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**Investigating the effects of dopamine and 3', 5'-
cyclic adenosine monophosphate-regulated neuronal
phosphoprotein, 32 kDa (DARPP-32) deletion on
adaptive reward-based learning and performance**

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Investigating the effects of dopamine and 3', 5'-cyclic adenosine monophosphate-regulated neuronal phosphoprotein, 32 kDa (DARPP-32) deletion on adaptive reward-based learning and performance.

Dopamine and 3',5'-cyclic adenosine monophosphate-regulated neuronal phosphoprotein (DARPP-32) is a critical mediator of neuroplasticity in striatal medium spiny neurons (MSNs). The work presented in this thesis used a global gene knockout (KO) construct to investigate the role of DARPP-32 in reward-based learning and performance.

Global deletion of the DARPP-32 gene disturbed performance during the intertemporal (delay) discounting procedure. DARPP-32 KO mice were less sensitive than their wildtype (WT) littermates during long delays to reinforcement. In comparison to WT mice, DARPP-32 KO mice also developed a risk-sensitive pattern of choices during a probability discounting task. Unlike the effects of DARPP-32 deletion on reinforcement along dimensions of time and risk, DARPP-32 knockout did not affect the degree of effort that subjects were willing to invest during food-reinforced progressive ratio testing. DARPP-32 KO mice also failed to exhibit Pavlovian-to-instrumental transfer and this impairment could not be rescued by administering methylphenidate prior to test. Finally, DARPP-32 KO mice were indistinguishable from WT mice during an amphetamine psychomotor sensitisation study.

Overall, the data in this thesis suggest DARPP-32 is involved in adaptive reward-based learning and performance.

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TABLE OF CONTENTS

Page

CHAPTER 1

INTRODUCTION

1.1 Introduction	1
1.2 Incentive motivation.....	2
1.2.1 Basic Pavlovian learning mechanisms in incentive motivation.....	2
1.2.2 Instrumental learning	3
1.3 Pavlovian mechanisms and the attribution of incentive salience.....	5
1.3.1 Glutamatergic and dopaminergic modulation of incentive salience	7
1.4 Action-outcome learning and the attribution of reward value	8
1.4.1 Cost-benefits computations and action-outcome like instrumental choices.....	9
1.4.2 Cost-benefits computations are not wholly guided by A-O principles	11
1.4.3 Glutamatergic and dopaminergic modulation of cost-benefits computations	12
1.5 Conclusion	13
1.6 Motivational circuits: a simplified description of incentive motivational neurocircuitry and its functions.....	14
1.6.1 The connections of the striatum	16
1.6.2 Amygdaloid connections.....	18
1.6.3 Conclusion	19
1.7 Prefrontal cortex function in motivated behaviour	19
1.7.1 Ventral striatal function in motivated behaviour	20
1.7.1.1 Nucleus accumbens: the core vs shell distinction.....	21
1.7.2 Caudate-putamen function	22
1.7.3 Ventral pallidum function in motivated behaviour	23
1.7.4 Ventral tegmental area function	24
1.7.5 Amygdala function in reward learning	24
1.7.6 Conclusion	25
1.8 DARPP-32: a complex neuronal phosphoprotein	26

1.8.1 DARPP-32 is influenced by protein kinases and protein phosphatases	28
1.8.2 DARPP-32: a molecular interface for postsynaptic neurotransmission	29
1.8.3. Thr ³⁴ -DARPP-32 phosphorylation and PP-1 inhibition.....	30
1.8.4 Dopamine and glutamate mediated dephosphorylation of Thr ³⁴ -DARPP-32.....	31
1.8.5 Thr ³⁴ -DARPP-32 phosphorylation is fine-tuned by activity at serine residues	31
1.8.6 DARPP-32 mediates nuclear processes	32
1.8.7 Thr ⁷⁵ -DARPP-32 phosphorylation and PKA inhibition	32
1.8.8 Summary	33
1.9 DARPP-32 is a molecular regulator of MSN receptor phosphorylation and electrophysiology	34
1.9.1 DARPP-32 and AMPA receptor phosphorylation	34
1.9.1.1 DARPP-32 modulates AMPA channel currents	35
1.9.2 DARPP-32 and NMDA receptor phosphorylation	36
1.9.2.1 DARPP-32 modulates NMDA channel currents	36
1.9.3 DARPP-32 and GABA _A receptors.....	37
1.9.4 DARPP-32 and ion channel regulation	37
1.9.5 DARPP-32 is a critical mediator of striatal synaptic plasticity.....	38
1.9.6 DARPP-32 is located in MSN spine heads and necks	39
1.9.7 Conclusion	39
1.10 DARPP-32 a common striatal substrate for drugs of abuse: behavioural and molecular insights from acute and repeatedly administered drugs of abuse	40
1.10.1 DARPP-32 and psychomotor sensitisation	42
1.10.2 DARPP-32 is a critical mediator of drug-induced gene transcription.....	43
1.10.3 DARPP-32 and drug reinforced learning	43
1.11 DARPP-32 and natural reward.....	44
1.11.1 DARPP-32: instrumental learning and behavioural flexibility.....	45
1.12 DARPP-32 and human reinforcement learning	46
1.13 The DARPP-32 knockout mouse	47
1.14 Thesis aims	48

CHAPTER 2

FLUORESCENCE IMMUNOHISTOCHEMISTRY

2.1 Introduction	52
2.2 Methods.....	54
2.2.1. Genotyping.....	54
2.2.2 Subjects	55
2.2.3 Fluorescent immunohistochemical (fIHC) detection of DARPP-32	55
2.2.4 Imaging	56
2.3 Results	57
2.3.1 Telencephalon	58
2.3.2 Prefrontal, motor and somatosensory cortices	59
2.3.3 Anterior olfactory nucleus (AON)	60
2.3.4 Amygdala	61
2.3.5 Basal ganglia	63
2.4 Discussion	65

CHAPTER 3

INTERTEMPORAL DISCOUNTING

3.1 Introduction	70
3.2 Methods.....	73
3.2.1 Subjects	73
3.2.2 Apparatus	73
3.2.3 Procedure.....	74
3.2.3.1 Magazine training.....	74
3.2.3.2 Lever training	74
3.2.3.3 Trial initiation training	74
3.2.3.4 Delay discounting.....	76
3.2.3.5 Reinforcer devaluation	78
3.2.4 Data analysis	79
3.3 Results	81
3.3.1 Lever training	81
3.3.2 Trial initiation training	81
3.3.3 Delay discounting.....	82

3.3.3.1 Sessions 8-12.....	82
3.3.3.2 Sessions 33-37.....	83
3.3.3.3 Sessions 47-51.....	85
3.3.3.4 Sessions 64-68.....	87
3.3.3.5 Sessions 69-70.....	88
3.3.3.6 Sessions 71-78 and 81-92.....	89
3.3.3.7 Sessions 71-75 vs sessions 88-92	
stability analysis	91
3.3.3.8 Sensory-specific reinforcer devaluation.....	92
3.3.3.9 General motivational reinforcer devaluation	94
3.4 Discussion	97

CHAPTER 4

PROBABILITY DISCOUNTING

4.1 Introduction	106
4.2 Methods.....	110
4.2.1 Subjects	110
4.2.2 Apparatus	110
4.2.3 Procedure.....	110
4.2.3.1 Magazine training.....	110
4.2.3.2 Lever training	111
4.2.3.3 Trial initiation training	111
4.2.3.4 Probability discounting	112
4.2.3.5 Reinforcer devaluation	114
4.2.4 Data analysis	114
4.3 Results	117
4.3.1 Lever training	117
4.3.2 Trial initiation training	117
4.3.3 Probability discounting	117
4.3.3.1 Sessions 30-34	118
4.3.3.2 Sessions 35-39	120
4.3.3.3 Sessions 40-44	121
4.3.3.4 Stability analysis.....	123
4.3.3.5 Sensory specific devaluation	124

4.3.3.6 General motivational devaluation.....	126
4.4 Discussion	127

CHAPTER 5

PROGRESSIVE RATIO

5.1 Introduction	134
5.2 Methods.....	137
5.2.1 Subjects	137
5.2.2 Apparatus	138
5.2.3 Procedure	138
5.2.3.1 Magazine training.....	138
5.2.3.2 Phase one: PR responding for sucrose	138
5.2.3.2.1 Continuous reinforcement	138
5.2.3.2.2 Fixed ratio training	139
5.2.3.2.3 Progressive ratio training.....	139
5.2.3.3 Phase two: PR responding for milk.....	141
5.2.3.3.1 Continuous reinforcement	141
5.2.3.3.2 Fixed ratio training	141
5.2.3.3.3 Progressive ratio training.....	141
5.2.4 Data analysis	142
5.3 Results	143
5.3.1 Phase one	143
5.3.1.1 Instrumental training	143
5.3.1.2 $j = 0.2$ schedule (240 min sessions 30 min TO)	143
5.3.1.3 $j = 0.2$ schedule (240 min sessions 30 min TO)	
pre-feeding manipulation.....	144
5.3.1.4 PR x 2 (240 min sessions 30 min TO).....	145
5.3.1.5 $j = 0.2$ schedule (60 min sessions 10 min TO)	146
5.3.1.6 $j = 0.2$ schedule (60 min sessions no TO)	147
5.3.2 Phase 2: Nosepokes	148
5.3.2.1 Instrumental training	148
5.3.2.2 $j = 0.2$ schedule (60 min sessions no TO)	148
5.3.2.3 $j = 0.12$ schedule (60 min sessions no TO)	
pre-feeding manipulation.....	149

