to enter the zebrafish CNS (see Porsolt et al. 2002).

Drugs were tested at the same concentrations used in CPP assessment. Ten fish were used per drug dose; the administration order of drug dosing was pseudo-randomised.

Drug administration was conducted using pre-treatment in drug-treated aquarium water. Pre-treatment lasted 20 minutes. Following this exposure, fish were netted out of the drug-treated water and placed in a large (22cm x 16cm x 27cm; WxHxL) tank, containing un-treated aquarium water. Fish were allowed to habituate to the assay tank for 4 minutes (due to observations of stress-like behaviour upon first introduction to the assay tank). Immediately following this, locomotion was assessed over a 16-minute period. This was monitored via a ceiling-mounted camera suspended above the tanks, using EthoVision XT 9 software (Noldus).

Table 6.1: Details of drugs tested. Drugs were obtained from the stated commercial supplier. Dose ranges were based on previous studes, referenced here.

Compound	Supplier	Code	Dose Range μM (mg/L)	References
Atomoxetine-HCl	Sequoia	SRP07328a	2 - 8.6 (0.6 - 2.5)	Cantilena et al. 2012 Wee and Woolverton 2004
Bupropion-HCl	Sequoia	SRP03446b	5.4 - 45.5 (1.5 - 12.5)	Tella et al. 1997 Vermoesen et al. 2011
Caffeine	Johnson Matthey	A10431	26 - 260 (5 - 50)	Richendrfer et al. 2012 Wong et al. 2010
Ethanol	VWR	-	100 - 300mM (4.6 - 13.8)	Grant and Samson 1985 Kily et al. 2008
Fentanyl citrate	Sigma	F3886	0.008 - 0.303 (0.004 - 0.16)	Nishida et al. 1989 O'Connor et al. 2011
Ketamine-HCl	Sigma	K2753	18.2 - 72.9 (5 - 20)	Riehl et al. 2011 Rocha et al. 1996
Morphine sulphate	Sigma	M8777	0.7 - 7.9 (0.5 - 6)	Lau et al. 2006 Bretaud et al. 2007
Naloxone-HCl	Sequoia	SRP00860n	3.13 - 25 (1.25 - 10)	Collins et al. 1983 O'Connor et al. 2011
Nicotine hemisulphate	Sigma	N1019	0.5 - 10 (0.2 - 4.2)	Bencan and Levin 2008 Kily et al. 2008
Phencyclidine-HCl	Sigma	P3029	0.36 - 3.6 (0.1 - 1)	Kyzar et al. 2012 Marquis et al. 1989
Procaine-HCl	Sigma	P9879	92 - 733 (25 - 200)	Johanson and Aigner 1981 Woolverton and Balster 1979
Δ^9 tetrahydrocannabinol	Sigma	T2386	0.16 - 0.64 (0.05 - 0.2)	O'Connor et al. 2011 Mansbach et al. 1994

6.2.4 Drugs and doses

Dose ranges were selected based on previous research of CPP and/or SA, using zebrafish, rodents or NHPs (see Figure 6.1). Maximum concentrations (in the assay tanks, mg/L) did not exceed 2x the mammalian effective dose (mg/kg). All drugs were used at pH 6.95 - 7.5.

Drug were assessed for toxic effects on the fish, prior to experimentation. Beginning with the lowest concentration, fish (n=3) were placed in individual tanks containing the drug in 200mL aquarium water. Fish were exposed to the drug for one-hour, during which they were monitored for signs of toxicity (difficulty swimming, exaggerated breathing, haemorrhaging gills, internal bleeding). Fish were re-checked at regular intervals over the six hours following exposure, as well as on the following morning. If no toxicity was detected, the process was repeated using a higher concentration of drug, until the highest intended dose had been assessed. If toxicity was detected before the highest dose was assessed, the highest non-toxic concentration was used as the highest dose.

6.2.5 CPP data analysis

Data were output from EthoVision as *time spent in zone* (for each of the two stimulus-presenting zones), and the proportion of time spent in the CS-presenting zone was calculated by dividing this time by the total time the fish was tracked. '*Change in preference*' was calculated by deducting each subject's CS preference during the baseline trial, from that of the probe trial.

Inferential statistical analyses were performed using SPSS® Version 21 for Mac (IBM®). In order to determine the most appropriate statistical model (for the fit of each dose-response curve), *regression analyses* were employed. Both *linear* and *quadratic* regression models were performed to determine the shape of the curve (linear or 'inverted U'); the model which produced the highest *t-ratio* was adopted for subsequent analysis.

The *residuals* from the *regression analysis* were plotted (SPSS *Legacy Boxplot*); outliers (1.5x IQR) were removed. These outliers were generally caused by tracking errors; the maximum number of subjects removed from an experiment was 4% (for any one drug, across all doses). The data were then analysed using polynomial *general linear model*. Post-hoc Dunnett's test was performed, comparing the dosed groups to the control (vehicle) group (*two-sided*).

6.2.6 Locomotion data analysis

Locomotion data were extrapolated as '*Distance moved*' in 2-minute time bins. In order to account for spurious data points (caused by problems with the tracking or acute environmental factor which caused stress-like 'freezing' behaviour), any time bin in which the subject moved less than 100cm was removed.

Data were then fitted to a *linear mixed effects* model, using *distance travelled* as the dependent variable, with *time* and *concentration* as fixed effects. The *residuals* of this model were plotted (SPSS *Legacy Boxplot*), and outliers were removed at 1.5x IQR. The *linear mixed effects* model was then run again, without outlying data.

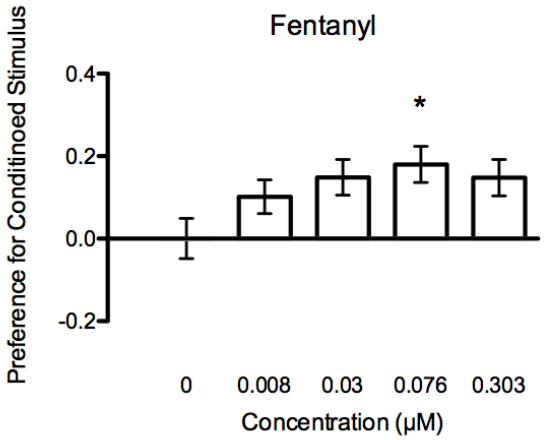

Figure 6.4: CPP assay validation. The conditioning of zebrafish with fentanyl (as an US) resulted in a quadratic regression. No effect was found in the linear term ($F_{1,129}$=1.698, p=0.195); however a significant preference change for the CS-presenting arena was found in the quadratic term ($F_{1,129}$=5.233, p=0.024) (n=130). By one-way ANOVA, then one-sided Dunnett's test; *, p<0.05. Data plotted as mean ± SEM.

6.3 Results

6.3.1 Conditioned place preference

Initially, an experiment to validate the CPP paradigm was conducted. Fentanyl was chosen as the US, as this opiate has previously been reported as reinforcing in humans, rodents and zebrafish (Stewart et al. 2015; see Section 6.1.2). The analysis found that the drug induced significant CPP in zebrafish (see Figure 6.4).

Subsequently, a range of 'neuroprotective' compounds with known reinforcing properties was assessed for their ability to induce CPP in zebrafish. Of the 10 drugs tested, six are reported here to be *true positives* (i.e. induce CPP in zebrafish; see Figure 6.5). Four were found to be *false negatives* (i.e. failed to induce significant CPP in zebrafish; see Figure 6.6).

Additionally, two negative controls were included in the study - atomoxetine and naloxone. These neuroprotective drugs have all been found to have no reinforcing effects on humans. Neither of these drugs were found to induce CPP in zebrafish (see Figure 6.7). Thus, there were no *false positive* results.

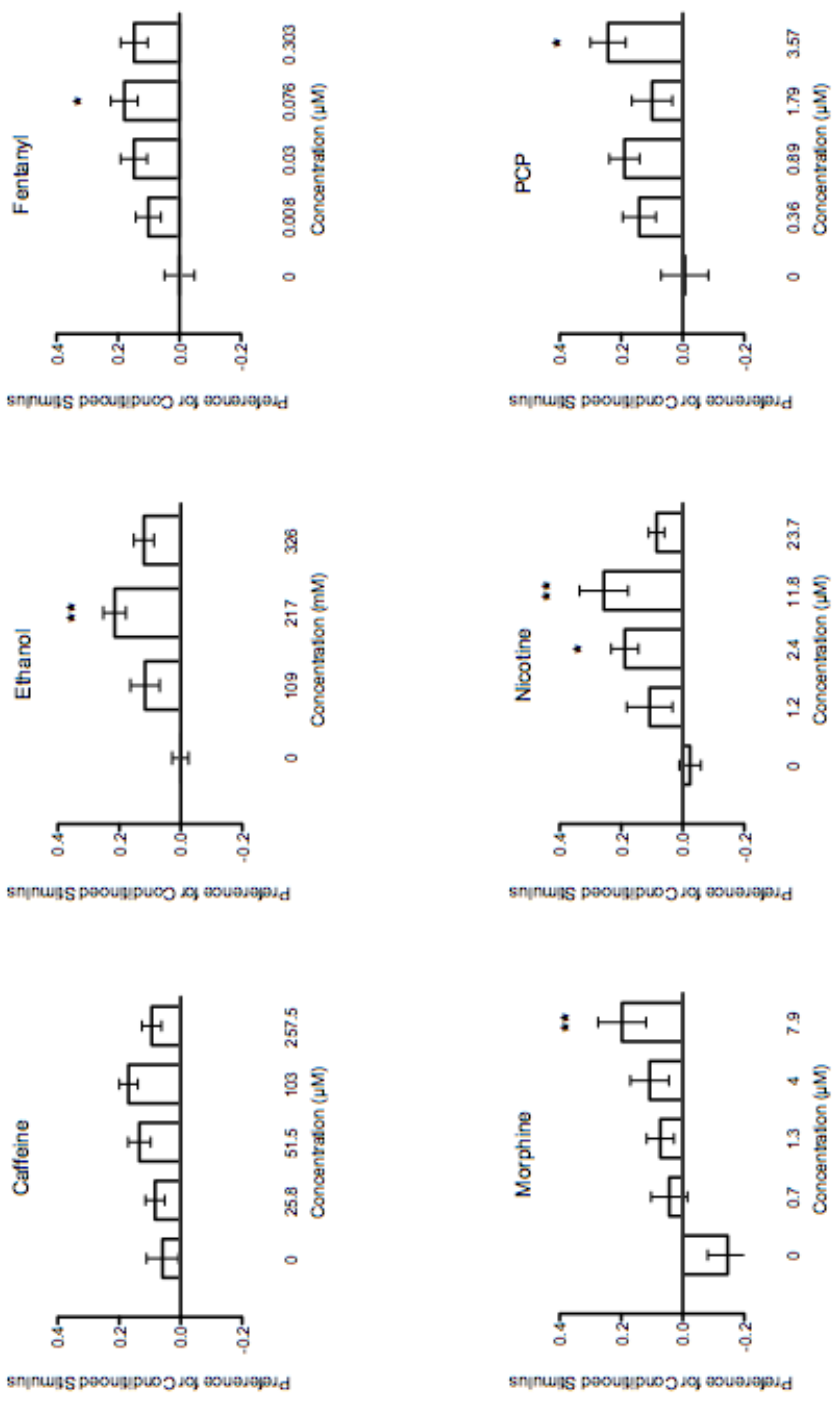

Figure 6.5: Neuroprotective drugs with conserved reinforcing properties in zebrafish. Six neuroprotective drugs - reported to exert reinforcing effects in humans - were found to induce significant CPP in zebrafish. Caffeine produced a quadratic dose-response curve, with no effect in the linear term ($F_{1,86}$=0.153, p=0.696); however a significant effect was found in the quadratic term ($F_{1,86}$=5.497, p=0.002) (n=89). Ethanol produced a quadratic dose-response curve, with effects for both the linear term ($F_{1,42}$=8.006, p=0.007) and the quadratic term ($F_{1,42}$=8.007, p0.007) (n=45). Fentanyl treatment produced a quadratic dose-response curve, with no effect found for the linear term ($F_{1,129}$=1.698, p=0.195); however a significant effect was found for the quadratic term ($F_{1,129}$=5.233, p=0.024) (n=130). Morphine produced a linear dose-response curve ($F_{1,37}$=8.704, p=0.006) (n=39). The quadratic dose-response curve for nicotine failed to reach significance in the linear term ($F_{1,47}$=1.279, p=0.264); however there was a significant effect in the quadratic term ($F_{1,47}$=11,516, p=0.001) (n=50). PCP treatment produced a significant linear dose-reponse curve ($F_{1,77}$=5.01, p=0.028) (n=78). By polynomial one-way ANOVA, then one-sided Dunnett's test; *, p<0.05; **, p<0.01. Data plotted as mean ± SEM.

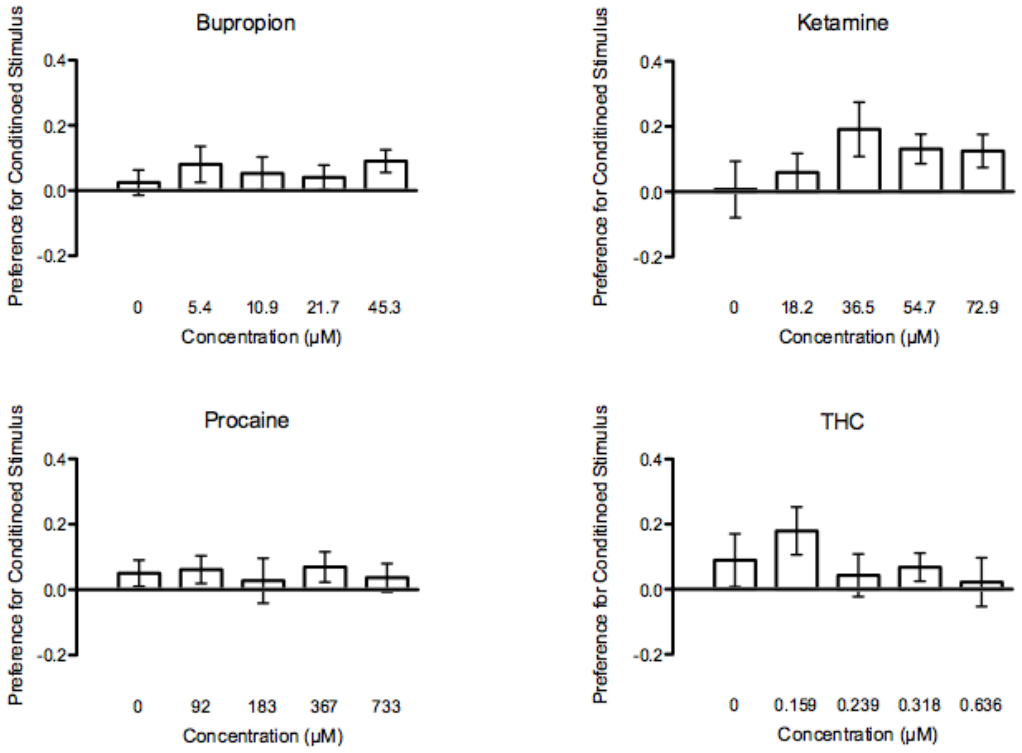

Figure 6.6: Neuroprotective drugs without reinforcing properties in zebrafish. Four neuroprotective drugs - reported to exert reinforcing effects in humans - failed to induce significant CPP in zebrafish. All of these drugs produced linear dose-response curves which failed to reach significance. Bupropion ($F_{1,81}$=0.587, p=0.446) (n=83). Ketamine ($F_{1,75}$=2.272, p=0.136) (n=76). Procaine ($F_{1,79}$=0.017, p=0.896) (n=137). THC ($F_{1,82}$=1.115, p=0.294) (n=83). By one-way ANOVA. Data plotted as mean ± SEM.

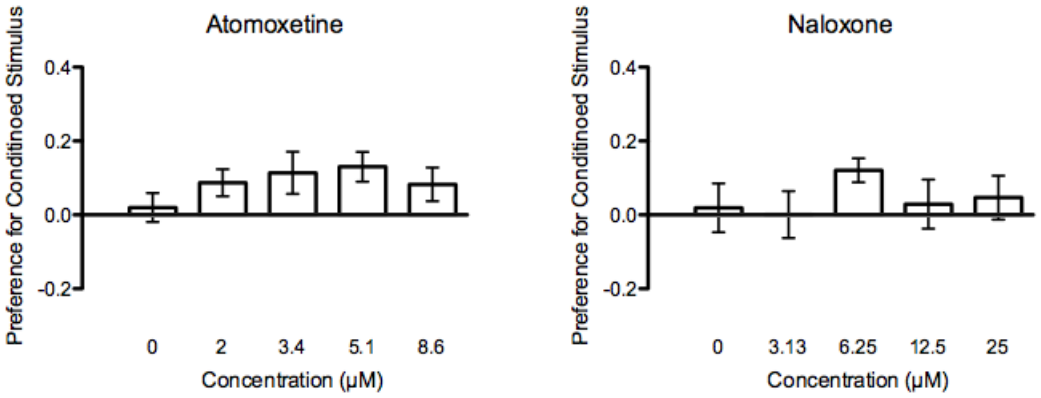

Figure 6.7: Neuroprotective drugs with no reinforcing properties. Two drugs which are not reinforcing in humans were included in the study as negative controls. Neither of these induced significant CPP in zebrafish. Atomoxetine produce a quadratic dose-response curve which failed to reach significance in both the linear ($F_{1,84}$=0.746, p=0.39) and quadratic terms ($F_{1,84}$=2.566, p=0.113) (n=88). Similarly, the quadratic dose-response curve for naloxone failed to reach significance in both term - linear ($F_{1,55}$=0.037, p=0.848); quadratic ($F_{1,55}$=0.202, p=0.655) (n=56). By linear regression. Data plotted as mean ± SEM.

6.3.2 Locomotion

For drugs which failed to induce CPP a locomotion assay was conducted, as a proxy measure to indicate whether the drug crossed the BBB. All of these '*false negative*' drugs - bupropion, ketamine, procaine, and THC induced significant changes in locomotion (see Figure 6.8).

Additionally, the negative control compounds used in the CPP assessment (atomoxetine and naloxone) were assessed for their affects on locomotion. Both of these compounds induced significant changes in locomotor activity (see Figure 6.9).

6.4 Discussion

The aim of this chapter was to test the hypothesis that a range of drugs known to be reinforcing in humans would induce CPP in zebrafish. Of the ten drugs assessed which are reinforcing in humans, six were found to induce significant conditioned changes in zebrafish preference, demonstrating a conservation of the relative signalling mechanisms.

Both of the *stimulants* (caffeine and nicotine) significantly induced a change in

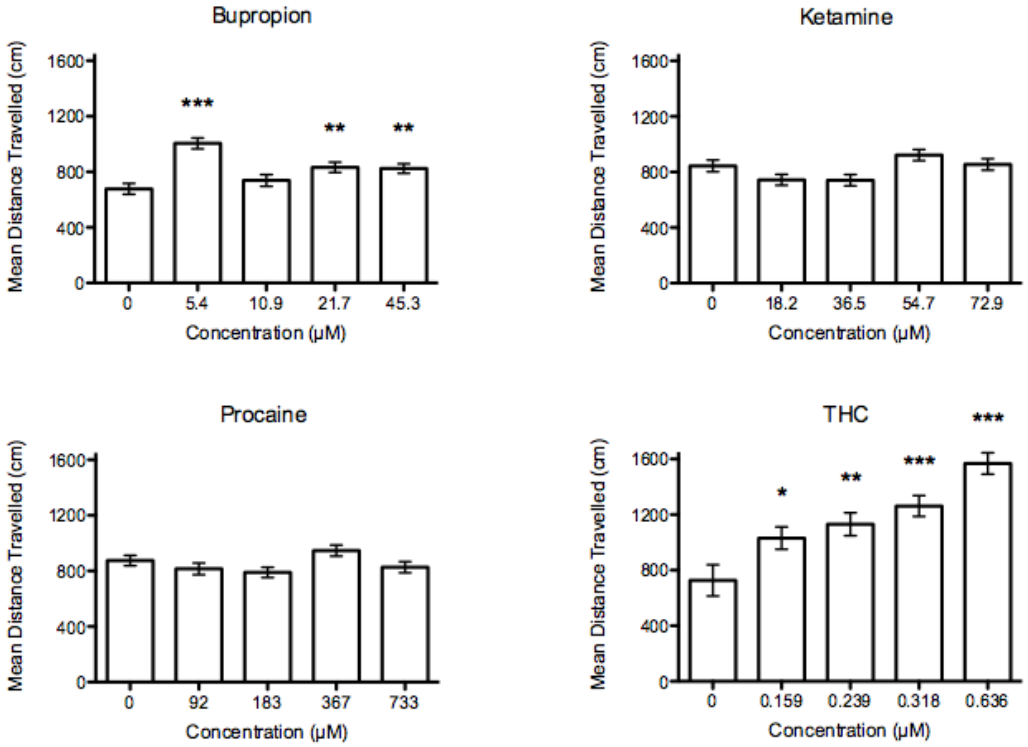

Figure 6.8: Locomotion assessment of drugs which failed to induce CPP in zebrafish. Neuroprotective drugs which exert reinforcing effects on humans, but failed to induce zebrafish CPP, were assessed for their affects on locomotion. Bupropion ($F_{4,279}$=9.904, p<0.0005), ketamine ($F_{4,305}$=3.74, p<0.0005), procaine ($F_{4,297}$=2.517, p=0.042) and THC ($F_{4,240}$=11.588, p<0.0005) all induced significant effects on *distance travelled* (n=50 per drug). By linear mixed effects model; *, p<0.05; **, p<0.01; ***, p<0.001. Data plotted as mean ± SEM.

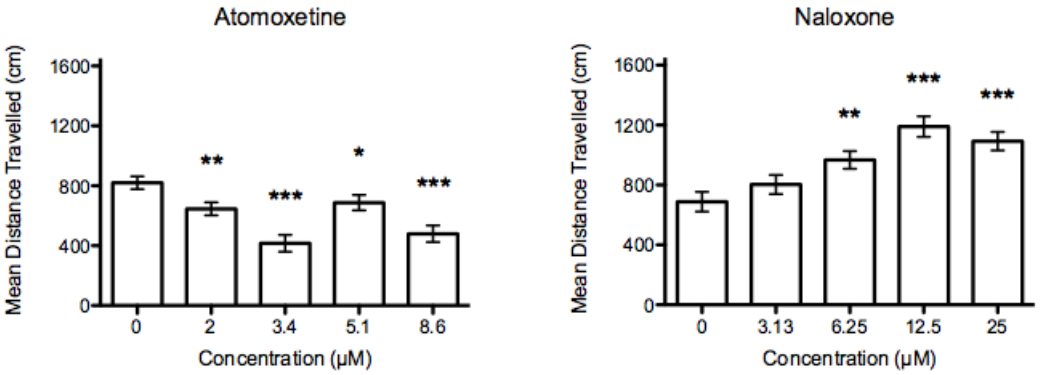

Figure 6.9: Locomotion assessment of CPP '*negative controls*'. Neuroprotective drugs which have no reported reinforcing affects on humans (and were found to have no affect on zebrafish CPP, above) were assessed for their affects on locomotor activity. Both of these drugs caused significant changes in *distance travelled*. Atomoxetine ($F_{4,231}$=10.642, p<0.0005). Naloxone ($F_{4,253}$=9.758, p<0.0005) (n=50 per drug). By linear mixed effects model; *, p<0.05; **, p<0.01; ***, p<0.001. Data plotted as mean ± SEM.

conditioned preference. The fact that these two compounds exert their reinforcing effects via distinct molecular pathways indicates that both of these mechanisms may be conserved in the zebrafish. With regard to nicotine, the fact that the α7 subunit has been implicated in modulating reward signalling suggests that this receptor's function may be conserved (Besson et al. 2012; Marquis et al. 1989). This has important implications in relation to neuroprotection, as the α7 subunit has been strongly associated with facilitating nicotine-induced neuroprotective signalling (e.g. Arias et al. 2005; Jonnala and Buccafusco 2001; Liu et al. 2007a; Shimohama 2009). Thus, as elucidating the specific functions of α7 is of importance to both of these neurobiological phenomena, future investigations may wish to assess the extent that α7 signalling is conserved in zebrafish.

Caffeine's reinforcing effects have been associated with antagonism of the A_1 receptor subtype (Solinas et al. 2002). Thus, the current finding of conserved caffeine reward in zebrafish implies a conservation of A_1 signalling. In contrast, the neuroprotective properties of caffeine have been associated with antagonism of A_{2A} (Gomes et al. 2011). The balance of neuronal excitation and inhibition - as affected by these two opposing receptors - has important implications for both reward and neuroprotective signalling. Therefore, the extent to which this balance is conserved in zebrafish is important to understanding the efficacy of this organism to model aspects of these neurological disorders. It is thus suggested that future research investigate the extent of adenosine signalling conservation in zebrafish on a more holistic scale.

As with the stimulants, both of the *opiates* (fentanyl and morphine) caused a conditioned change in preference. The fact that both opiates used here are μ-opioid receptor agonists strengthens the argument of conserved opioid signalling functioning in zebrafish. Further, in light of findings that μ-opioid receptor antagonism induces neuroprotection (in a rodent model of ischaemia), it would be of importance for future research to investigate the conservation of this opposing action of μ-opioid (Liao et al. 2003).

Further, it has been reported that the μ-opioid receptor is necessary for nicotine-induced reward (Walters et al. 2005). Thus, the extent to which this relationship between nAChRs and μ-opioid receptors is conserved in zebrafish would present an interesting facet of reward signalling in this organism.

The *alcohol* tested (ethanol) caused CPP. This is in-keeping with previous research reporting this effect, supporting assertion that key reinforcing aspects of EtOH reward are conserved in zebrafish (Collier et al. 2014; Kily et al. 2008).

The *antidepressant* tested (bupropion) failed to induce CPP; however locomotion analysis indicates that it does indeed cross the BBB. Thus it appears that any effect it has on increasing DA in the zebrafish Vd is insufficient to cause reinforcement (as occurs in the mammalian NAcc), at the concentrations used here. Additionally, as the effect of this drug on DA and norepinephrine is weak, it is possible that increased exposure is necessary to induce the biochemical adaptations necessary to facilitate reward. However, further research is needed to test this hypothesis. Thus, within the context of the assay used here, exposure failed to induce CPP.

Similarly, no effect was found for the *local anaesthetic* procaine. The locomotor analysis found a significant effect of the drug, indicating that it crosses the BBB. This suggests that its modulation of DA is insufficient to induce reward signalling. However, the locomotor analysis failed to find any single dose significantly different from controls. Indeed, it appears that the significance of the model may be driven by the difference between 183μM and 367 μM. This suggests that the effects of increasing procaine concentration may be countered by homeostatic signalling in the zebrafish brain, with a non-significant trend towards decreasing locomotion (up to 183μM) ameliorated at higher concentrations. However research on a molecular level is necessary to investigate this hypothesis. Further, it is possible that the drug does not, in fact, cross the BBB, and that the subtle, significant effect on locomotion results from a pharmacological action on peripheral tissues; however, again, further research is needed to investigate this.

Conditioning with the *cannabinoid* THC failed to induce a significant change in

preference. This suggests that the conservations of functioning in the zebrafish PTN and Dc are insufficient to model this function of the mammalian VTA and PFC, in this respect. However, as THC is not reinforcing in rodents, the lack of an effect does not diminish the utility of zebrafish as a pre-screening refinement to the use of rodents in the pharmaceutical industry (e.g. Wakeford et al. 2016).