5.3.2.4 $j = 0.12$ (120 min sessions 30 min TO)	150
5.3.2.5 PR-1 (90 min sessions no TO).....	151
5.3.2.6 PR-1 (90 min sessions no TO) pre-feeding manipulation.....	152
5.4 Discussion	152

CHAPTER 6

PAVLOVIAN-TO-INSTRUMENTAL TRANSFER

6.1 Introduction	156
6.2 Methods	159
6.2.1 Subjects	159
6.2.2 Drug	160
6.2.3 Apparatus	160
6.2.4 Procedure.....	160
6.2.4.1 Magazine training	160
6.2.4.2 Pavlovian training	160
6.2.4.3 Instrumental training	161
6.2.4.4 Pavlovian-to-instrumental transfer test	161
6.2.5 Data analysis	162
6.3 Results	164
6.3.1 Instrumental training	164
6.3.2 Pavlovian training	165
6.3.3 Pavlovian to instrumental transfer test.....	167
6.3.3.1 Active lever change scores	167
6.3.3.2 Inactive lever change scores.....	168
6.3.3.3 Magazine entries	168
6.4 Discussion	169

CHAPTER 7

AMPHETAMINE PSYCHOMOTOR SENSITISATION

7.1 Introduction	175
7.2 Methods.....	178
7.2.1 Subjects	178
7.2.2 Apparatus	178
7.2.3 Drug	179

7.2.4 Procedure.....	179
7.2.5 Data analysis	180
7.3 Results	181
7.3.1 Habituation.....	181
7.3.2 Acquisition of amphetamine sensitisation	182
7.3.3 Amphetamine sensitisation acquisition slope values	183
7.3.4 Dose-effect test habituation	184
7.3.5 Dose-effect test following 7 day wash-out period	185
7.4 Discussion	186

CHAPTER 8

SUMMARY OF FINDINGS AND GENERAL DISCUSSION

8.1 Introduction	193
8.2 DARPP-32 and intertemporal discounting.....	194
8.3 Probability discounting	195
8.4 Progressive ratio.....	196
8.5 Pavlovian-to-instrumental transfer.....	196
8.6 Amphetamine psychomotor sensitisation	197
8.7 DARPP-32 and the effect of motivational shifts	198
8.8 Behavioural conclusion.....	199
8.9 Biological considerations and limitations	200
8.10 Disturbances in the electrophysiological properties of DARPP-32 expressing neurons	201
8.11 Limitations	203
8.12 Human relevance.....	204
8.13 Recommendations for future research	204
8.13.1 Regional specific targeting of DARPP-32	204
8.13.2 DARPP-32 and intertemporal choices	205
8.13.3 DARPP-32 and probabilistic reinforcement choices	205
8.13.4 DARPP-32 and the exertion of effort.....	206
8.13.5 DARPP-32, incentive salience and incentive sensitisation.....	206
8.13.6 DARPP-32 and behavioural flexibility	207
8.14 Conclusion	208

References209

LIST OF ABBREVIATIONS

5-HT	Serotonin
5-HTRs	Serotonin receptor
6-OHDA	6-hydroxydopamine
ARs	Adenosine receptor
AC	Adenylyl cyclase
ACC	Anterior cingulate cortex
ADHD	Attention deficit hyperactivity disorder
AMPA	α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPH	Amphetamine
ANOVA	Analysis of variance
A-O	Action-outcome
AON	Anterior olfactory nucleus
ATP	Adenosine triphosphate
AUC	Area under the curve
BLA	Basolateral nucleus of the amygdala
Ca ²⁺	Calcium
CAMKII	Calcium and calmodulin dependent protein kinase
cAMP	Cyclic adenosine monophosphate
cdk5	Cyclin dependent protein kinase 5
CeA	Central nucleus of the amygdala
cGMP	Cyclic guanosine monophosphate
CK1	Casein kinase 1
CK2	Casein kinase 2

CPP	Conditioned place preference
CPu	Caudate putamen
CR	Conditioned response
CRf	Conditioned reinforcement
CS	Conditioned stimulus
DA	Dopamine
DARs	Dopamine receptors
DARPP-32	Dopamine and 3',5'-cyclic adenosine monophosphate-regulated neuronal phosphoprotein
DAT	Dopamine transporter
DHPG	3,5 dihydroxyphenylglycine
DLS	Dorsolateral striatum
DMS	Dorsomedial striatum
DOI	2,5-dimethoxy-4-iodoamphetamine
EEG	Electroencephalography
ERK	Extracellular signalling regulated protein kinase
fIHC	fluorescence immunohistochemistry
FR	Fixed ratio schedule of reinforcement
GABA	Gamma aminobutyric acid
GluRs	AMPA glutamate receptor subunit
GPe	Globus pallidus external
GPi	Globus pallidus internal
HET	Heterozygous type
HIPP	Hippocampus
ICj	Islands of Calleja
IEG	Immediate early gene

IP	Intraperitoneal
ITI	Intertrial interval
K ⁺	Potassium
KO	Knockout
LiCl	Lithium Chloride
LLR	Larger later reward
LTD	Long-term depression
LTP	Long-term potentiation
LU	Large uncertain reward
Mag	Magnification
mGluR _N	Metabotropic glutamate receptor
MPEP	2-methyl-6-(phenylethynyl)pyridine hydrochloride
mPFC	Medial prefrontal cortex
MPH	Methylphenidate
MSN	Medium spiny neuron
Na ⁺	Sodium
NAc	Nucleus accumbens
NAcC	Nucleus accumbens core
NAcSh	Nucleus accumbens shell
NMDA	N-methyl-d-aspartate
NRs	NMDA glutamate receptor subunit
NO	Nitric oxide
OCD	Obsessive compulsive disorder
OFC	Orbitofrontal cortex
OT	Olfactory tubercle

PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PIT	Pavlovian-to-instrumental transfer
PKA	cAMP-dependent protein kinase
PKG	cGMP-dependent protein kinase
PLC	Phospholipase C
PP-1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PP2B	Protein phosphatase 2B
PP2C	Protein phosphatase 2C
PR	Progressive ratio schedule of reinforcement
R	Response
RI	Random interval schedule of reinforcement
R-O	Response-outcome
SAL	Saline
SC	Small certain reward
SEM	Standard error of the mean
Ser	Serine
SNc	Substantia nigra pars compacta
SNP	Single nucleotide polymorphism
SNr	Substantia nigra pars reticulata
S-R	Stimulus response
SSR	Smaller sooner reward
STED	Stimulated emission depletion microscopy

STN	Subthalamic nucleus
TO	Timeout
UR	Unconditioned response
US	Unconditioned stimulus
THAL	Thalamus
Thr	Threonine
VI	Variable interval schedule of reinforcement
VP	Ventral pallidum
VTa	Ventral tegmental area
WT	Wildtype

Chapter 1

Introduction

Introduction 1.1

Disturbances in brain circuits underpinning motivated behaviours are thought to be implicated in the pathogenesis of addiction (Robinson & Berridge, 1993/2001/2003), pathological gambling (Reuter et al., 2005), eating disorders (Broft et al., 2012; Oberndorfer, Kaye, Simmons, Strigo & Matthews, 2011; Wagner et al., 2010), schizophrenia (Abi-Dargham et al., 1998; Das et al., 2007; Sorg et al., 2013; Weinberger, Berman & Zec, 1986), bipolar disorder (Strakowski, DelBello & Adler, 2005), obsessive compulsive disorder (OCD) (Menzies et al., 2008) and depression (Rogers et al., 2004). Elucidating the biopsychological basis of these disorders is therefore a major aim of affective neuroscience. Historically, great emphasis has been placed on disturbances in neurotransmission and the history of psychiatry during the past 60 years is founded on the discovery of effective drugs to ameliorate debilitating psychiatric symptoms. Indeed, in the modern age, it is not unusual to encounter popular media outlets discussing the efficacy of selective-serotonin reuptake inhibitors in the treatment of depression, the contribution of dopamine (DA) receptor antagonists to the treatment of schizophrenia or the potential development of a ‘silver bullet’ to liberate compulsive drug users from the burden of addiction. Much insight and clinical benefit has been gained from such compounds. However, with the aim of stimulating novel treatments, increasing emphasis is being placed on the intracellular pathways that reside downstream from the receptors where traditional psychiatric treatments exert their clinical effects. The work contained in this thesis aims to contribute to our understanding of how a DA and glutamate sensitive intracellular signalling molecule, dopamine and 3', 5'-cyclic adenosine monophosphate-

regulated neuronal phosphoprotein, 32 kDa (DARPP-32) contributes to incentive motivational processes that are relevant to addiction and impulse control disorders.

1.2 Incentive motivation

In its normal state, brain incentive motivation circuits facilitate adaptive motivated behaviours by allowing animals to flexibly modify their behaviour under environmentally and physiologically changing conditions in order to acquire food, water and to gain access to mates or to overcome aversive states by evading threat. Complex motivated behaviours can be guided by prior experience (learning) and physiological demand states which channel appropriate motivated behaviours towards relevant goals by enhancing the salience of reward-predictive cues (Berridge, 2004; Dickinson & Balleine, 2002; Toates, 1986). In the disturbed state, however, incentive motivation circuits are thought to underpin behavioural aberrations such as drug addiction by hyper-inflating the incentive value of drugs and drug-associated cues and by predisposing individuals to damaging reward-based decision making/cognitive styles (e.g. impulsivity/cognitive control) (Carter & Tiffany, 1999; Jentsch & Taylor, 1999; Robinson & Berridge, 1993/2001). Thus, understanding how reward value is psychologically and biologically encoded is of great interest to affective neuroscientists.

1.2.1 Basic Pavlovian learning mechanisms in incentive motivation

Two distinct, though often interacting, learning mechanisms are routinely recruited in laboratory settings to isolate the biological and psychological determinants of reward value. The first, Pavlovian/associative learning, is normally described in text book fashion as a mechanistic process involving the contingent pairing of initially neutral stimuli with biologically relevant stimuli such as food or water - termed unconditioned stimuli (US). Through their repeated pairing, these initially neutral stimuli are

incrementally transformed into conditioned stimuli (CS) capable of eliciting conditioned responses (CRs) resembling the unconditioned response (UR) (Pavlov, 1927). Konorski, however, fractionated the conditioned response topography by distinguishing between consummatory and preparatory CRs (Konorski, 1967). Consummatory CRs are US specific responses, such as a reflex or consuming food. However, preparatory CRs are not as US specific and might include conditioned approach behaviour or conditioned potentiation of instrumental responding (Dickinson & Balleine, 2002).

CSs are motivationally rich phenomena which can contain representations of the temporal, hedonic, sensory, and affective and response eliciting properties of USs (Delamater & Oakeshott, 2007). The representational topography of CSs, therefore, provides sufficient information to guide responses adaptively in an experience-dependent fashion. In fact, CSs are themselves capable of acquiring rewarding properties, such that they become highly valued reinforcers, capable of sustaining high levels of work in the absence of the presentation of the US with which they were previously paired. Thus, Pavlovian/associative learning is a potent psychological mechanism that dynamically stimulates and sustains sophisticated goal-directed behaviour.

1.2.2 Instrumental learning

The second kind of learning mechanism, instrumental learning, involves the experience-dependent modification of one's behaviour in response to the consequences produced by one's actions. For example, a rodent in an operant box that receives a food reward in return for manipulating a response device will increasingly direct responses toward that device and increase its reward seeking behaviour when placed in the box. This form of reinforcement is termed positive reinforcement on the basis that the positive outcome strengthens lever responding. On the other hand, negative reinforcement involves the omission of responses that produce negative outcomes. For instance, an

animal in an operant box may learn to omit a response at a particular time to avoid receiving a mildly aversive stimulus such as shock. Thus, the behavioural output of the animal is conditioned to avoid negative outcomes. The distinction between these two forms of instrumental learning was first formalised by the behaviourist Edward Thorndike in his “law of effect” which states that actions which produce desirable outcomes are likely to re-occur during similar circumstances, whereas actions which produce undesirable outcomes are likely to be omitted during similar circumstances (Thorndike, 1911). Owing to the nature of the studies that form the basis of this thesis, we will limit our discussion to positive reinforcement. This is not to neglect the importance of negative reinforcement or to devalue the influence such studies have had in informing our understanding of basic learning mechanisms.

Initial explanations of instrumental learning rules relied heavily on stimulus-response (S-R) theories to account for the increasing behavioural output of the animal when placed in close proximity to response-paired stimuli (Thorndike, 1911). For example, the box in which the animal is placed to perform the response, or the response device (lever/chain etc.) itself, are stimuli that precede reward receipt. Through the repeated pairing of the response with the rewarding outcome, the stimulus association between the stimulus and the response is strengthened and the likelihood of the subject reproducing that behaviour in the presence of the stimulus increases. Although such theories have provided substantial insights into how learning and habit formation occur, S-R accounts fail to capture the entire repertoire of learning that can occur during instrumental associations. For example, Balleine and Dickinson (1998a) suggest that S-R explanations of learning claim that animals lack knowledge about the outcomes produced by their actions. However, animals can use information to guide responses following explicit changes in the reward value of the reinforcer (e.g. reinforcer devaluation) suggesting that animals do

acquire knowledge about the relations between their actions and the outcomes they produce (Adams, 1982; Balleine & Dickinson, 1991). This form of learning, termed action-outcome/response-outcome learning, suggests that animals can encode multiple attributes about the relations between the response and its outcome and use that information to flexibly modify behaviour. In addition to this, many species of animal can use information about the relative costs of reinforcement (e.g. time, risk and effort) to make subjective reward value decisions and guide their behavioural output accordingly in order to optimise reward acquisition under environmentally fluctuating conditions (Cardinal & Howes, 2005; Evenden & Ryan, 1996; Floresco, Tse & Ghods-Sharifi, 2008). We will revisit this idea in greater depth below. For the time being let us return to the proposal that incentive value can be encoded via associative principles.

1.3 Pavlovian mechanisms and the attribution of incentive salience

Kent Berridge, in expanding on the work of Robert Bolles, Dalbir Bindra and Frederick Toates, has developed an incentive salience model of motivated behaviour to elaborate the psychological mechanisms by which conditioned stimuli become attractive and compelling motivational magnets which energise and captivate behaviour. In this model, conditioned stimuli, by virtue of their predictive relationship with highly valued USs, acquire motivational properties through stimulus-stimulus associations. Motivational states/deficit signals interact with CSs to enhance their incentive salience by inducing shifts in the hedonic and motivational properties of incentive stimuli (e.g. alliesthesia) in order to drive behaviour in a goal-directed fashion (Berridge, 2000; Berridge 2004; Robinson & Berridge, 1993; Toates, 1986). Berridge extended this principle by fractionating the hedonic aspects of conditioned reward which he terms “liking” from the motivational aspects of conditioned reward which he refers to as “wanting”. In this formulation, conditioned incentive stimuli are highly valued,

hedonically appealing incentives in their own right. This model of incentive motivation has been adapted to provide a highly influential model of addiction to drugs of abuse (Robinson & Berridge, 1993).

Animal behaviour assays such as conditioned reinforcement (CRf), the Pavlovian-to-instrumental transfer (PIT) test or the autoshaping/sign-tracking procedure provide significant supporting evidence for Berridge's claim that conditioned stimuli do acquire incentive motivational properties in the manner proposed by him and others (Berridge, 2000/2004; Toates, 1986). Berridge (2000) lists these as follows: 1) In the CRf assay, a discrete CS is repeatedly paired with a US during a training phase until animals acquire asymptotic responding to the presentation of the CS. Then, during a test phase, animals are placed into a conditioning chamber in the presence of an instrumental device which, when manipulated, generates the presentation of the CS but never the US. Animals will exert significant levels of effort to acquire the presentation of the CS, even though it no longer predicts the US. Thus the CS is imbued with conditioned reward value to such a profound extent that the animal will work for it, even though it has no inherent biological value. 2) During the autoshaping procedure, a stimulus such as a lever is used as a CS and repeatedly paired with an appetitive stimulus. During training, animals will come to approach the CS and direct US modality appropriate consummatory CRs (e.g. biting food predictive cues, licking water predictive cues) toward the CS even though such responses are unnecessary to achieve reinforcement. Thus CSs become highly attractive stimuli that animals will approach and even attempt to consume. 3) Reward associated stimuli will potentiate instrumental responding in a purely motivation driven fashion (e.g. in the general PIT test). During PIT, animals are trained in one phase to associate a CS with an appetitive US and in a separate phase to manipulate a response device (e.g. a lever, a chain pull or a nosepoke) to acquire the same US/reinforcer. Then, during an extinction

test, the CS is superimposed over the presence of the lever. When this occurs, animals will significantly increase the number of lever responses they make relative to when the cue was not present. Thus, CSs acquire incentive salience properties that can enhance reward value in multiplicative fashion. In an outcome-selective variation of this procedure, subjects are trained to associate 2 distinct CSs, CS1 & CS2, with 2 distinct USs, US1 & US2 and, in a separate phase of training to make 2 distinct responses, R1 & R2, to acquire those reinforcers. In the test phase, outcome-selective PIT is established when CS1 elevates R1 significantly more than R2 and when CS2 potentiates R2 more than R1. It was recently shown that pre-feeding rodents prior to a PIT test abolishes the general motivating effects of associative cues on instrumental responding (e.g. the general PIT effect) providing additional support for the idea that deficit signals interact with conditioned associations to enhance incentive salience and cue-driven reward seeking (Corbit, Janek & Balleine, 2007).

1.3.1 Glutamatergic and dopaminergic modulation of incentive salience

Significant focus has been placed on the biological basis of the abovementioned behaviours. We will discuss this in greater detail below. However, one consistent finding is that pharmacological or genetic manipulations aimed at disrupting normal DA or glutamate tone can enhance, attenuate or even abolish incentive salience. For example, acute or repeated amphetamine treatment enhances PIT (Wyvell & Berridge, 2000; Wyvell & Berridge, 2001) and similar findings have been observed in the autoshaping (Doremus-Fitzwater & Spear, 2011) and CRf assays (Mead, Crombag & Rocha, 2004; Robbins, Watson, Gaskin & Ennis, 1983). Mice with genetic mutations to glutamate receptor subunits additionally display disturbances in conditioned reward behaviours (Crombag, Sutton, Takamiya, Holland, et al., 2008; Crombag, Sutton, Takamiya, Lee, et al., 2008) and acute treatment with glutamate receptor antagonists disrupts many

Pavlovian incentive motivational processes including autoshaping (Dalley et al., 2005), PIT (George, Huston & Stephens, 2009) and CRf (O'Connor, Crombag, Mead & Stephens, 2010). Repeated administration of drugs of abuse stimulates long-term changes in DA and glutamate neurotransmission with the behavioural consequences of these changes being the sensitisation of locomotor output (Vanderschuren & Kalivas, 2000) and, in certain instances, the sensitisation of incentive salience/conditioned reward (Wyvell & Berridge, 2000; Mead et al., 2004). The findings of these studies are consistent with the predictions outlined in the incentive-sensitisation theory of addiction (Robinson & Berridge, 1993/2001/2003) in which it is postulated that stimuli consistently present during drug administration acquire incentive salience in the manner proposed by Berridge and that, through repeated use of drugs, disturbances in mesolimbic DA signalling promote the sensitisation of incentive salience.