Additionally, it has been reported that pre-exposure to THC (in the absence of conditioning stimuli) leads to significant CPP in rodents (Valjent and Maldonado 2000). The authors commented that first-time exposure in drug-naïve subjects induces a dysphoria (often leading to conditioned place aversion), which is attenuated in subsequent exposures. Thus, it is possible that pre-exposure in zebrafish would result in a significant CPP; although further research is necessary to establish the existence of this conservation. However, within the context of the assay utilised in the current research, it must be concluded that THC fails to induce rewarding behaviour in zebrafish.

In relation to neuroprotection, it is worth mentioning that many phytocannabinoids have prolonged activity in the body (compared with endocannabinoids, due to decreased cellular uptake) (see Mechoulam et al. 2002). This suggests that such compounds may enhance and prolong the neuroprotection exerted by endocannabinoid. In this regard, the non-psychoactive phytocannabinoid CBD delays onset of ALS (Weydt et al. 2005). As CBD is an indirect agonist of the cannabinoid receptors, this suggests that direct receptor activation may not be required to initiate of these neuroprotective mechanisms. Thus, future translational research into the neuroprotective consequences of cannabinoid signalling may wish to target other (down-stream) molecules in the signalling pathways, as an alternative to developing compounds with abuse potential.

The two *general anaesthetics* tested produced non-uniform results - PCP induced CPP, whilst ketamine failed to do so. As the primary pharmacological action - namely, antagonism of NMDA-Rs - is common to both of these compounds, it appears that the non-specific actions of ketamine inhibit reward signalling. Alternatively, it is possible that these secondary actions induce a mild aversive response, which counteracts the reward signalling of NMDA-R antagonism.

It is unlikely that this would be facilitated by ketamine's weak agonism of opioid receptors, as its higher affinity for μ- over κ -receptors (rewarding and aversive, respectively) suggest a facilitation of reward signalling. However it is possible that the increase in 5-HT may be responsible for this effect. Indeed, it has been found that 5-HT signalling in the periaqueductal grey matter facilitates aversion in rats (Nogueira and Graeff 1995). In this regard, modulatory serotonergic projections have been reported to extend from the DRN to the periaqueductal grey matter, a mechanism associated with "defensive behaviour" (Stezhka and Lovick 1994; Stezhka and Lovick 1997; Vianna and

Brandao 2003). Further, the close proximity of the periaqueductal grey matter to the DRN (in the mammalian brain) suggests that this may indeed be a strong candidate mechanism to explain the failure of ketamine to induce CPP. However, currently no zebrafish neuroanatomical region has been reported to be orthologous to the mammalian periaqueductal grey matter; thus, further research is required to elucidate the signalling which drives this phenomenon.

In summary, the results presented in this chapter suggest that zebrafish may have a utility as a model of human abuse potential for the assessment of stimulants, opiates and alcohol. Using the current paradigm it appears that their neurological complexity may be insufficient to model the human abuse of anaesthetics, antidepressants and cannabinoids. However, analysis of a more comprehensive range within these drug classes is necessary to understand the full potential of this organism as a model for human reward. Additionally, it is possible that a refinement on the assay protocol (e.g. pre-exposure in the assessment of cannabinoids) may lead to the induction of CPP in the *false negative* compounds reported here. Thus, these data serve as an encouraging pilot study into the utility of zebrafish CPP as a model of human abuse potential.

The current investigation was primarily focussed on assessment of behavioural responses, as a measure of conserved reward mechanisms. This was, in part, due to the involvement of elements of neuroprotective pathways, in reward signalling. Thus, the implications of conserved reward-related behaviours suggests the relative neuroprotective mechanisms may, too, be conserved. This is especially true of caffeine, nicotine, PCP, and possibly EtOH, for which the primary reward pharmacology of the drugs are also targets for the protective signalling; however this may have little bearing on those drugs for which the relationship between the two facets of this dual action is unclear (namely, morphine and fentanyl). Thus, future research may wish to establish the conservation of these neuroprotective mechanisms, as well as elucidating any interactions with reward signalling pathways.

In conclusion, the data reported here suggest that certain fundamnetal aspects of mesocorticolimbic reward signalling are conserved in the zebrafish, although the extent of this conservation appears limited. It is possible that the lack of induced rewarding behaviour by certain compounds relates to the relative simplicity of zebrafish neuroanatomical structures, secondary pharmacological mechanisms, or possibly inadequacies of the assay. Despite this, there is clear evidence that zebrafish present an efficacious tool for modelling at least some of the rewarding aspects of human drug abuse. It is now necessary for further research to investigate a more extensive range of compounds which induce CPP in zebrafish.

Chapter 7

Development of a Drug Discrimination Assay

7.1 Introduction

In addition to CPP (explored in Chapter 6) and SA assays, *drug discrimination* paradigms are commonly used to assess the abuse potential of novel compounds. These typically involve multiple training sessions in a '*two-choice*' assay; conditioning an animal to make one response following drug treatment, and the alternate response following saline treatment (e.g. Colpaert 1999; Solinas et al. 2006).

An animal (commonly rodent or NHP) is administered a drug or saline, then placed in an operant conditioning chamber presenting two levers. Following drug administration, presses on one of the levers is reinforced with a reward (food pellet). Following saline administration, presses on the other lever is reinforced. Thus, the psychoactive '*state*' produced by the drug administration is paired with one conditioned response, whilst saline is paired with the alternative conditioned response. A novel compound can then be administered and the animal placed in the chamber (with neither lever administering reinforcement); the ability of a novel drug to pharmacologically mimic the drug used in conditioning is then assessed by the animal's response (i.e. choice of lever presses).

However, the requirement of food reinforcement poses two potential problems. Chiefly, the motivation to continually make responses is subject to the appetite of the animal. This may be partly controlled for by limiting or ceasing the animal's feeding outside conditioning, as well as introducing *variable* or *random interval schedules* (which also facilitates repetitive responding in probe trials, where no reinforcement is administered) (Millenson 1963; Pietras et al. 2010; Young et al. 2009). However, drugs which dampen appetite may result in fewer responses and thus require a greater number of conditioning sessions to achieve sufficient 'learning'. This is particularly pertinent to the development of a zebrafish assay, as the quantities of food consumed are much lower than that of (larger) mammals. The reliable control of food release in these small quantities is problematic in generating an automated assay system. Thus, it is possible that non-appetitive (i.e. aversive) conditioning may present a more efficacious paradigm in this model organism.

Regardless of these potential limitations, drug discrimination allows direct, highly specific pharmacological comparison of the interoceptive cues induced by a novel compound, with those of a drug whose effects are known (Solinas et al. 2006). Thus, whilst not directly indicating reinforcing properties, drug discrimination assesses the ability of a novel compound to mimic the pharmacological action of a drug with known abuse potential (e.g. Appel et al. 1983; Kamien et al. 1993).

In addition to the '*screening out*' of drugs with high abuse potential, it is possible that a

researcher may intend to identify drug which mimic abused compounds. For example, the identification of drugs with low abuse potential (e.g. methylphenidate) which '*substitutes for*' (not discriminated from) a drug with high abuse potential (e.g. amphetamine) may be utilised as a treatment for individuals with dependence on this compound (e.g. Pena et al. 2011; Lile et al. 2006). In this regard, the establishment of methadone as a non-discriminated substitute for heroin - whilst highly controversial - has the potential to facilitate the attenuation of opiate dependence (e.g. Donny et al. 2005; Leri et al. 2004; Newman et al. 2002).

Similarly, comparisons between cocaine and procaine found that, whilst both are self-administered in isolation, when given the choice cocaine is preferred, even when procaine was delivered at a concentration sixteen times that of cocaine (Johanson and Aigner 1981; Woolverton and Balster 1979). Whilst procaine was less rewarding, its ability to *substitute* for cocaine demonstrates some extent of pharmacological similarity.

7.1.1 Neurobiology of drug discrimination

Understanding the neurobiological structures involved in *drug discrimination* tasks is somewhat complex. This may be due to the distinct mechanisms of action by which many of the drugs which induce this behavioural phenomenon affect the brain (for a selection relative to neuroprotective mechanisms, see Chapter 6, Section 6.1.2). As such, it has received little research attention. However logic dictates that this involves an interaction between the structures involved in basic learning (in fear conditioning, the amygdalae, as well as hippocampi in more complex tasks; see Chapter 3, Section 3.1.2) and the pharmacological targets (primary or subsidiary) of the relevant drug. Thus, the involvement of amigdalae, hippocampi, VTA, NAcc, PFC, and raphe nuclei may all be involved, to some extent.

With regard to the conservation of these structures in zebrafish, more extensive descriptions have been discussed earlier in this thesis. However, in brief, the PTN (VTA), Vd (NAcc), Dc (PFC), Dm (amygdala), Dl (hippocampus) have all been associated with conserved functioning (of the relative mammalian structures).

Drug discrimination is essentially a '*state-dependent learning*' paradigm, in which the psychoactivity caused by a drug provides the 'state'; learning which occurs in this state is better retreived when in the same state (Overton 1972; Overton 1984). These psychological states - induced by drugs including antidepressants, antipsychotics, barbiturates, benzodiazepines, opiates, and stimulants - have been found sufficient to facilitate retrieval of conditioned responses in mammals, when learned under the influence of these compounds (Colpaert et al. 1976; Hill et al. 1971; Petersen and

Table 7.1: Neuroprotective drugs which are discriminated from saline. Previous research has found that the following neuroprotective drugs induce *state-dependent learning* in mammals. The drug-conditioned responses have been found to 'generalise to' a substituted compound(s) (as listed).

Compound	Human abuse potential	Mammalian reinforcement (substitution with)	References
Atomoxetine	Low/ Negligable	Human (methylphenidate) Rat (cocaine)	Lile et al. 2006 Upadhyaya et al. 2013
Bupropion	Significant	Rhesus monkey (cocaine) Rat (nicotine, methamphetamine)	Kleven et al. 1990 Wilkinson et al. 2009
Caffeine	Significant	Human (none) Rat (cocaine)	Oliveto et al. 1992 Harland et al. 1989
Ethanol	Significant	Human (N/A) Rats (ketamine, PCP)	Duka et al. 1998 Shelton and Balster 1994
Fentanyl	Significant	Squirel monkey (morphine) Rat (morphine)	Schaefer and Holzman 1977 Colpaert et al. 1980
Ketamine	Significant	Squirel monkey (ketamine) Rat (ethanol)	Brady and Balster 1981 Shelton and Balster 1994
Morphine	Significant	Squirel monkey (fentanyl) Rat (fentanyl)	Schaefer and Holzman 1977 Colpaert et al. 1980
Naloxone	Low/ Negligable	Human (none) Rat (diprenorphine, naltrexone)	Preston et al. 1987 Smurthwaite et al. 1992
Nicotine	Significant	Human (N/A) Rat (anabasine, cytisine)	Perkins 1999a Stolerman et al. 1984
Phencyclidine	Significant	Squirel monkey (ketamine) Rat (ethanol)	Brady and Balster 1981 Shelton and Balster 1994
Procaine	Significant	Rhesus monkey (cocaine) Rat (cocaine)	La Garza and Johanson 1983 Silverman and Schultz 1989
Δ^9 THC	Significant	Rhesus monkey (WIN 55,212-2) Rat (CP-55,940, methanandamine)	Wiley et al. 1995 Burkey and Nation 1997

Ghoneim 1980). However, within drug discrimination paradigms, retrieval when the subject is in the same state (drug vs saline) as conditioning was conducted under must be quantifiably different from retrieval in the non-same state. In this regard, several '*neuroprotective*' compounds have been found to induce discriminatory states.

7.1.2 Drug discrimination of neuroprotective compounds

As mentioned above, the specific mechanisms by which drug discrimination is facilitated varies (dependent on the drug used, as well as the specificities of the paradigm). However there is evidence that many drugs with neuroprotective properties induce this manner of state-dependent discriminative learning in mammals. This evidence is summarised in Table 7.1:

However, despite this wealth of research into discriminative state-dependent learning in mammals, zebrafish research has paid little attention to this area of investigation. The current research intends to address this gap in the literature, investigating the phenomenon of zebrafish *drug discrimination* by designing and developing an automated assay.

7.2 Aims

The aim of this chapter is to develop a drug discrimination paradigm. This will be undertaken with the following objectives:

1. Develop a paradigm which induces an immediate (short-term) conditioned response (aversion of the CS)
2. Develop a paradigm which induces prolonged (longer-term) aversion (i.e. the conditioned response is retained over a longer period of time)
3. Test the hypothesis that zebrafish can discriminate between drugs which exert different interoceptive cues on humans
4. Assess the ability of zebrafish to discriminate a range of (paired) compounds, discriminated by mammalian model organisms

7.3 Methods

7.3.1 Subjects

Wild-type Tubingen zebrafish (*Danio rerio*) were bred in-house, and raised in the fish facility at Queen Mary, University of London, according to standard protocols. All experimentation was conducted on fish 4-6 months post fertilisation. All experiments were conducted in accordance with the Animals (Scientific Procedures) Act, 1986, under license by the Home Office (UK).

7.3.2 Assay set-up and design

All equipment was designed and set up as used in Chapter 3 (see Figure 3.3). In brief, an infrared-transmitting screen was placed on top of a LCD monitor, with transparent

'assay' tanks placed on top. Stimuli were presented via the LCD monitor - visible to the subjects through the transparent tanks. Fish were monitored via a camera suspended from the ceiling, with an infrared filter over the lens (to allow tracking of the fish without interference from the stimuli). Zebrafish behaviour was monitored in real-time, using EthoVision XT 9 software (Noldus).

7.3.3 '*Passive*' classical fear conditioning

All *passive classical fear conditioning* experiments were conducted using a thirty minute habituation period (both stimuli presented, alternating every five minutes). Basal preferences were recorded for thirty minutes, immediately following habituation (unless multiple habituation session were conducted, as in Section 7.4.1).