1.4 Action-outcome learning and the attribution of reward value

Although Pavlovian principles provide fascinating insights into the associative basis of reward, the processes by which animals come to attribute reward value is complex, and other researchers such as Dickinson & Balleine have emphasised the role of instrumental action-outcome incentive learning in guiding how an animal can come to learn that an action leads to a specific outcome (Dickinson & Balleine, 2002; Dickinson & Balleine, 1994). Dickinson and Balleine suggest that in certain instances, it is not enough to simply alter the motivational state of an animal or to reduce the incentive value of the food in order for these manipulations to affect behaviour, animals must first learn that reward value has been altered in order to modify their responses. For instance, research has shown that when a reinforcer is paired with illness by administering lithium chloride (LiCl), an animal will continue to work for that reinforcer until it re-experiences the reinforcer following its pairing with illness (Balleine & Dickinson, 1991). Pre-feeding

subjects prior to test, instead of pairing the reinforcer with LiCl, produces similar reinforcer devaluation effects (Balleine & Dickinson, 1998b). Studies such as these suggest that, under certain conditions, incentive learning is required for animals to flexibly alter their behaviour following changes in incentive value.

The prevailing view is that A-O learning is evident during the formative phases of learning, though it transitions to a more habitual S-R profile as a function of experience (Dickinson, 1985; Yin & Knowlton, 2006). For example, with experience, animals become increasingly rigid and insensitive to devaluation as a function of the amount of training/experience they have between the action and the outcome. Animals that have been relatively ‘overtrained’ will persist in responding on a lever associated with the delivery of food, even though the reward value of that food has been devalued by being paired with illness or by the animal being pre-fed prior to the test (Adams, 1982; Coutureau & Killcross, 2003). DAergic lesions of the substantia nigra pars compacta and dorsal striatum disrupt habit formation when rodents are subjected to variable interval instrumental training (Faure, Haberland, Conde, Massioui, 2005) and repeated exposure to DAergic drugs such as amphetamine speeds habit formation during random interval schedule instrumental training (Nelson & Killcross, 2006).

1.4.1 Cost-benefits computations and action-outcome like instrumental choices

In addition to using sensory associations to ascribe subjective reward value, animals can use a multitude of reward-related cost-benefit representations, such as the delay to reinforcement, the probability of reinforcement, and the effort needed to achieve reinforcement to make goal-directed instrumental cost-benefits computations about the subjective value of incentives. For example, many species, including humans (Bickel, Odum & Madden, 1999; Field, Christiansen, Cole & Goudie, 2007; Johnson & Bickel, 2002), non-human primates (Freeman, Green, Myerson & Woolverton, 2009;

Woolverton, Myerson & Green, 2007), rats (Cardinal, Robbins & Everitt, 2000; Evenden & Ryan, 1996; Winstanley, Dalley, Theobald & Robbins, 2003), mice (Mitchell, Reeves, Li & Phillips, 2006; Oberlin & Grahame, 2009; Wilhelm, Reeves, Phillips & Mitchell, 2007) and birds (Green, Myerson, Holt, Slevin & Estle, 2004) can perform complex computations about subjective reward value as a function of the length of time that one must wait in order to receive that reward. For example, when provided with an option of choosing between a small reward and a large reward that are both delivered immediately, subjects will ordinarily prefer the large reward. However, if a delay to reinforcement is associated with the large reward, subjects will choose that reward less and less as the delay increases, even when it is beneficial to continue to choose that reward. In other words, delays to reinforcement devalue the incentive value of the large reward. Human (Rachlin, Ranieri & Cross, 1991; Rasmussen, Lawyer & Reilly, 2010) and rodent (St Onge & Floresco, 2009; St Onge, Stopper, Zahm & Floresco, 2012) subjects can also perform complex probabilistic calculations to decide whether to choose a large but probabilistically fluctuating reward or a small certain reward. In similar fashion to the effects of delay on reinforcement choices, animals will prefer the large reward when it is certain but will come to choose it less when the probability of reinforcement diminishes. In addition to time and risk investment costs, animals incur motivational (e.g. effort) costs for achieving reinforcement. Instrumental motivation can be directly assessed by establishing the willingness of an animal to progressively increase its motivation to press for reinforcement as the effort costs associated with reinforcement are progressively increased (Crombag, Ferrario & Robinson, 2008; Richardson & Roberts, 1996; Zhang, Balmadrid & Kelley, 2003) or by providing subjects with a choice between a low-effort small reward and a high-effort large reward (Botvinick, Huffstetler & McGuire, 2009; Bardgett, Depenbrock, Downs, Points & Green, 2009; Floresco et al., 2008; Ghods-

Sharifi & Floresco, 2010; Salamone, Cousins & Bucher, 1994). As with delay to reinforcement or uncertain reinforcement, animals will initially prefer the large reward when effort costs are low but, as effort costs are increased, they will begin to modify the subjective reward value and increasingly direct their choices toward the low-effort choice. Inefficient choice patterns in cost-benefits tasks, though particularly inefficiencies in delay-based reinforcement choices, are considered an endophenotype for impulse-control disorders such as addiction and pathological gambling (Gray & MacKillop, 2014; MacKillop, 2013).

1.4.2 Cost-benefits computations are not wholly guided by A-O principles

The extent to which behaviour in such tasks is wholly guided by action-outcome principles is controversial. For example, animals will take a significant number of sessions to assimilate the contingencies in cost-benefits choice tasks in accordance with the idea that they are modifying their actions in response to unfamiliar outcomes until an optimal pattern of responding is established. However, if the task parameters are reorganised, animals can take as long to adjust their responses following changes in task parameters as they did to initially learn the task. This suggests that both S-R and A-O principles may be present during discounting tasks. In being cognisant of this perspective, Cardinal, Robbins & Everitt (2003) developed a composite model of non-human discounting, in which it is asserted that Dickinsonian A-O principles are present and that animals are able to make decisions about instrumental relations and their relative reward value. However, it is suggested that weak S-R associations are also present and that these associations strengthen with experience. The authors also propose that stimuli present in the experimental arrangement enter into Pavlovian-to-instrumental interactions to modify choices. The extent to which such processes are present in other forms of instrumental choice arrangements such as the probability discounting task is unknown, however, it is

entirely likely that S-R and A-O learning occurs similarly during this task and that uncertainty may influence choices in a similar fashion to how delay to reinforcement does. This is not to suggest, however, that delay and uncertainty are indistinct psychological processes.

1.4.3 Glutamatergic and dopaminergic modulation of cost-benefits computations

As with the attribution and/or enhancement of incentive salience, reward value and/or its relationship with the investment costs (e.g. time, risk, effort etc.) associated with achieving reinforcement is/are sensitive to pharmacological interference with the DA and glutamate systems. Systemic amphetamine bi-directionally alters intertemporal reinforcement choices, promoting delay tolerance in some studies (Cardinal et al., 2000; Wade, de Wit & Richards, 2000) or delay aversion (Cardinal et al., 2000) in others, and systemically administered DA antagonists increase delay aversion (Koffarnus, Newman, Grundt, Rice & Woods, 2011; Wade et al., 2000). Systemically administered glutamate receptor antagonists have also recently been shown to increase delayed choices (Yates, Batten, Bardo & Beckmann, 2014). There is some evidence to suggest that nucleus accumbens (NAc) DA transmission and its interactions with the serotonin (5-HT) system are important aspects of delayed reinforcement choices (Winstanley, Dalley, Theobald & Robbins, 2003). Probabilistically constrained reinforcement choices are affected by discrete administration of DA antagonists into the medial prefrontal cortex (mPFC) (St Onge, Abhari & Floresco, 2011) or the NAc (Stopper, Khayambashi & Floresco, 2013) and by systemically administered glutamate antagonists (Yates et al., 2014). A wealth of data have established a role for DA (Aberman & Salamone, 1999; Aberman, Ward & Salamone, 1998; Denk, Walton, Jennings, Sharp, Rushworth & Bannerman, 2005; Ishiwari, Weber, Mingote, Correa & Salamone, 2004; Floresco et al., 2008; Sokolowski & Salamone, 1998; Trifilieff et al., 2013; Zhang et al., 2003) and glutamate (Floresco et

al., 2008; Paterson & Markou, 2005; Stephens & Brown, 1999) transmission in effortful procedures. Evidence has identified a role for nucleus accumbens DA in the provision of instrumental motivation (Aberman & Salamone, 1999; Aberman et al., 1998; Ishiwari et al., 2004; Sokolowski & Salamone, 1998; Trifilieff et al., 2013; Zhang et al., 2003).

1.5 Conclusion

In summary, we have discussed how Pavlovian associative mechanisms can transform (initially) motivationally neutral stimuli into highly valued incentive stimuli capable of prompting hedonic responses and sustaining high levels of work, and also how they are capable of multiplicatively enhancing motivation to seek reward. We have also discussed how animals can use action-outcome like representations to direct instrumental choices on the basis of reward value and also on the basis of the investment costs (e.g. costs benefits computations). Lastly, we have explored how DA and glutamate transmission in forebrain regions including the accumbens and prefrontal cortex support many of these processes. In subsequent sections of this introduction we will examine the neurobiological circuits which facilitate Pavlovian conditioned reward behaviour and complex intertemporal and probabilistic choice procedures and how DA and glutamate signalling converge in striatal regions at DARPP-32.