Conditioning sessions - when not conducted immediate following baseline recordings - involved the presentation of a blank screen for one minute, followed by nine cycles of conditioning (1.5 seconds CS presentation, terminating with administration of US; then 8.5 seconds of non-CS presentation) unless stated otherwise.

All probe preferences (those which did not immediately follow conditioning) were conducted by netting fish into the assay tanks at the stated time following conditioning. A blank screen was presented for one minute, followed by a 2-minute probe trial (both stimuli presented, one to each half of the screen; counterbalanced).

7.3.4 '*Escape*' fear conditioning

All *escape fear conditioning* experiments were conducted without an habituation period, *per se* (i.e. in distinction to baseline recordings), unless stated otherwise (i.e. Section 7.4.1).

Baseline recordings were taken over 30 minutes, with both stimuli presented ('split' across the screen, as per Figure 3.4). Stimuli presentation was switched (i.e. which side presented CS vs non-CS) after fifteen minutes.

Conditioning sessions involved the presentation of both stimuli (in the 'split-screen' fashion). Every time the fish swam into the 'zone' presenting the CS it was allowed two seconds to escape. Failure to relocate to the non CS-presenting 'zone' resulted in the administration of a 9V electric shock, delivered every second the fish was in the CS-presenting zone (unless stated otherwise). Each discrete conditioning trial lasted 55

seconds, followed by presentation of a blank screen for five seconds, unless stated otherwise. The side to which each stimulus was presented (CS vs non-CS) was pseudo-randomised between discrete trials. Thirty discrete (one-minute) trials were conducted (unless stated otherwise). Immediately following conditioning, fish were exposed to a 'dark-out' period (blank screen, no stimuli presented) for one minute, unless stated otherwise. Conditioning which was not conducted immediately following baseline recordings involved the presentation of a blank screen for one minute prior to stimuli exposures.

Probe trials were designed to exactly mimic baseline trials. If this was not immediately following conditioning, a one-minute habituation period (blank screen) was included prior to stimuli exposure.

7.3.5 Data analysis

Data were extrapolated from EthoVision as *time spent in 'zone'* (for each of the two stimulus-presenting 'zones'). The proportion of time spent in the CS-presenting zone was calculated by dividing the time in the CS zone by the total time for which the fish was tracked.

Data were input into SPSS® Version 21 for Mac (IBM®). They were fitted to a *linear mixed effects model*, with '*distance*' as a covariate (as discussed in Section 3.4, to account for any variance caused by differences in locomotion) and '*subject ID*' as a random factor, using the relevant fixed factors (as reported in the results). The residuals of the model were plotted (SPSS *Legacy Boxplot*), and outliers (1.5x IQR) were removed. The data were then analysed using the same model. Where necessary, *paired one-tailed t-tests* were conducted post-hoc.

7.4 Results

7.4.1 '*Passive*' classical fear conditioning

Pilot - titration of discrete trials

Initially, an experiment was conducted to assess whether the basic *classical fear conditioning* assay (as developed in Chapter 3) induces aversion 24-hours following

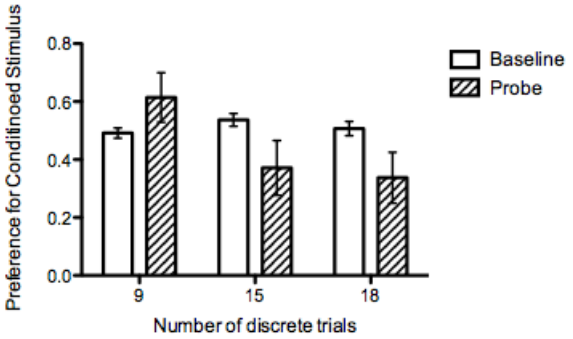

Figure 7.1: Shock titration. The number of electric shocks delivered (paired with CS exposure) was titrated to establish the most effective conditioning parameters, when *probe* trials were conducted at 24 hours. A main effect was found for the *number of shocks* delivered ($F_{2,21.670}$=16.535, p<0.0005). No main effect was found for *trial* ($F_{1,21.562}$=1.953, p=0.176); however there was a significant *trial* * *number of shocks* interaction ($F_{2,21.317}$=16.827, p<0.0005). Post-hoc analyses of *baseline*s vs *probe*s for each condition failed to find significant differences at any number of shocks (by paired one-tailed t-test with Bonferroni correction applied). Data plotted as mean ± SEM.

conditioning. As this manner of aversion was not previously investigated, a titration of the number of discrete trials was included - 9 trials (1.5-minutes total, as used previously in this thesis); 15 trials (2.5-minutes total); or 18 trials (3-minutes total).

Following a 30-minute habituation period, basal preferences were recorded as described in Section 3.2.2 (also thirty minutes). Immediately following conditioning, a blank screen was presented and trials were terminated. Fish were netted into individual housing tanks and housed on a circulating system for 24 hours. Following this, fish were netted into the assay tanks; a blank screen was presented for one minute, then both stimuli presented and probe preferences recorded. No significant aversion of the CS was found (see Figure 7.1).

Increased habituation, titration of conditioning trials

As no aversion was observed in the initial pilot, it was hypothesised that increased habituation to the assay setup and stimuli may facilitate retention. Zebrafish were netted into the assay tanks and exposed to the two stimuli (CS and non-CS, 'split screen') for two sessions of thirty minutes (one per day, on the two days preceding baseline recordings and conditioning). The day following the second habituation session, the assay was conducted as described in above (Section 7.4.1). '*Time of probe*' was included in data analysis as a between-subjects factor (0 hours as the positive control, 24 hours as the experimental group). Probes conducted immediately following conditioning

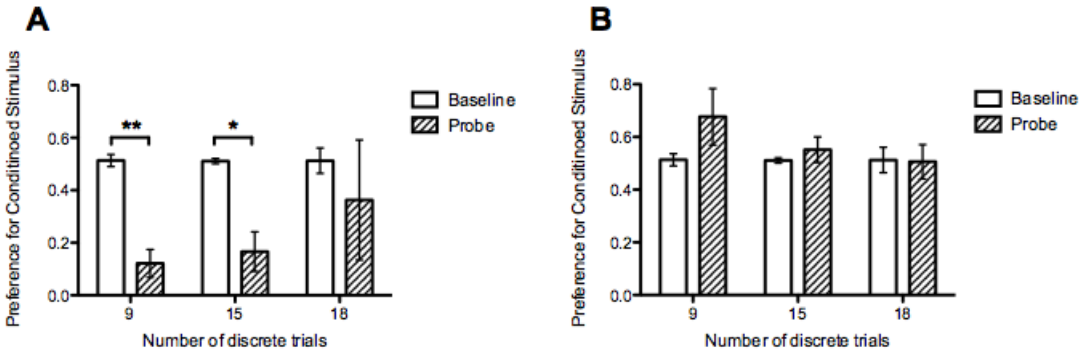

Figure 7.2: Increased habituation. Zebrafish were exposed to the stimlui (as per *baseline* trials) on two separate days prior to conditioning. (A) When probe trials were conducted immediately following conditioning, a significant main effect was found for *number of shocks* ($F_{2,7.806}$=9.491, p=0.008). Pairwise comparisons found *9-shocks* to be significantly different from *18-shocks* (p=0.043). (B) However, when probe trials were conducted 24 hours after conditioning, no significant main effect was found for *number of shocks* ($F_{2,11}$=3.509, p=0.066) *, $p<0.05$; **, $p<0.01$; (by paired one-tailed t-test with Bonferroni correction applied). Data plotted as mean ± SEM.

recorded significant aversion of the CS, using 9 and 15 discrete trials (see Figure 7.2a). However, at 24 hours after conditioning, no significant aversion of the CS was recorded with any number of discrete trials used (see Figure 7.2b).

Length of recall assessment

As no aversion was observed at 24 hours following conditioning, an experiment was conducted to assess the length of time following conditioning, for which CS aversion is observable. Results indicate that significant aversion is only observable immediately following conditioning, with extinction happening within thirty minutes of this (see Figure 7.3).

7.4.2 '*Escape*' fear conditioning

As the '*passive*' *fear conditioning* assay failed to induce an observable long-term aversion of the CS, it was decided to progress with a more 'active' form of conditioning. In the following trials, zebrafish were given the choice of both stimuli (CS and non-CS) during conditioning; every time the fish swam onto the zone presenting the CS, an electric shock was administered (within parameters stated).

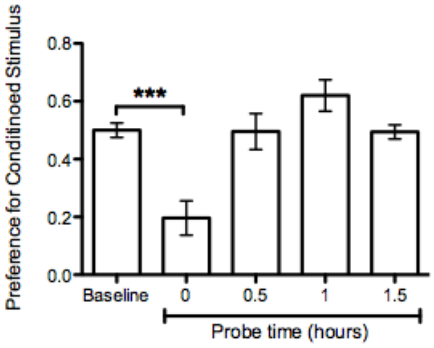

Figure 7.3: Length of recall. Zebrafish were assessed for their aversion of the CS at set time following conditioning. A significant effect was found for *trial* ($F_{4,41}$=4.693, p=0.003). Pairwise comparisons found that *baseline* was only significantly different from *Probe 0-hours*; ***, p<0.0005. Data plotted as mean ± SEM.

Initial pilot trial

An initial pilot experiment was designed, consisting of three phases - baseline, conditioning and probe.

During ***baseline*** recordings, zebrafish were exposed to both stimuli (using the 'split screen', as per Figure 3.4), for 10 minutes

During ***conditioning*** sessions, again both stimuli were presented. Each time a fish swam over the *zone* presenting the CS, the fish had two seconds to '*escape*'. Failure to do so within 2 seconds resulted in the administration of a 9V electric shock, delivered every second that the fish remained on the CS-presenting *zone*, until it escaped to the non CS-presenting *zone* (or the discrete trial ended).

Probe trials were conducted in the same manner as baseline trials, except that a one minute 'dark-out' period was included prior to presentation of stimuli.

The entire paradigm was repeated on two consecutive days. Thus, the baseline on Day 2 serves to assess whether aversion of the CS occurs at 24-hours following conditioning, as well as providing a comparison for the probe on that day.

Initial analysis of this pilot found no effect for *trial* or *day* (see Figure 7.4a). A plot of CS preference against time indicated that extinction may occur in the first 1.5 minutes of the probe trial (Figures 7.4b), with a significant difference in preference between the two days (suggesting that extinction occurs more rapidly on the second day; Figure 7.5a).

However, subsequent analysis of these data found no significant aversion on either day (Figure 7.5b). This demonstrates that the parameters of the paradigm are insufficient to induce a conditioned aversion of the CS.

Manipulation of baseline (habituation) and 'dark-out' period

In order to establish efficacious parameters, an experiment was conducted to assessed whether increased exposure to the stimuli ('habituation') during baseline monitoring would facilitate aversion of the CS in the probe trial. Thus, the length of baseline was manipulated in this experiment (10 vs 30 minutes). Additionally, the 'dark-out' period was manipulated to assess the short-term length of aversion.

Initially a qualitative assessment was conducted. This indicated that increasing pre-conditioning (baseline) exposure to 30 minutes faciliatated immediate CS aversion, but that this aversion failed to be retained at 30 minutes following conditioning (see Figure 7.6). Subsequently, a quantitative experiment was conducted, empirically confirming this finding (see Figure 7.7).

Increased number of discrete trials

Following this establishment of an escape assay which induces immediate CS aversion, an experiment was conducted to assess whether increasing the number of discrete trials within the 30-minute conditioning session would increase aversion. Thus, the previously used conditioning parameters of thirty trials (30x 60-second discrete trials) was compared to ninety trials (90x 20-second discrete trials).

The result of this analysis found that both conditioning parameters induced significant aversion of the CS (see Figure 7.8). However no difference between the two conditions was found. As the aversion produced by the original parameters was of higher significance (despite this not being different from the new paradigm), the assay development proceeded around the thirty-trial paradigm.

Manipulation of '*dark-out*' period

Using the parameters established thus far (30-minute baseline; 30x 60-second conditioning trials), the 'dark-out' period was titrated to establish the short-term length of retention. This analysis failed to find significant aversion past a one-minute 'dark-out'

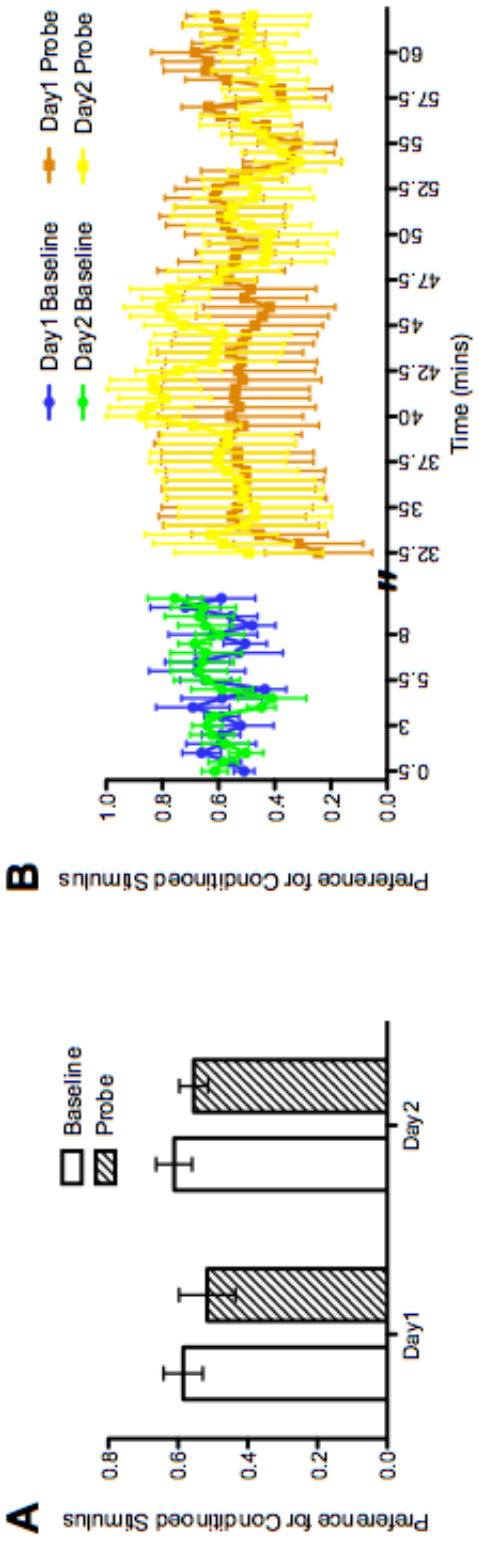

Figure 7.4: Assessment of pilot '*escape*' fear conditioning assay. (A) The mean CS preferences were not significantly different between *baselines* and *probes* ($F_{1,8}$=0.835, p=0.388). The preferences were not significantly different on *Day 1* from *Day 2* ($F_{1,8}$=0.094, p=0.768). No *trial* * *day* interaction was found ($F_{1,8}$=0.02, p=0.890). (B) CS preferences were plotted against time, indicating that aversive behaviour may extinguish in the first 1.5 minutes of probe trial. Data plotted as mean ± SEM.