1.6 Motivational circuits: a simplified description of incentive motivational neurocircuitry and its functions

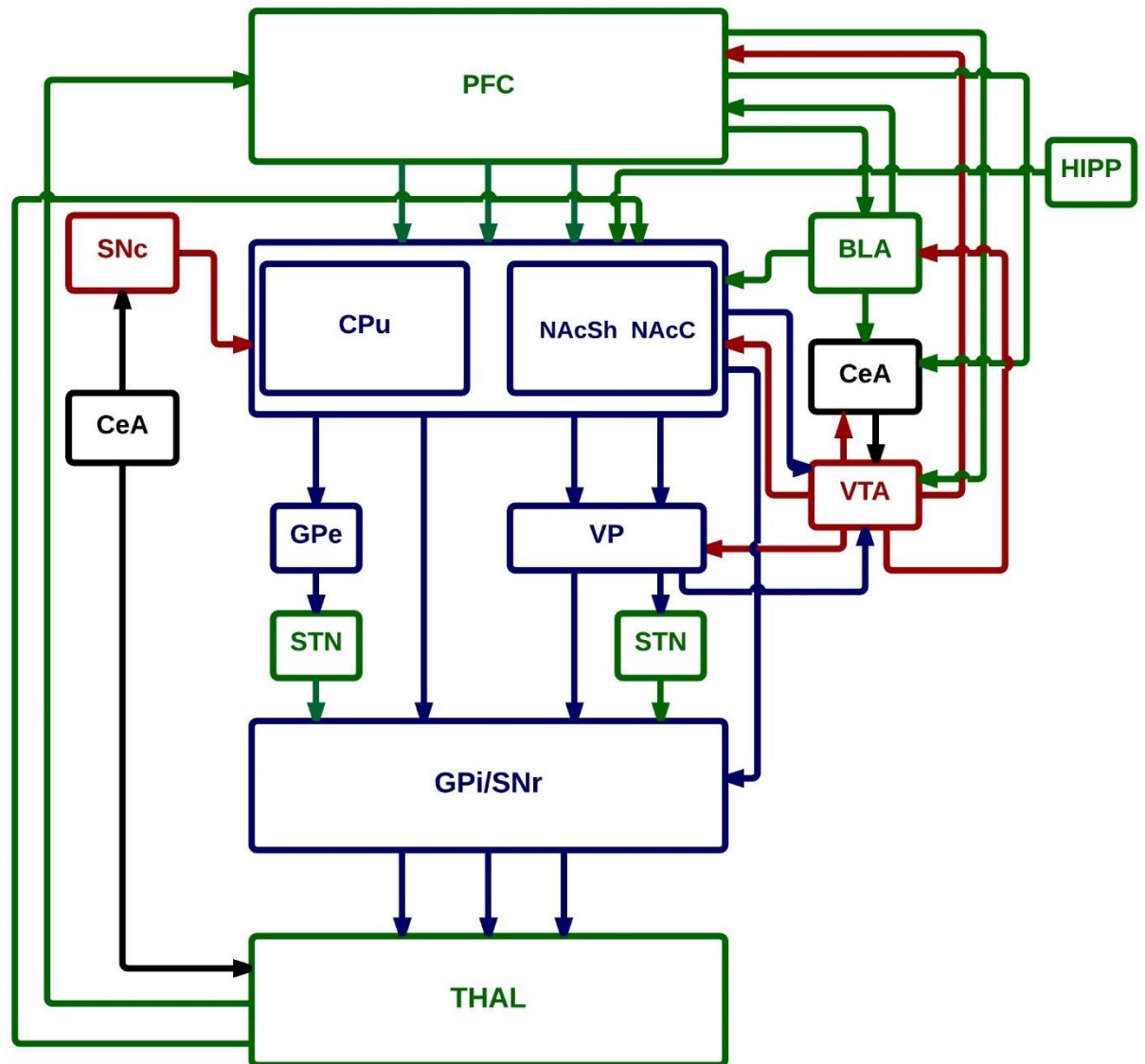


Fig. 1.1. Simplified schematic of incentive motivational circuits.

Black = unknown transmitter profile of projection neurons; **Blue** = inhibitory GABA projections; **Green** = excitatory glutamate projection; **Red** = neuromodulatory dopamine projection. Abbreviations: BLA basolateral nucleus of the amygdala; CeA central nucleus of the amygdala; CPu caudate putamen; GPe globus pallidus externa; GPi globus pallidus interna; HIPP hippocampus; PFC prefrontal cortex; SNc substantia nigra pars compacta; SNr substantia nigra pars reticulata; STN subthalamic nucleus; THAL thalamus; VP ventral pallidum; VTA ventral tegmental area.

Motivational assays, including a variety of tasks designed to assess incentive salience attribution (e.g. CRf, autoshaping and PIT) and tasks aimed at examining reinforcement choices along dimensions of time and uncertainty, and also the performance of instrumental motivation, are underpinned by topographically organised re-entrant circuits consisting, in part, of the prefrontal cortex (PFC), the striatum, the basal ganglia, the amygdala, the hippocampus, the thalamus and the midbrain. The rodent PFC, consisting of the orbitofrontal cortex (OFC), the mPFC (consisting of the infralimbic and prelimbic cortices) and the anterior cingulate cortex (ACC), is comprised of principally pyramidal glutamatergic neurons. Some of the most important glutamatergic projections from the PFC in the area of incentive motivation extend to the striatum (Sesack, Deutch, Roth & Bunney, 1989; Vertes, 2004), the amygdala (McDonald, 1998; Sesack et al., 1989) and regions of the midbrain, including the DAergic cells of the ventral tegmental area (VTA) (Carr & Sesack, 2000; Sesack & Pickel, 1992). The PFC in turn receives DA innervation from the VTA, thus these 2 regions are engaged in a reciprocal modulatory relationship (Wise, 2004).

Disturbances in glutamate and DA innervated circuits downstream of the PFC, particularly the basal ganglia, which consists of the dorsal (caudate putamen) and ventral striatum (NAc and the olfactory tubercle (OT)), globus pallidus interna (GPi), the globus pallidus externa (GPe), the ventral pallidum (VP), the substantia nigra pars compacta (SNc), the substantia nigra pars reticulata (SNr) and the subthalamic nucleus (STN), are associated with a range of neuropsychiatric and neurodegenerative disorders including obsessive compulsive disorder, schizophrenia, pathological gambling, drug addiction, Parkinson's disease, and Huntington's disease.

Basal ganglia circuits have traditionally been segregated on the basis of opioid peptide expression patterns and DA receptor subtypes that are present on striatal neurons within

distinct striatal pathways. The predominant (~90%) neuronal morphology of the striatum is the GABAergic medium spiny neuron (MSNs). MSNs expressing the opioid peptide substance P and the DA D₁ receptor project directly to the internal capsule of the globus pallidus and the substantia nigra pars reticulata (SNr) (Gerfen, 1992; Gerfen et al., 1990). SNr cells are also GABAergic and their projections to the thalamus cast an inhibitory influence on thalamic glutamate projections to cortical regions. Activity within direct striatal MSNs disinhibits the influence that the SNr has on the thalamus, resulting in the generation of goal-directed behaviour (Gerfen, 1992). In contrast, MSNs within the so-called indirect striatopallidal pathway express the opioid peptide enkephalin, the DA D₂ and adenosine A_{2A} receptors and activity within this circuit strengthens the inhibitory role of the thalamus leading to a reduction in motor behaviour (Gerfen, 1992). For example, indirect striatal neurons project to the globus pallidus externa which, in turn, projects to subthalamic nucleus of the thalamus; the STN then extends projections back to the SNr. Activity within the indirect pathway inhibits behavioural responding. Thus, the traditional view of the direct and indirect striatal pathways are as behavioural opponent processes. Additionally, and more recently, some studies have identified a complementary or even synergistic role of these pathways in mediating the acute behavioural effects of different drugs of abuse, and their ability to induce plasticity (Badiani et al., 1999; Badiani, Belin, Epstein, Calu & Shaham, 2011; Lobo & Nestler, 2011).

1.6.1 The connections of the striatum

The striatum consists of the caudate and putamen (CPu) in its most dorsal region and the NAc and OT in its ventral portion. The ventral striatum has been described as an interface between frontal, limbic and motor regions which allows these regions to integrate high-order computations relating to the planning and affective components of goal-directed behaviour (Day & Carelli, 2007; Mogenson, 1987; Sesack & Grace, 2010).