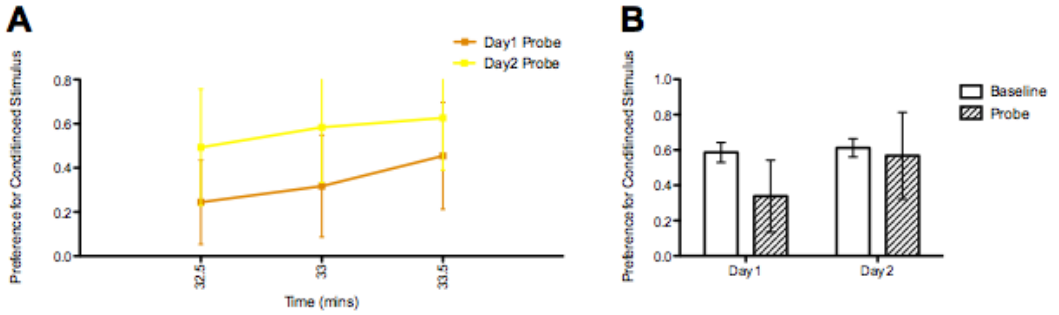

Figure 7.5: Analyses using 1.5-minute probes. (A) Comparison of the initial 1.5-minutes of probes (for each day) found a main effect was found for *day* ($F_{1,14.807}$=5.074, p=0.04). (B) Assessment of the full data set found no main effects; neither for *trial* ($F_{1,8.569}$=0.269, p=0.617) nor *day* ($F_{1,9.77}$=4.399, p=0.063). No *trial* * *day* interaction was found ($F_{1,8.769}$=1.91, p=0.201). Data plotted as mean ± SEM.

interval (see Figure 7.9). Whilst the aversion at five minutes may have reached significance with increased power (the baseline is lower than the other groups), it clearly does not continue past this time interval. Thus, it was deemed insufficient for the requirements of a *drug discrimination* assay.

Manipulation of US frequency

The trials conducted thus far have utilised a US administration frequency of one shock per second. Therefore, it was hypothesised that, by increasing the frequency of shock administration, increased aversion would be observed. However, the results of this experiment failed to support this hypothesis. Further, the increase in US frequency appears to trend towards a decrease in aversion (see Figure 7.10). Thus, the delivery of one shock per second was continued for assay development.

Repeated conditioning sessions

As the objective of the current chapter is the development of a *drug discrimination* assay, multiple conditioning sessions, over several days, will be required. Thus, the assay from this point will be developed in this manner.

Initial pilot

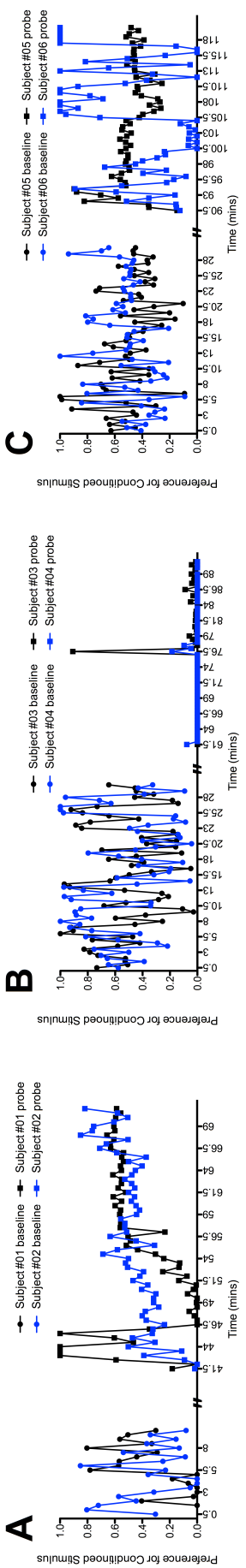

Figure 7.6: Qualitative manipulation of baseline (habituation) and '*dark-out*' periods. Two fish (per group) were assessed for their CS-aversion following different periods of habituation ('*baseline*') and '*dark-out*' (between conditioning and probe). (A) Ten minutes of habituation may have produced learning in one of the fish (Subject #01), however this appears to extinguish rapidly over the course of the probe trial. (B) By increasing the habituation time to thirty minutes, the fish appear to avoid the CS for the entirety of the probe trial. (C) Increasing the 'dark' period (between conditioning and probe) from 1-minute to 30-minutes appears to inhibit CS aversion. Data plotted as raw preference scores.

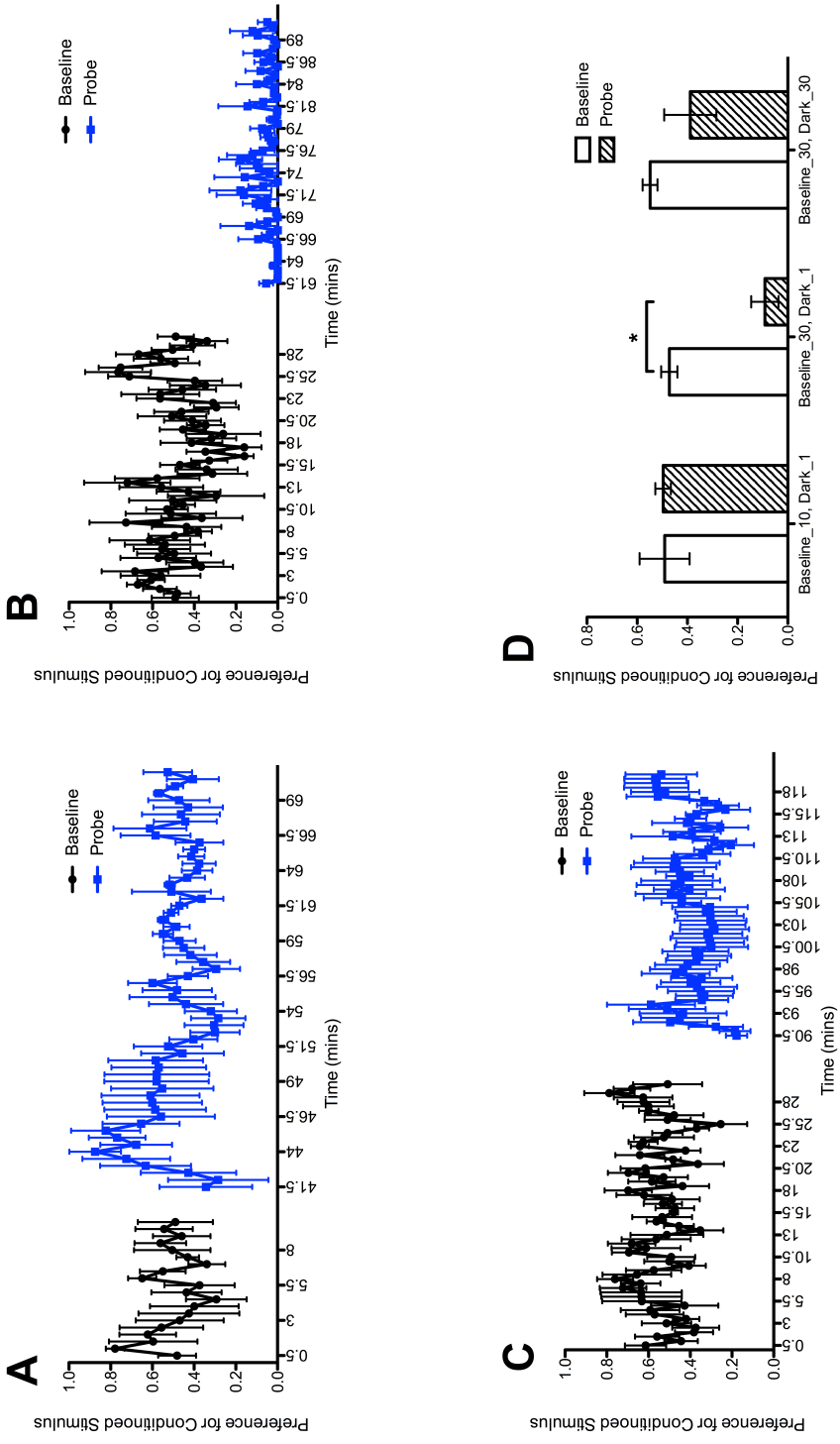

Figure 7.7: Quantitative manipulation of baseline (habituation) and '*dark-out*' periods. (A) Following ten minutes of baseline and one minute 'dark-out', no main effect was found for *trial* ($F_{1,313.902}$=1.673, p0.197). (B) Following thirty minutes of baseline and one minute of 'dark-out', a main effect was found for *trial* ($F_{1,433.116}$=32.095, p<0.0005). (C) Following thirty minutes baseline and 30 minutes 'dark-out', a significant main effect was found for *trial* ($F_{1,450.873}$=21.478, p<). (D) Direct comparison of these parameters found significant main effects for *trial* ($F_{1,19}$=5.454, p=0.031) and *length of baseline* (i.e. 10 vs 30 minutes) ($F_{1,19}$=4.958, p=0.038). No significant effect was found for *length of 'dark-out'* ($F_{1,19}$=3.281, p=0.086). By *linear mixed effects model*; *, p<0.0005. Data plotted as mean ± SEM.

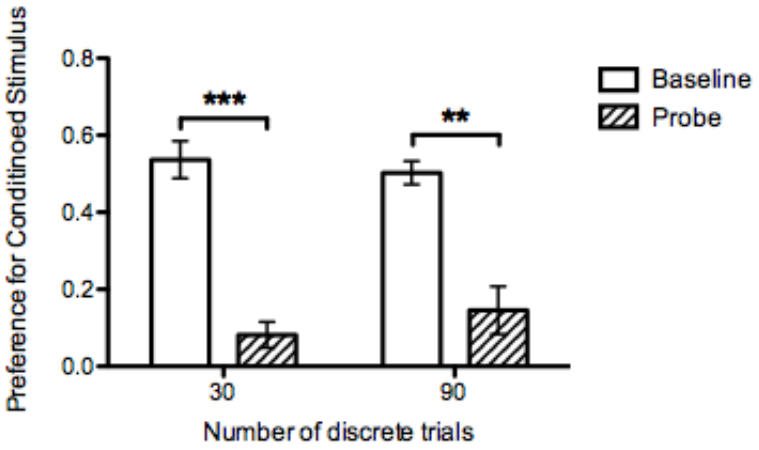

Figure 7.8: Increased number of discrete trials. Fish were exposed to the either 30 trials (30x 60seconds) or 90 trials (90x 20seconds). No significant effect was found for *number of discrete trials* ($F_{1,9.322}$=0.338, p=0.575). No *number of discrete trials* * *trial* interaction was found ($F_{1,9.753}$=1.433, p=0.26). A significant main effect was found for *trial* ($F_{1,15.729}$=55.488, p<0.0005). By *linear mixed effects model*, then *one-tailed* paired samples t-test with Bonferonni correction applied; **, p<0.01; ***, p<0.001 Data plotted as mean ± SEM.