The NAc has been anatomically segregated into 3 subdivisions, comprising a core (NAcC) region, a shell (NAcSh) region and a rostral pole region. Most focus has been directed toward the core and shell subdivisions which, in turn, have largely functionally segregated behavioural roles (see below) (Zahm & Brog, 1992). The NAc receives dense DA innervation from the VTA and a modest DA input from the substantia nigra, as well as significant glutamate innervation from the basolateral nucleus of the amygdala (BLA), the thalamus, the hippocampus and the pre-limbic, infralimbic, medial orbital frontal and anterior cingulate cortices (Beckstead, Domesick & Nauta, 1979; Sesack & Grace, 2010; Voorn, Vanderschuren, Groenewegen, Robbins & Pennartz, 2004). The precise nature of the glutamate inputs to the subdivisions of the NAc differ in a subtle but important way. For example, the NAcC receives glutamatergic innervation from the prelimbic cortex, the anterior cingulate, the dorsal subiculum, the thalamus and the basolateral nucleus of the amygdala (BLA), whereas the NAcSh receives dense glutamate inputs from the prelimbic, infralimbic and orbito frontal cortices, the ventral subiculum, the thalamus and the BLA (Sesack & Grace, 2010; Yin, Ostlund & Balleine, 2008; Vertes, 2004; Voorn et al., 2004). The NAcC projects to the dorsolateral VP, the SNr and also the entopeduncular nucleus (Sesack & Grace, 2010). Thus whilst the NAcSh projects to the lateral hypothalamus, the ventromedial VP, the VTA, the SNc and the brainstem, the NAcC projects to basal ganglia output nuclei (Deniau, Menetrey & Thierry, 1994; Kelley, 2004; Sesack & Grace, 2010). NAc circuitry therefore maintains a direct and indirect-like segregation, with the NAc extending a direct-like projection to the SNr and an indirect projection to the VP which, in turn, extends to the STN and from there to the SNr (Sesack & Grace, 2010). The OT is another ventral striatal nuclei which has an important role in mediating motivated behaviour. The OT receives dense DAergic innervation from the VTA and significant glutamate innervation from cortical regions. The dorsal striatum

receives its primary DA input from the SNc and its principle glutamate inputs from the mPFC and sensory and motor cortices (Yin et al., 2008). Overall, corticostriatal glutamate projections from the OFC, infralimbic, prelimbic and anterior cingulate cortices convey information to the striatum relating to higher order cognitive functions and reward value appraisals to allow for the generation of appropriate and efficient behavioural responses. However, rather than exerting a simple hierarchical top-down influence on striatal tone, the re-entrant nature of fronto-striato-limbic motivational circuits provides basal ganglia nuclei with a mechanism to influence activity within cortical regions by a feedback mechanism involving the mediodorsal thalamus.

1.6.2 Amygdaloid connections

The amygdala interacts, mainly via its central (CeA) and BLA nuclei, with cortical, striatal and midbrain regions to modulate reward related behaviours by conveying conditioned affective and sensory information. The CeA has a predominantly medium spiny-like neuronal morphology, although there are aspiny neurons, whereas the cells of the BLA are principally pyramidal (Sah, Faber, Lopez De Armentia & Power, 2003). The CeA receives sensory input from the major sense modalities, as well as inputs from PFC, thalamus, hypothalamus and brainstem (Sah et al., 2003). The BLA similarly receives sensory and PFC inputs, as well as thalamic and hypothalamic inputs. In addition to these inputs, the CeA and BLA receive DA inputs from the VTA (Beckstead et al., 1979; Sah et al., 2003). The CeA extends to the bed nucleus of the stria terminalis, the hypothalamus, the brainstem, the substantia nigra pars compacta, the VTA and the thalamus whilst the BLA extends projections to the infralimbic, prelimbic and orbitofrontal cortices, the nucleus accumbens, the hippocampus, the bed nucleus of the stria terminalis and the CeA (Conzaes & Chesselet, 1990; Robbins & Everitt, 2002; Stamatakis et al., 2014; Sun, Yi & Cassell, 1994). Although the CeA has a significant population of GABA expressing

neurons, it also has populations of neurons expressing peptides such as corticotrophin releasing factor (Beckerman, Van Kempen, Justice, Milner & Glass, 2013).

1.6.3 Conclusion

In summary, glutamate inputs from the PFC, the amygdala converge with DA inputs originating from the substantia nigra pars compacta or the ventral tegmental area at striatal GABAergic MSNs to coincidentally modulate the throughput of motivational signals through the basal ganglia. The next section therefore examines some of the more specific motivational functions mediated by the abovementioned circuit.

1.7 Prefrontal cortex function in motivated behaviour

The rodent PFC is involved in high order executive functions such as planning and working memory (Dalley, Cardinal & Robbins, 2004) and also in updating reward value and optimising reinforcement choices (Churchwell, Morris, Heurtelou, & Kesner, 2009; Dalley et al., 2004; Schoenbaum, Roesch, Stalnaker & Takahashi, 2009; St Onge & Floresco, 2010; Walton, Bannerman, Alterescu & Rushworth, 2003; Winter, Dieckman & Schwabe, 2009). The OFC signals outcome-expectancy information relating to changes in reward value by processing salient motivational features such as the sensory properties of reinforcers and relevant conditioned outcome-expectancy information (Schoenbaum et al., 2009). For example, lesions to or pharmacological inactivation of the OFC disrupts selective PIT (Ostlund & Balleine, 2007), Pavlovian reinforcer devaluation (Gallagher, McMahan & Schoenbaum, 1999), intertemporal (Winstanley, Theobald, Cardinal & Robbins, 2004) and probabilistic reinforcement choices (Abela & Chudasama, 2013), and reversal learning (Boulougouris, Dalley & Robbins, 2007), thus suggesting that the OFC is critically involved in modifications of reward value and the mediation of reinforcement choices by using reward value information to make optimal

choices. Some of the motivational functions of the rodent mPFC include reinforcer devaluation (Corbit & Balleine, 2003; Ostlund & Balleine, 2005), delay discounting (Churchwell et al., 2009) and probability discounting (St Onge & Floresco, 2010). Interference with the ACC impairs autoshaping, indicating that this PFC nucleus has a facilitative role in stimulus reward learning (Cardinal et al., 2002). The ACC also has a role in mediating effort-related reinforcement choices (Floresco & Ghods-Sharifi, 2007; Holec, Pirot & Huston, 2014; Hosking, Cocker & Winstanley, 2014; Schweimer & Hauber, 2005; Walton et al., 2003). In summary, the PFC is involved in modifying complex reward-based decision making processes along dimensions of time, uncertainty/risk and effort and, also in using conditioned sensory associations to guide instrumental actions.

1.7.1 Ventral striatal function in motivated behaviour

The NAc is a motivational hub involved in the provision of drug and food-associated behaviours or in drug-stimulated modifications to motivated behaviour. The behavioural functions of the NAc include unconditioned feeding responses (Maldonado-Irizarry, Swanson & Kelley, 1995; Stratford & Kelley, 1997), autoshaping (Cardinal et al., 2002), Pavlovian approach behaviour (Parkinson, Olmstead, Burns, Robbins & Everitt, 1999), pharmacological potentiation of CRf (Parkinson et al., 1999; Wolterink et al., 1993), PIT (Corbit & Balleine, 2011), intertemporal discounting (Acheson et al., 2006; Cardinal et al., 2001), probability discounting (Cardinal & Howes, 2005; Stopper & Floresco, 2011), probabilistic reversal learning (Dalton, Phillips & Floresco, 2014) and a multitude of effortful behaviours including effort-based choice (Cousins, Atherton, Turner & Salamone, 1996), effort discounting (Ghods-Sharifi & Floresco, 2010) and food-reinforced progressive ratio schedules of reinforcement (Aberman et al., 1998). The NAc also has a well-defined role in mediating drug related behaviours such as psychomotor

sensitisation (Vanderschuren & Kalivas, 2000), drug self-administration (Hutcheson, Parkinson, Robbins & Everitt, 2001; Zito, Vickers & Roberts, 1985) and conditioned drug reward (Sellings & Clarke, 2003).

NAc injections of indirect DA agonists such as amphetamine can enhance PIT (Wyvell & Berridge, 2000) and CRf (Taylor & Robbins, 1984), and intra-accumbal DA antagonists can attenuate PIT (Lex & Hauber, 2008), CRf (Wolterink et al., 1993) and autoshaping (Di Ciano, Cardinal, Cowell, Little & Everitt, 2001; Saunders & Robinson, 2012). Intra-accumbal injections of glutamate antagonists have been shown to disrupt autoshaping (Di Ciano et al., 2001) and the response potentiating effects of amphetamine during CRf (Burns, Everitt, Kelley & Robbins, 1994). In addition to this, intra-accumbal injections of DAergic compounds modify choices in probabilistic (Stopper, Khayambashi & Floresco, 2013) and effort-based (Farrar et al., 2010) cost-benefits tasks and the motivation to exert effort in progressive ratio schedules (Zhang et al., 2003). Furthermore, NAc DA release occurs in response to reward associated cues conveying information about the temporal and effort associated costs of reinforcement (Day, Jones, Wightman & Carelli, 2010). Overall, these findings suggest that accumbal DA and glutamate transmission interact to coincidentally modulate many important incentive motivational processes.

1.7.1.1 Nucleus accumbens: the core vs shell distinction

Many of the behavioural functions of the nucleus accumbens can be dissociated along anatomical lines. The NAcSh underpins conditioned drug associations such as conditioned place preference (CPP), as well as unconditioned responses to primary reinforcers (e.g. feeding) (Ikemoto, 2007; Kelley, 2004). Ikemoto (2007) suggested that the NAcSh is involved in stimulus-outcome learning and it has additionally been suggested that the NAcSh is involved in response suppression during conditions of

instrumental uncertainty (Floresco, 2015). NAcSh lesions abolish the outcome-selective form of PIT (Corbit & Balleine, 2011), as well as the response-potentiating effects of amphetamine on CRf (Parkinson et al., 1999), and inactivation of the NAcSh impairs reinforcement choices constrained by probabilistic contingencies (e.g. probability discounting, probabilistic reversal learning) (Dalton et al., 2014; Stopper & Floresco, 2011). The nucleus accumbens core, on the other hand, mediates distinct aspects of CS-US associations (autoshaping, incentive salience), response vigour and effort in instrumental tasks and, in addition to this, promotes delay tolerance in intertemporal choice procedures. For example, lesions of or pharmacological interference with the NAcC disrupts autoshaping (Parkinson, Willoughby, Robbins & Everitt, 2000), interferes with performance on effortful tasks (Sokolowski & Salamone, 1998; Ghods-Sharifi & Floresco, 2010), promotes delay aversion in intertemporal choice tasks (Cardinal et al., 2001), disrupts general PIT (Corbit & Balleine, 2011), and enhances context-specific psychomotor sensitisation (Kelsey, Gerety & Guerriero, 2009).