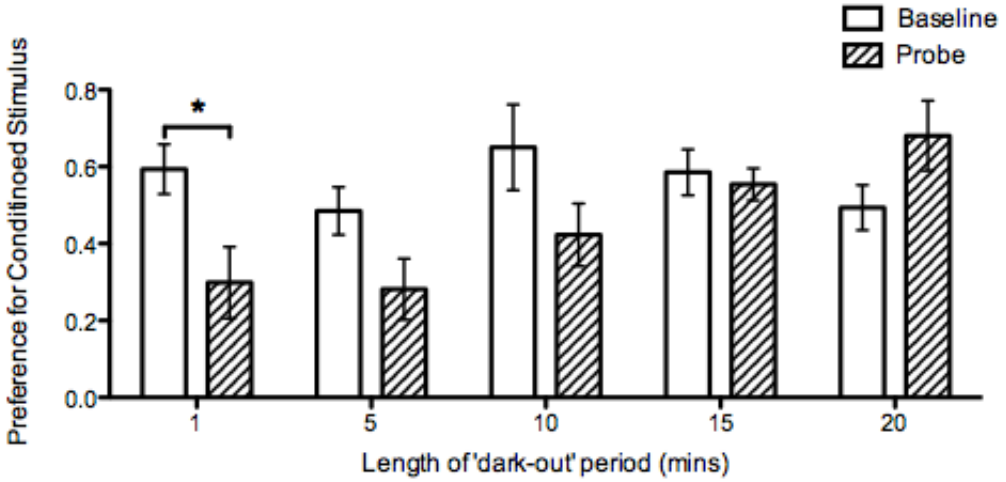

Figure 7.9: Titration of '*dark-out* period. The '*dark-out*' period between conditioning and probe trial was titrated to investigate the length of time for which aversion is observable. A main effect was found for *trial* ($F_{1,20.681}$=10.619, p=0.004). No main effect was found for the '*dark-out*' period ($F_{4,18.891}$=1.821, p=0.167); however a significant *trial* * *dark-out* interaction was found ($F_{4,18.834}$=6.234, p=0.002). By *linear mixed effects model*, then *one-tailed* paired samples t-test with Bonferroni correction applied; *, p<0.05. Data plotted as mean ± SEM

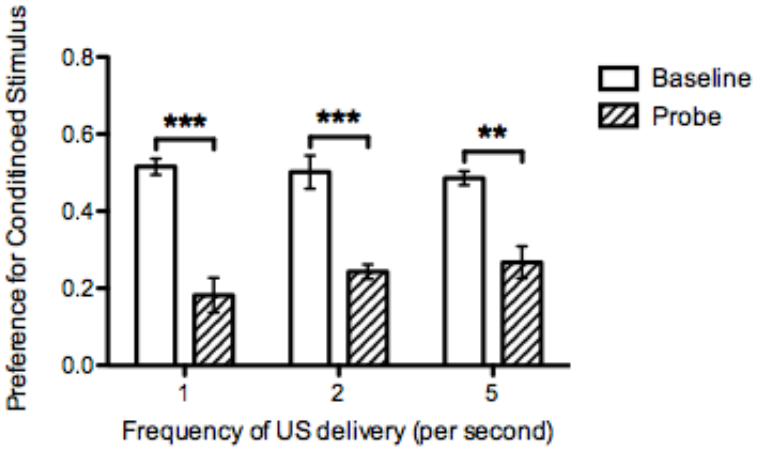

Figure 7.10: Manipulating the frequency of US delivery. During conditioning, zebrafish were exposed to US (9V electric shock) whilst swimming inside the '*zone*' presenting the CS (following the two seconds escape time at the start of each discrete trial). Shocks were delivered at a frequency of 1-, 2- or 5-shocks per second (as labelled). No main effect was found for *shock frequency* ($F_{2,47}$=0.219, p=0.804). A significant main effect was found for *trial* ($F_{1,47}$=45.255, p<0.0005). No *shock frequency* * *trial* interaction was found ($F_{2,47}$=1.389, p=0.259). By *linear mixed effects model*, then *one-tailed* paired samples t-test with Bonferroni correction applied; **, p<0.01; ***, p<0.001. Data plotted as mean ± SEM.

A pilot study was conducted to assess the prolonged retention of CS aversion, following multiple conditioning sessions. The following protocol was designed:

Day	Trial	Time
Day 1 (Monday)	Baseline	31 mins
	Conditioning (session 1)	31 mins
Day 2-4 (Tuesday - Thursday)	Conditioning (sessions 2-4)	31 mins
Day 5 (Friday)	Probe	31 mins

Results of this experiment found no significant aversion of the CS at 24 hours following the final conditioning session (see Figure 7.11).

Repeated conditioning sessions; manipulation of habituation exposure

It was hypothesised that increasing exposure to the conditioning stimuli may facilitate attention to them, resulting in increased aversion of the CS. Thus, prior to each conditioning session, zebrafish were exposed to both stimuli ('split' across the screen, as

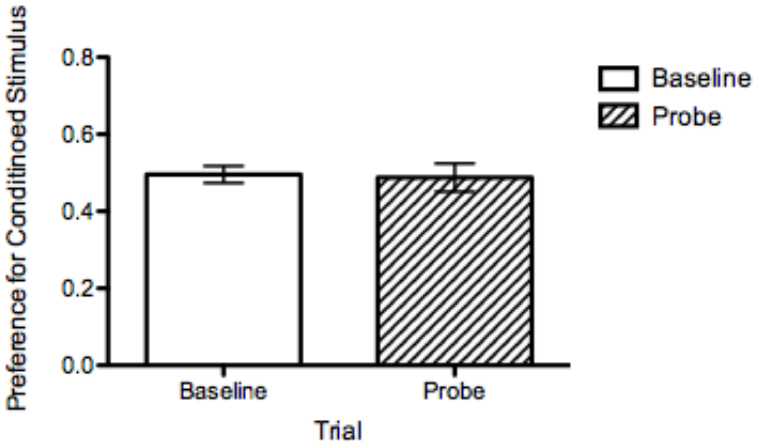

Figure 7.11: Repeated conditioning sessions pilot. Zebrafish were exposed to four conditioning sessions (one per day, on four consecutive days), then probed one day following the final conditioning session. No significant effect was found for *trial* ($F_{1,12}$=0.383, p=0.548). Data plotted as mean ± SEM.

in trial sessions), for thirty minutes. As a control, a group were also exposed to a blank (black) screen (as opposed to the stimuli), in the same manner. The results of this experiment found no significant aversion of the CS in either group (see Figure 7.12).

Repeated conditioning sessions; voltage titration

Finally, it was hypothesised that the CS may be too strong for repeated conditioning session (possibly due to induced stress). Thus, a voltage titration was conducted to assess whether a lower level of electric shock would facilitate longer-term aversion. The results of this titration experiment demonstrate that none of the voltages utilised induced an observable delayed aversion (see Figure 7.13).

7.5 Discussion

The results presented here failed to establish a paradigm of long-term conditioned responding - an essential precursor to the development of a *drug discrimination* assay.

As discussed in Chapter 3, simplistic *delay fear conditioning* is largely reliant on amygdala functioning; however with increased complexity of these paradigms, the hippocampus becomes involved (see Section 3.1.2). Thus, the establishment of a *delay*

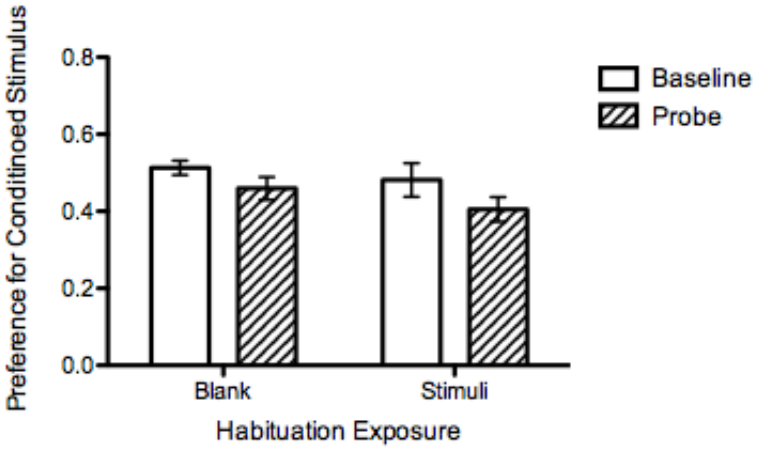

Figure 7.12: Manipulation of habituation exposure. Subjects were exposed to either a blank screen or the conditioning stimuli during the habituation period. No significant effects were found for *trial* ($F_{1,13}$=0.588, p=0.457) or *habituation exposure* ($F_{1,13}$=4.051, p=0.065). No *trial* * *habituation exposure* interaction was found ($F_{1,13}$=0.005, p=0.946). By *linear mixed effects model*. Data plotted as mean ± SEM.

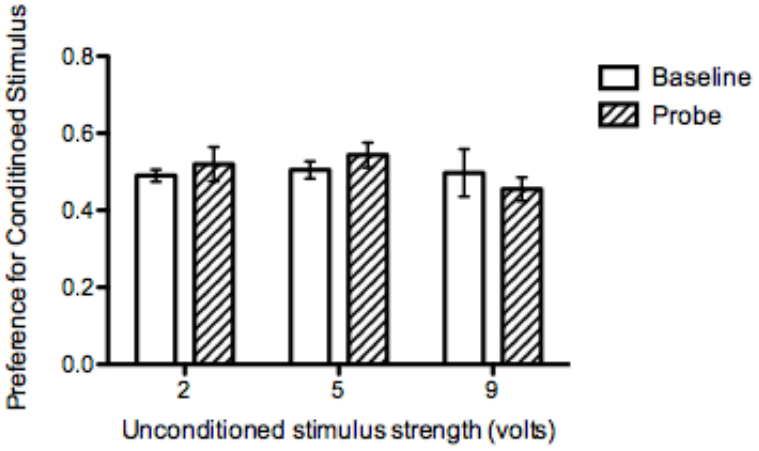

Figure 7.13: Voltage titration. Zebrafish were conditioned on four consecutive days, using 2V, 5V, or 9V US. No main effects were found for *trial* ($F_{1,19.539}$=0.074, p=0.789) or *voltage* ($F_{2,12.711}$=0.662, p=0.533). No *trial* * *voltage* interaction was found ($F_{2,12.352}$=1.023, p=0.388). Data plotted as mean ± SEM.

fear conditioning assay (developed in Chapter 3, and used in all subsequent chapters) indicated that the amygdala functioning which facilitates this manner of conditioning is conserved in zebrafish (likely in the Dm). However, in the current chapter, the requirement of more complex conditioning (to develop a *drug discrimination* assay) failed to produce significant aversion (when measured at a more delayed time point). Thus, it is possible that the conservation of hippocampal functioning (likely in the zebrafish Dl) is insufficient to reproduce more complex *fear conditioned* behaviours. In this regard, it would be of interest for future research to investigate *trace fear conditioning* paradigms in zebrafish. Indeed, by titrating the length of the *trace* period, the extent of hippocampal functional conservation may be indicated.

Prior research (in which amygdala functioning was inhibited in rats) has demonstrated the amygdala's involvement in the acquisition of *classical fear conditioning*, but not its consolidation (Wilensky et al. 2000). Thus, the failure of the current research to observe aversive behaviour at a delayed point in time (i.e. beyond one minute post conditioning) suggests a lack of consolidation. In this regard, rats with hippocampal lesions acquire conditioning, however the rate of extinction is accelerated (Winocur 1990). This supports the argument - in light of the current findings - that amygdala functioning is conserved in zebrafish to a much greater extent than hippocampal functioning. However the level of zebrafish 'hippocampcal-associated functioning' (i.e. memory consolidation) must now be investigated in order to predict the possibility of exploiting this function in the development of a *drug discrimination* paradigm.

It must be noted that others have reported longer-term retention of fear conditioning using simple operant paradigms. Zebrafish have been conditioned to cross a 'hurdle' in response to a CS, to avoid an electric shock (e.g. Pradel et al. 1999; Xu et al. 2007). Thus conditioned learning is assessed by a single operant response - crossing the hurdle from one compartment to the other. It may be possible to develop this paradigm into a two-choice assay for utility as a drug discrimination paradigm, by pairing the US to different CS (i.e. two different coloured lights). However undertaking this investigation would involve the construction and development of new hardware (i.e. new assay tanks with a hurdle and a dark compartment) which was not achievable in the time-frame of the current thesis. Thus, future research may wish to attempt this method of assay development.

Additionally, perineuronal nets (PNN; comprised of chondroitin sulphate proteoglycans, CSPG) have been shown to play an important role in inhibiting the extinction-induced loss of fear conditioned memory traces. In juvenile rats, prior to PNN development (<24 days), extinction of fear conditioning actually leads to the erasure of the fear memory (measured by a lack of '*spontaneous recovery*' of the conditioned response); however, following the formation of PNNs, extinction no longer erases the memory (e.g. Gogolla et al. 2009; Quirk et al. 2010; Wang and Fawcett 2012). This has

been associated with the function of PNNs in stabilising synapses, inhibiting neuroplastic changes (Wang and Fawcett 2012). In this regard, the function of CSPGs in zebrafish appears to be inverse from that of mammals; in zebrafish they have been shown to guide axonal regeneration, a function inhibited by CSPGs in mammals (Becker and Becker 2002; Kroehne et al. 2011). Thus, it seems logical that zebrafish PNNs may facilitate the breakage of synapses (as a mediator of synaptic plasticity), especially in light of the increase plasticity of the zebrafish brain (Kizil et al. 2012; Kyritsis et al. 2012). However research into this function of PNN in zebrafish is currently lacking. Thus, it may present an interesting area for future investigations into mechanisms of neuronal regeneration.

It is possible that the repeated administration of an aversive US results in '*learned helplessness*'. In this regard, it may be possible to exploit the social nature of zebrafish to avoid complications of both aversive (learned helplessness) and appetitive (satiation) reinforcement. The presentation of conspecifics has been utilised as a US, which may provide increased motivation to respond (e.g. Sison and Gerlai 2011). However, again, this investigation was not achievable within the time-frame of the current thesis.

In conclusion, the apparent lack of conservation of hippocampal functioning in zebrafish presents a problem in the development of a *drug discrimination* assay. However a greater elucidation of the extent of this conservation in necessary, in order to exploit these competencies for the development of state-dependent - and subsequently, *drug discrimination* - paradigms. Designing paradigms around operant behaviours, or else utilising a social (rewarding) US may lead to increased retention of learning, and may be more efficacious in the development of a *drug discrimination* assay.

Chapter 8

General Discussion

8.1 Thesis summary

The purpose of the research undertaken in this thesis was to evaluate the utility of zebrafish - as a pre-mammalian model organism - in translational neurobiological research settings. This focussed on two related neurological conditions - Alzheimer's-like diseases and drug reinforcement.

8.1.1 Alzheimer's-like diseases

The establishment of a *classical aversion* paradigm - and its validation using aluminium toxicity - presents an exciting tool for both pharmacological and genetic research. Indeed, the fact that this assay is so quickly implemented (the whole cycle, which may assess multiple fish simultaneously, takes 63.5 minutes) demonstrates its effective implementation for screening purposes. Further, the demonstration that zebrafish function in this AD-related behavioural paradigm, and that this learning is inhibited by neurotoxin-induced neurodegeneration, indicate the efficacy of zebrafish in this area of research.

To further regard the utility of zebrafish, the finding that transgenic $hAPP_{LON/SWE}$ expression causes an age-related decline in performance in the behavioural assay promotes the use of zebrafish as a refinement (or at least pre-screen) to rodents. Indeed, whilst doubts are cast over the role of amyloid in the aetiologies of AD, the data generated from the transgenic line suggest that zebrafish may be implemented in the stead of commonly used models of rodent amyloidopathies.

Additionally, the generation of new transgenic zebrafish was undertaken and achieved. Whilst it was not possible to characterise these lines in the current thesis, they present tools which may be efficacious for future investigations into Alzheimer's-like pathologies.

8.1.2 Safety pharmacology

A range of compounds with neuroprotective properties were assessed for their abilities to induce CPP in zebrafish. Both of the *stimulants* (caffeine and nicotine), both of the *opiates* (fentanyl and morphine) and the *alcohol* (ethanol) administered caused significant changes in stimulus preference. This demonstrates a conservation of their pharmacological targets (and their down-stream signalling cascades) within reward pathways, in zebrafish. Similarly, the significant effect for the *general anaesthetic*, PCP, demonstrates that its pharmacological action (NMDA-R antagonism) produces a conserved effect in zebrafish.

However, the other *general anaesthetic*, ketamine, failed to produce a significant effect, despite having the same primary pharmacological action. In this regard, its lower affinity for the receptor may demonstrate that NMDA-R-induced reward signalling is weaker in zebrafish than in mammals.

Similarly, the failure of the *antidepressant* (bupropion), *local anaesthetic* (procaine) and *cannabinoid* (THC) to induce CPP suggest a lower level of conservation in the signalling facilitating these reward mechanisms (DA and norepinephrine re-uptake inhibition, DA secretion and NMDA inhibition, and CB-induced DA release; respectively).

8.1.3 State-dependent learning

The failure to establish a *drug discrimination* assay does not indicate an inability of zebrafish to perform state-dependent learning tasks, as the assay development failed to induce prolonged retention of learning. This may be due to accelerated extinction (e.g. exposure to the stimuli causes a form of *retrieval-induced forgetting*). Alternatively, it may be that memory consolidation is less efficient in zebrafish (suggesting a lower conservation of hippocampal-related functioning).

In this regard, several different paradigms may be adopted - using either aversive and non-aversive US - as alternatives to the one utilised here. Thus, the failure to establish an assay in the current thesis should not be regarded as a failure of zebrafish to perform this task. Rather, it presents one method by which they do not retain long-term conditioned aversion. However, in light of the myriad different paradigm which can be adopted, it was not possible to fully explore the development of a *drug discrimination* assay in the time-frame of the current thesis.

8.2 Future directions

8.2.1 Neurodegeneration

The findings of impaired *fear conditioning* in transgenic $hAPP_{LON/SWE}$ fish appears to have positive implications for industrial pharmaceutical screening of novel AD-targetting drugs. However it is necessary for future research to attempt to rescue this phenotype via the administration of neuroprotective compounds. Whilst this investigation was beyond

the scope of the current research, it would provide further support to the implementation of zebrafish as a pre-mammalian organism in industrial drug discovery.

Additionally, the generation of new gene-manipulation strains of zebrafish may provide exciting tools for future investigations into the pathogenesis of AD. The hAβ_{40} transgenic line may provide insight into the downstream signalling consequences of this peptide, in the absence of aberrant neuronal apoptosis expected from Aβ42 expression, thus elucidating the consequences of dysregulated amyloid signalling. Perhaps more interestingly, the dapk1-overexpressing zebrafish line may provide some insight into AD-related dysregulations up-stream of amyloid dysregulation. Thus, the utilisation of these generated lines may further facilitate research, both in basic and in translational settings.

It must also be noted that there are potential implications for the fear conditioning assay (developed herein) in the research of other neurodegenerative conditions:

Parkinson's disease

The loss of DA-ergic neurons in the substantia nigra is the major pathological characteristic of PD (Bernheimer et al. 1973; Hornykiewicz 1975). However the amygdalae and hippocampi receive DA-ergic afferent signals from the substantia nigra, suggesting secondary (non-motor) dysfunctions to these structures (Gasbarri et al. 1994; Menegas et al. 2015; Swanson 1982).

In this regard, the presence of (PD-associated) Lewy bodies has been reported in the amygdala of PD patients, as well as a decreased amygdala size (Braak et al. 1994; Harding et al. 2002). Additionally, decreased DA activity in the amygdala has been associated with depression in PD (Remy et al. 2005). This implies that patients would exhibit impairments to behavioural / emotional responses related to amygdala functioning. Indeed, PD patients show reduced startle responses, as well as decreased amygdala activity when perceiving fear, which is slightly rescued by DA treatment (Bowers et al. 2006; Tessitore et al. 2002).

Rodent studies have demonstrated that the stimulation or selective destruction of DA-ergic neurons in the substantia nigra disrupts nociceptive responses (Barceló et al. 2012; Carey 1986; Takeda et al. 2005; Tassorelli et al. 2007; see Chudler and Dong 1995). This suggests an impairement in US detection, and subsequently CS pairing. Further, there is evidence that inhibition of DA-ergic neurons in the *pars compacta* of the substantia nigra - which project to the *central nucleus* of the amygdala - results in impaired 'surprise' learning (Lee et al. 2008)

Thus, the evidence of amygdala dysfunction in PD suggests an impairment to *fear conditioning*. However currently there is no data (clinical or animal) to support this hypothesis. Thus, it is proposed that future studies into non-motor aspects of PD investigate the possibility of impaired *fear conditioning*. This would allow the assay developed herein to be utilised in this area of research.

Huntington's disease

HD is caused by a mutation in the extremely ubiquitous *HTT* gene which, within neurobiology, is commonly investigated in relation to atrophy of the caudate nucleus, putamen, cortical, striatal and basal ganglia areas (e.g. Bernheimer et al. 1973; Jernigan et al. 1991; Kassubek et al. 2004; Roos et al. 1985; Thieben et al. 2002; Vonsattel et al. 1985; see Petrasch-Parwez et al. 2012; Burg et al. 2009). Given the crossover of these sites with PD, the same logical justifications of impaired amaygdala functioning - and subsequently *aversive conditioning* - may apply.

Indeed, a certain amount of attention has been paid to the amygdala in HD research. fMRI studies in patients has associated decreases in amygdala volume and connectivity with impaired recognition of emotional facial expressions (Kipps et al. 2007; Mason et al. 2015). Further, pre-symptomatic carriers of the mutated *HTT* gene display decreased amygdala activity during a task that induces 'irritability', suggesting that a test of amygdala function may be useful in early detection (Klöppel et al. 2010).

As with PD, there is a lack of studies that have investigated *aversive conditioning* in HD patients. However transgenic mice expressing a human mutant form of *HTT* display impaired fear conditioning, suggesting that implementing a mutant zebrafish model of HD in the assay developed herein would have implications for HD lead identification (Bolivar et al. 2003).

8.2.2 Safety pharmacology

The selection of neuroprotective drugs which induced CPP suggest that drugs developed with these pharmacological actions may be screened for their reinforcing properties in zebrafish, prior to mammalian (rodent) assays. In this light, a more comprehensive range of drugs and drug classes may be investigated in the future, providing greater insight into the conservation of reward-related pharmacological signalling consequences, as touched upon here.

Finally, the attempt to develop a *drug discrimination* assay utilising *fear conditioning* paradigms suggests that this mode of conditioning is insufficient for consolidation of learning. However the implementation of alternative paradigms (using aversive, appetitive or social US) has previously been reported to produce learning with longer-term retention. Thus, future investigations may wish to adopt these alternative paradigms, which may prove more efficacious in the development of a *state-dependent* learning assay.

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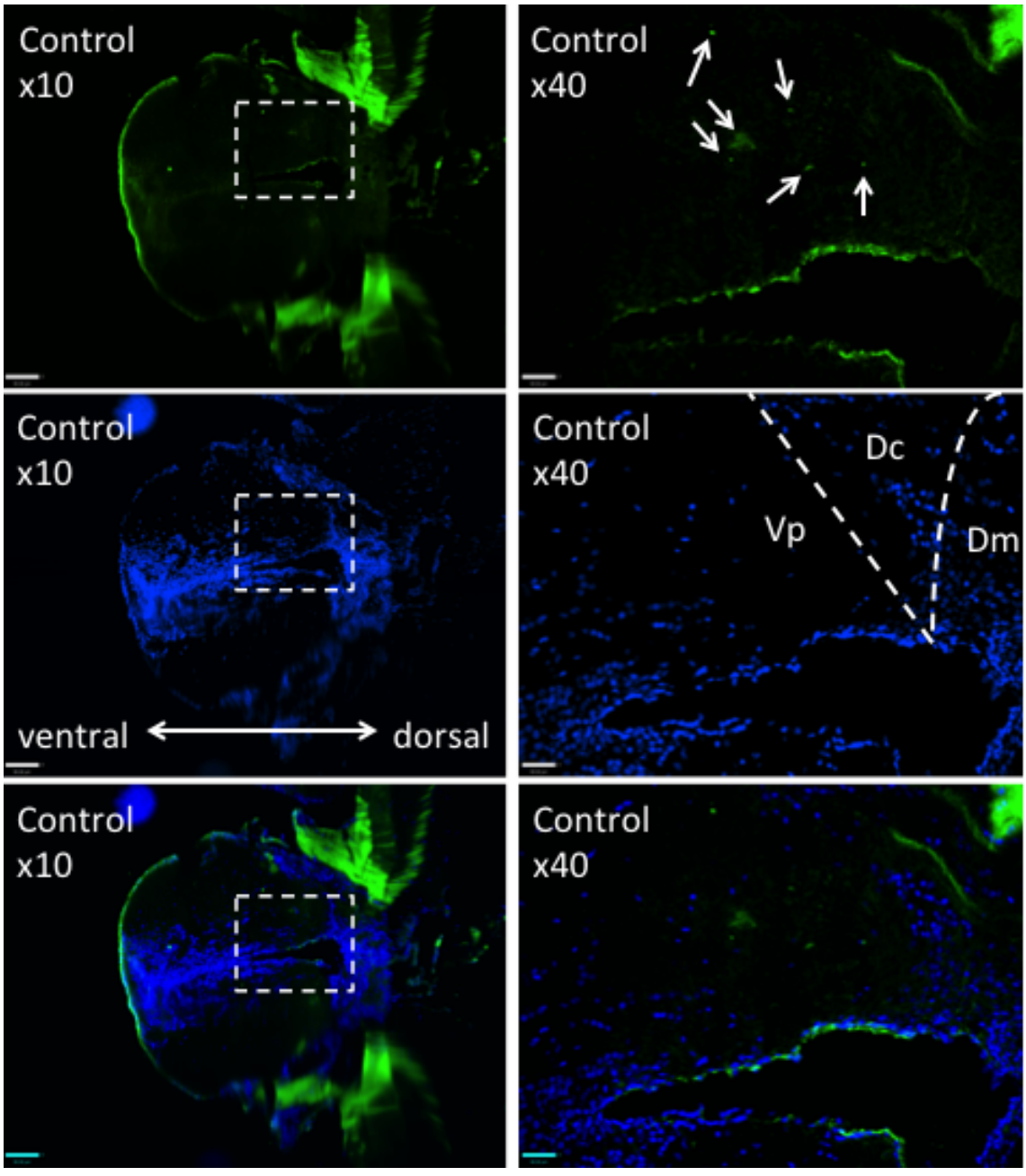
Chapter 9