In summation, the nucleus accumbens appears to promote purposeful motivated behaviour by integrating signals from cortical, midbrain and amygdaloid regions involved in the processing of reward value and associated affective signals.

1.7.2 Caudate putamen function

The caudate-putamen resides dorsally to the NAc and is functionally implicated in behavioural flexibility and both action-outcome and stimulus-response/habit learning. Lesions to the dorsomedial striatum impair behavioural flexibility during instrumental reversal learning (Castañé, Theobald & Robbins, 2010). Lesions to the dorsolateral striatum also impair habit formation (Yin, Knowlton & Balleine, 2004). For example, extended instrumental training for food reinforcers renders subjects insensitive to outcome devaluation manipulations but lesions of the dorsolateral striatum (DLS)

maintain outcome-devaluation in subjects trained under habit promoting variable interval schedules of reinforcement (Yin et al., 2004). Conversely, lesions to the dorsomedial striatum (DMS) impair the ability of subjects to maintain action-outcome relations following outcome devaluation (Yin, Ostlund, Knowlton, & Balleine, 2005). It was recently shown that connections between the BLA and the dorsomedial striatum are necessary for reinforcer devaluation; lesions or temporary inactivation of these connections prevents the devaluation effect (Corbit, Leung & Balleine, 2013). The CPu is also implicated in the Pavlovian enhancement of instrumental responding. Temporary inactivation of the dorsolateral striatum leaves the selective-outcome PIT effect intact, however, the magnitude of this effect is significantly attenuated (Corbit & Janak, 2007). When the dorsomedial striatum is temporarily inactivated, however, outcome-selective PIT is abolished (Corbit & Janak, 2007). The CPu, therefore, facilitates the acquisition of relevant instrumental processes by mediating action-outcome learning and the encoding of habits. In addition to this, the CPu mediates the modification of instrumental responding following changes in reward value by, more generally, integrating sensory information and reward value information to guide instrumental choices.

1.7.3 Ventral pallidum function in motivated behaviour

The VP is a basal ganglia node that has been described as a ‘common pathway’ for mesocorticolimbic projections which is involved in the generation of motivated behaviour (Smith, Tindell, Aldridge & Berridge, 2009). Reversible inactivation of the VP simultaneously reduces high-effort responses to obtain a highly palatable reward but increases consumption of a less-preferred, concurrently available reward (Farrar et al., 2008). The firing profile of VP neurons following CS presentation indicates that VP neurons encode the incentive-sensitisation of such stimuli in response to repeated amphetamine treatment (Tindell, Berridge, Zhang, Peciña, & Aldridge, 2005).

Furthermore, reversible inactivation of the VP blocks selective outcome PIT and this selective outcome promoting effect of Pavlovian stimuli most likely involves projections from the NAcSh to the ventromedial VP (Leung & Balleine, 2013; Root, 2013).

1.7.4 Ventral tegmental area function

The VTA provides the most substantial DA inputs to the ventral striatum, the amygdala and the frontal cortex. The functional basis of these connections is the focus of intense speculation. However, one general feature of its function is that it is involved in the encoding of stimulus-outcome associations. Investigators such as Schultz (2002) have suggested that VTA DA neurons forming connections with the NAc function as a teaching-signal that encodes the predictability of reward associated cues, whilst others such as Robinson & Berridge (1993) suggest that VTA DA neurons encode the incentive salience of conditioned associations. Although speculation surrounding the precise psychological functions of VTA DA remains, it is widely accepted that VTA DA is involved in associative processes. Consistent with this idea, temporary inactivation of the VTA disrupts both the general and outcome-selective forms of PIT (Corbit, Janak & Balleine, 2007).

1.7.5 Amygdala function in reward learning

Deep within the medial temporal lobe lies an almond-like structure, termed the amygdaloid complex, which has a well-defined role in reward learning. The amygdala, particularly the basolateral (BLA) and central (CeA) nuclei, are of particular importance to reward-related motivation and decision making. It has been suggested that, in the domain of fear conditioning, the BLA is involved in a serial circuit with the CeA. However, in the domain of appetitive motivation, the CeA and BLA exist in a parallel circuit to promote distinct motivational functions (Balleine & Killcross, 2006). In this

scheme then, the CeA adds affective tone to conditioned associations, imbuing conditioned stimuli with motivational resonance/incentive salience (Corbit & Balleine, 2005). The BLA, however, mediates the formation of sensory CS-US associations (Corbit & Balleine, 2005), as well as the mediation of instrumental reward devaluation following sensory-specific satiety (Balleine, Killcross & Dickinson, 2003). Lesions to, or pharmacological inactivation of, the BLA disrupts intertemporal (Winstanley et al., 2004), probabilistic and effort-based (Ghods-Sharifi, St. Onge & Floresco, 2009) reinforcement choices, selective outcome PIT (Corbit & Balleine, 2005), and both instrumental (Balleine et al., 2003; Johnson, Gallagher & Holland, 2009) and Pavlovian reinforcer devaluation (Johnson et al., 2009), whereas lesions of the CeA disrupt general PIT (Corbit & Balleine, 2005), autoshaping (Parkinson, Robbins & Everitt, 2000) and the ability of intra-accumbal injections of amphetamine to potentiate CRf (Robledo, Robbins & Everitt, 1996). Recent work has also revealed a critical role of the CeA in the development of habit formation (Lingawi & Balleine, 2012).

1.7.6 Conclusion

In summary, complex motivated behaviours are underpinned by cortical and amygdaloid glutamate fibres involved in the processing of cognitive and affective processes and midbrain DA fibres which converge at striatal GABAergic MSNs to modulate neural activity in the basal ganglia and, subsequently, the generation or inhibition of goal-directed behaviour. Inherently, motivated behaviour involves careful and seemingly complex cost-benefits computations, balancing risks, energy expenditure with expected gains based on integrating a variety of learned information about reward value and incentive salience signals to guide the selection of appropriate choices during any given moment. We will next explore how DARPP-32 is an important integrator of

DA and glutamate signalling in striatal regions involved in many of the aforementioned behaviours.

1.8 DARPP-32: a complex neuronal phosphoprotein

DA and glutamate axons form synapses with MSNs on dendritic spines and spine heads to modulate MSN excitability. For example, coincident activity at striatal DA and glutamate receptors can induce certain forms of electrophysiological plasticity such as long-term potentiation (LTP) and long-term depression (LTD) (Gerfen & Surmeier, 2011; Surmeier, Ding, Day, Wang & Shen, 2007). DA exerts opposite effects on MSN excitability in the direct and indirect pathways. D₁-family (D₁, D₅) receptors are G_s/G_{olf} G-protein coupled receptors which stimulate adenylyl cyclase (AC) resulting in the accumulation of cyclic adenosine monophosphate (cAMP) and the subsequent activation of cAMP-dependent protein kinase (PKA), whereas D₂-family (D₂, D₃, D₄) receptors are G_i/G_o G-protein coupled receptors which inhibit cAMP formation and PKA activation when stimulated. The intracellular distinction between D₁-family and D₂-family receptors has profound consequences for the excitability of MSNs. D₁ receptor stimulation enhances strong glutamate signals, promoting the so-called up-state in the direct pathway and the induction of LTP whereas D₂ receptor stimulation dampens the sensitivity of MSN populations in the indirect pathway to endogenous glutamate, thus promoting the induction of LTD (Gerfen & Surmeier, 2011; Surmeier et al., 2007). Gerfen & Surmeier (2011) suggest that these D₂-mediated up-state opposing events are orchestrated by reduced activity through Na⁺ and Ca²⁺ ion channels which promote membrane depolarisation, and via increased activity through K⁺ channels which promote membrane hyperpolarisation. D₁ receptors via PKA, on the other hand, increase depolarising Na⁺ and Ca²⁺ channels and diminish hyperpolarising K⁺ channels. D₁-mediated PKA activation also promotes gene transcription and the phosphorylation of glutamate receptor

subunits which modify the responsiveness of cells to glutamate, either by directly promoting the phosphorylation of glutamate receptor subunits, or by interacting with intracellular proteins such as DARPP-32 which also influence gene transcription and the phosphorylation of glutamate subunits.

DARPP-32 is a complex, bi-functional integrator of neurotransmission in the majority of dopaminergic brain regions, which is intimately involved in the regulation of key neuronal processes, including neurotransmitter receptor and ion channel phosphorylation, which are directed toward controlling neuronal excitability and gene expression (Svenningsson et al., 2004). DARPP-32 is abundantly enriched in striatal MSNs and discrete regions of the amygdala (Ouimet, Miller, Hemmings, Walaas & Greengard, 1984; Walaas & Greengard, 1984; Perez & Lewis, 1992). In the cortex, DARPP-32 is expressed less intensely and its profile is limited to sparse populations in layers III and layers VI (Ouimet et al., 1984). We will discuss the regional distribution of DARPP-32 within motivational circuits in greater detail in chapter 2.