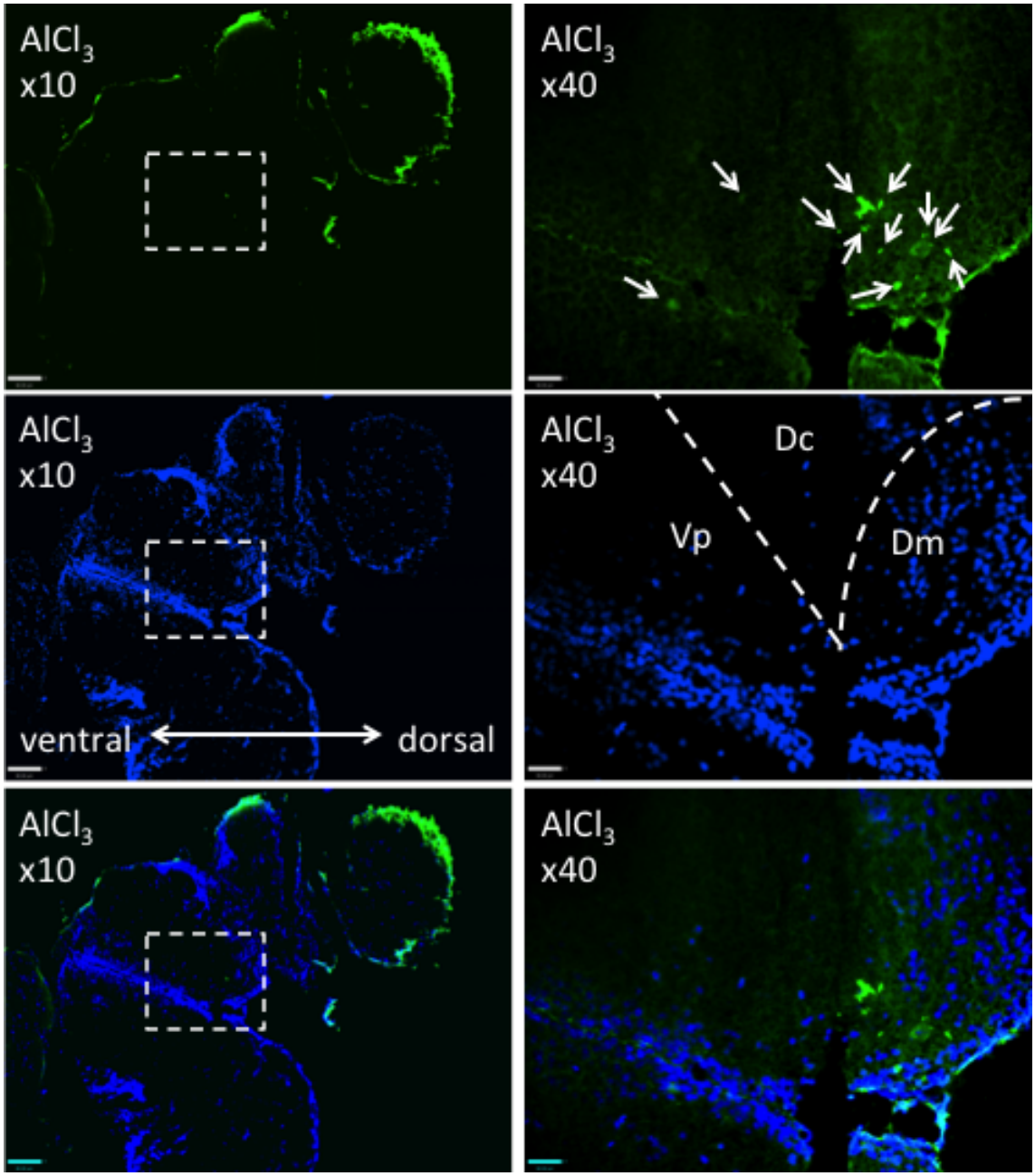
Appendices

9.1 FluoroJade® staining of $AlCl_3$ brains

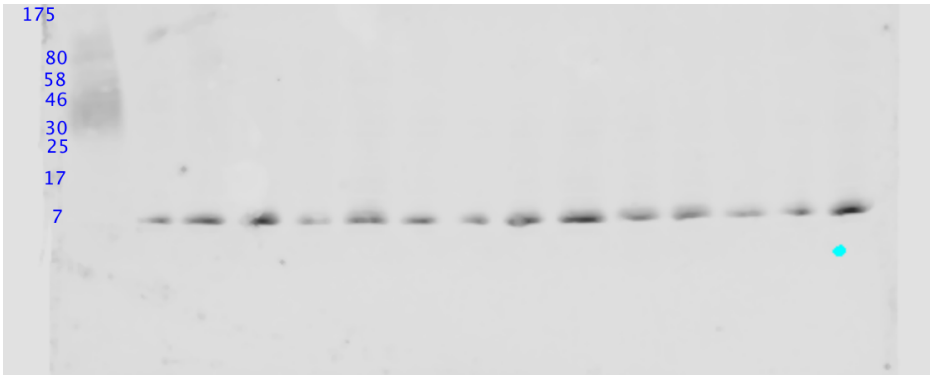
Control

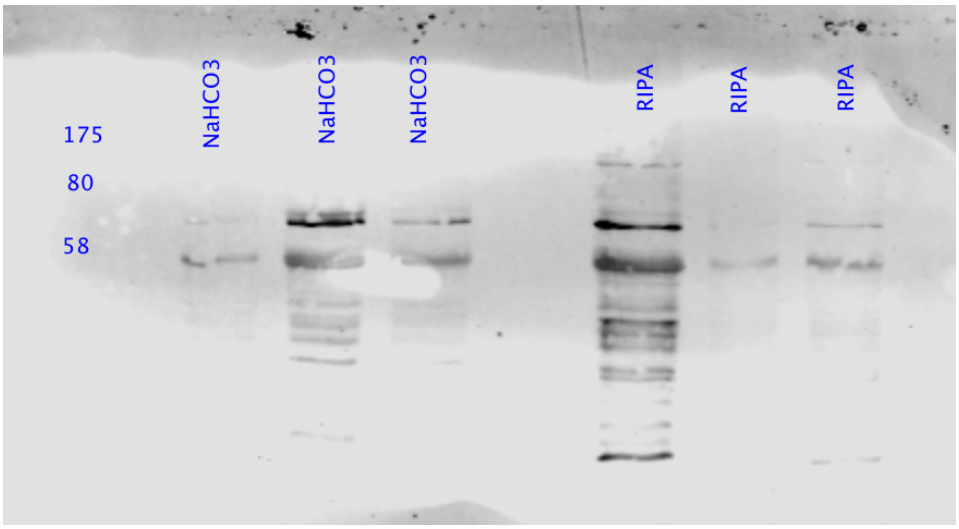


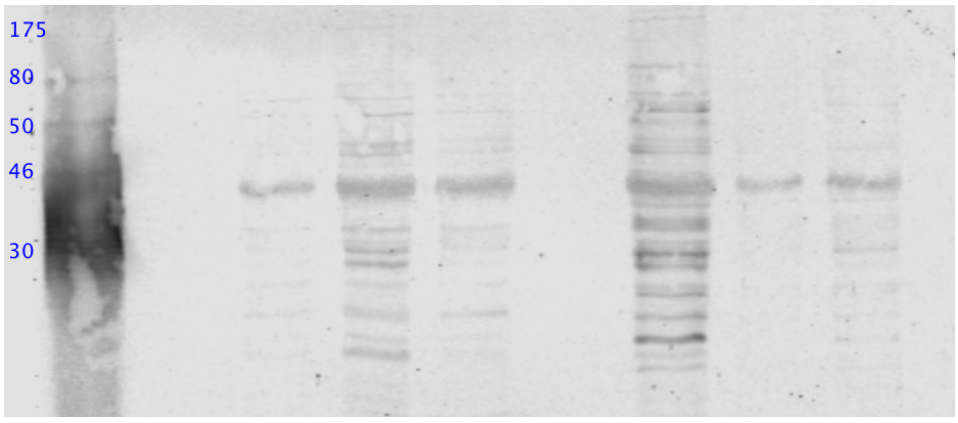
100μM


AlCl3
x10
AlCl3
x40
AlCl3
x10
AlCl3
x40
Dc
Vp
Dm
ventral
dorsal
AlCl3
x10
AlCl3
x40

9.2 Failed western blot detection of appb







Failed western blot detection of appb. Total protein extractions were run on SDS-PAGE and blotted onto nitrocellulose. The antibody used (Abcam ab17473) failed to produce consistent results, probing protein bands of varying sizes. Thus, these data were not included in the results of this thesis

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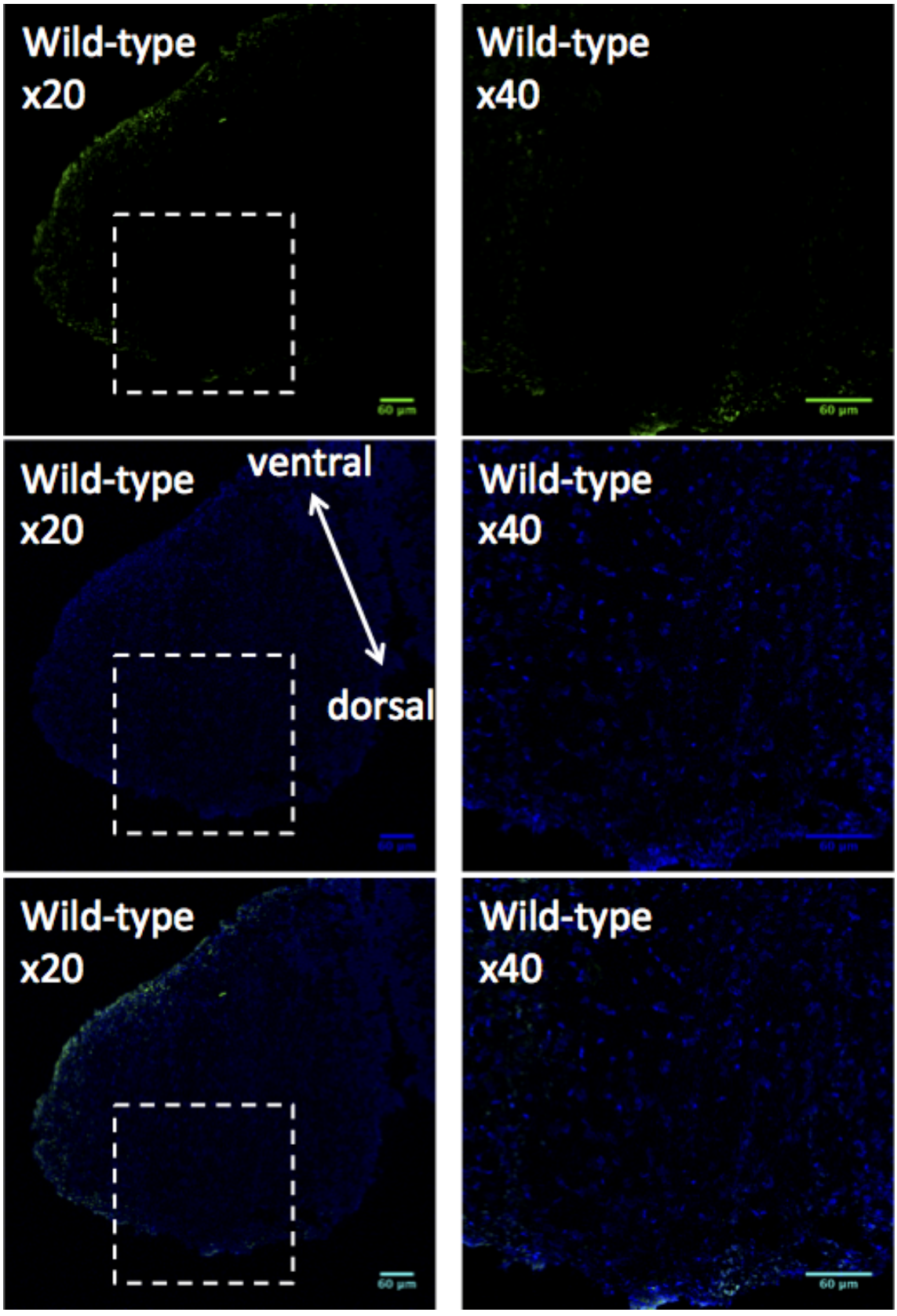
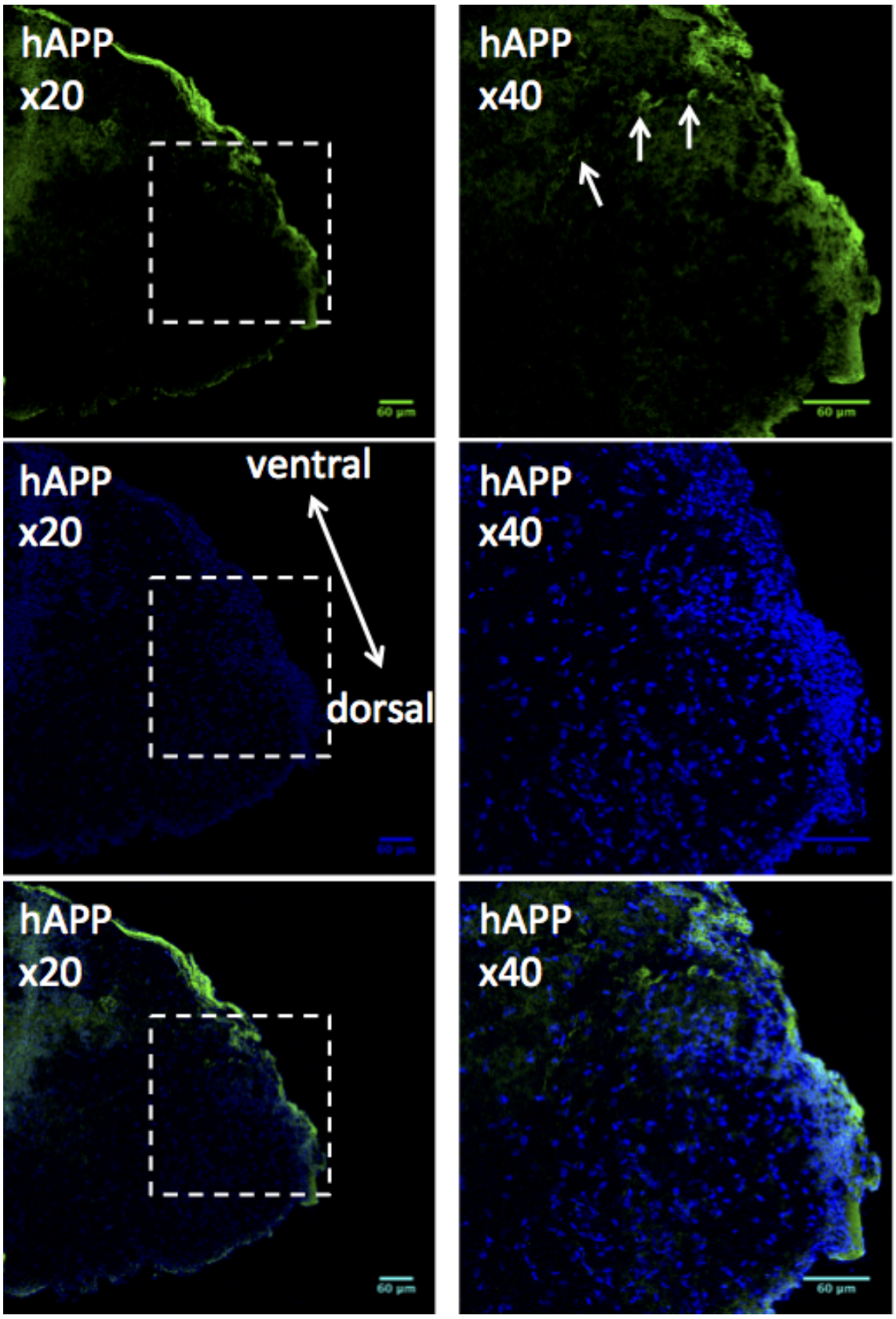
9.3 FluoroJade® staining of $hAPP_{LON/SWE}$ brains

Control



100μM


hAPP
x20
hAPP
x40
hAPP
x20
ventral
dorsal
hAPP
x40
hAPP
x20
hAPP
x40