1.8.1 DARPP-32 is influenced by protein kinases and protein phosphatases

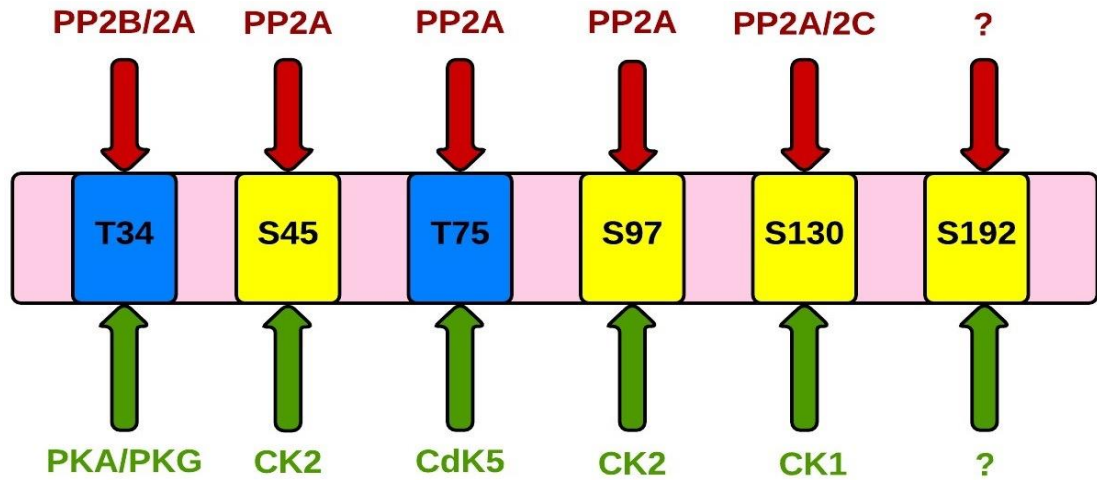


Fig. 1.2. DARPP-32 phosphorylation residues and their effectors. Adapted from Yger & Girault (2011).

DARPP-32 possesses an intricate phosphorylation profile consisting of both serine and threonine residues; these residues include Thr³⁴, Thr⁷⁵, Ser⁴⁵, Ser⁹⁷ (Ser¹⁰² rat), Ser¹³⁰ (Ser¹³⁷ rat) and a Ser¹⁹² in the mouse brain. Thr³⁴-DARPP-32 phosphorylation is stimulated most potently by PKA, however, cyclic-guanosine monophosphate-dependent protein kinase (PKG) additionally promotes Thr³⁴-DARPP-32 phosphorylation (Hemmings, Williams, Konigsberg & Greengard, 1984). Dephosphorylation of Thr³⁴ is most strongly influenced by Ca²⁺-dependent protein phosphatase 2B (PP2B/calcineurin) (King et al., 1984). However, protein phosphatase 2A (PP2A) also contributes to this event (Nishi, Snyder, Nairn & Greengard, 1999). Thr⁷⁵ phosphorylation is prompted by cyclin-dependent protein kinase 5 (cdk5) (Bibb et al., 1999) and its dephosphorylation is stimulated by PP2A (Nishi et al., 2000). Ser⁴⁵ and Ser⁹⁷ phosphorylation are prompted by casein kinase II (CK2) and both of these residues are dephosphorylated by PP2A (Girault, Hemmings, Williams, Nairn & Greengard, 1989). PP2A-mediated dephosphorylation of Ser⁹⁷ has been shown to involve a cAMP-PKA dependent mechanism (Stipanovich et al., 2008). Ser¹³⁰ phosphorylation is stimulated by casein kinase 1 (CK1) (Desdouits, Cohen, Nairn, Greengard & Girault, 1995). Ser¹³⁰ dephosphorylation is prompted by PP2A and

protein phosphatase 2C (PP2C) *in vitro*, however, dephosphorylation of Ser¹³⁰ is not disturbed by the PP2A inhibitor okadaic acid *in vivo*, indicating that PP2C may have a greater role in dephosphorylating this residue than PP2A in the brain (Desdouits, Siciliano, Nairn, Greengard & Girault, 1998).

1.8.2 DARPP-32: a molecular interface for postsynaptic neurotransmission

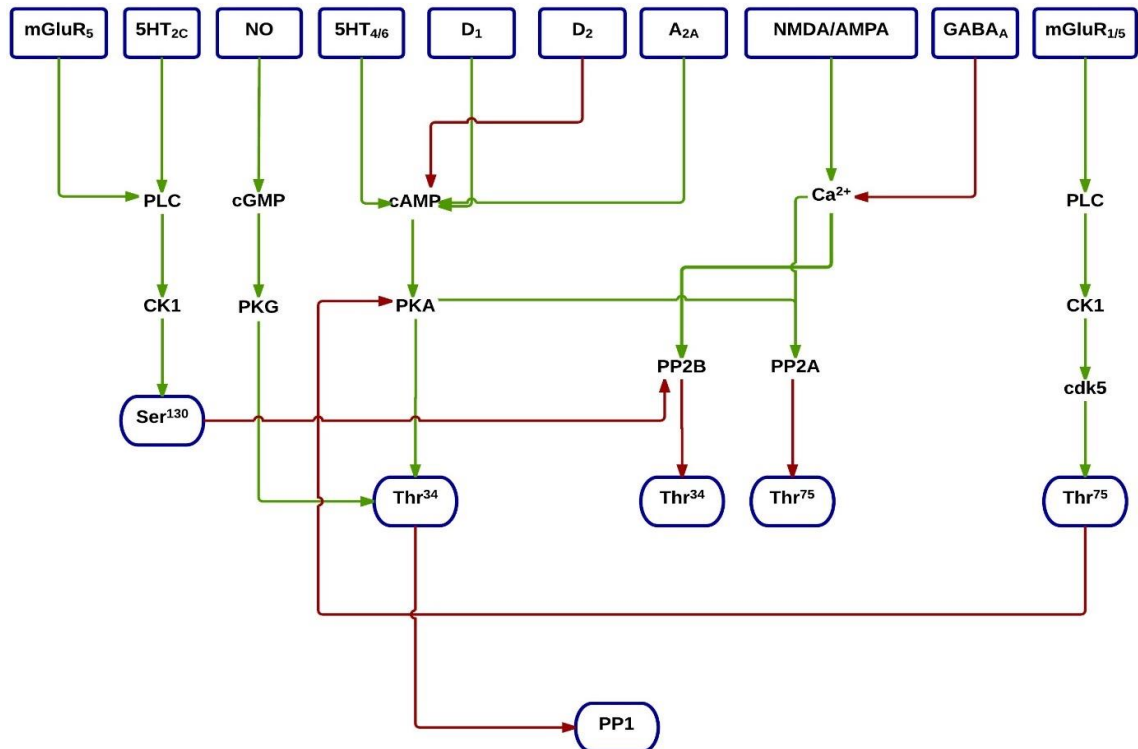


Fig.1.3. Simplified diagram of major signalling pathways involved in Thr³⁴ & Thr⁷⁵ phosphorylation. Green indicates an increase in activity whilst red equates to a reduction in activity. Not all pathways represented. Adapted from Nishi et al, 2005; Svenningsson et al, 2002/2004.

DARPP-32 integrates postsynaptic signals from the DA, glutamate, serotonin, GABA, norepinephrine and opioid systems, in addition to neuromodulators such as adenosine and nitric oxide (NO) (Svenningsson et al., 2004). We will, however, primarily focus on the effects of those neurotransmitter systems that are the most significant effectors of DARPP-32, such as DA or glutamate, or those systems which have been identified as making a significant and consistent contribution to the behaviours contained in subsequent chapters (e.g. 5-HT & adenosine) (see Fig. 1.3). With regards to the

contribution made by these other neurotransmitter and neuropeptide systems, the reader is referred to any of the numerous excellent reviews to have elaborated all of the signalling pathways and their direct effects on DARPP-32 phosphorylation and its downstream targets (Greengard, Allen & Nairn, 1999; Svenningsson et al., 2004; Yger & Girault, 2011).

1.8.3. Thr³⁴-DARPP-32 phosphorylation and PP-1 inhibition

D₁ receptor activation promotes the PKA-stimulated phosphorylation of Thr³⁴-DARPP-32 and the subsequent inhibition of protein phosphatase 1 (PP-1) (Hemmings, Greengard, Tung & Cohen, 1984; Nishi, Snyder & Greengard, 1997). PP-1 inhibition, as we will later discuss, has important consequences for transcriptional events and the electrophysiological properties of MSNs. Glutamate induced nitric oxide signalling, via either α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-d-aspartate (NMDA) or metabotropic glutamate (mGluR) type 5 receptors, stimulates cyclic guanosine monophosphate (cGMP) and the subsequent activation of PKG which also elevates Thr³⁴-DARPP-32 phosphorylation (Nishi et al., 2005). Thr³⁴-DARPP-32 phosphorylation is also affected by adenosine transmission in the indirect pathway. Adenosine A_{2A} receptors form heteromers with D₂ receptors (Fuxe et al., 2005) and their activation promotes Thr³⁴-DARPP-32 phosphorylation in indirect striatopallidal MSNs. The adenosine A_{2A} receptor agonist CGS 21680 stimulates increases in Thr³⁴-DARPP-32 via a mechanism involving cAMP and PKA (Svenningsson et al., 1998). A_{2A} mediated increases in Thr³⁴-DARPP-32 phosphorylation require the co-activation of mGluR₅ receptors by glutamate (Nishi et al., 2003). mGluR₅ receptor activation also stimulates increases in Thr³⁴-DARPP-32 phosphorylation and it does so via an extracellular signal-regulated protein kinase (ERK)/AC/cAMP/PKA pathway (Nishi et al., 2005). mGluR₅-stimulated increases in Thr³⁴ phosphorylation require the co-activation of A_{2A} receptors

(Nishi et al., 2003). GABA activation increases Thr³⁴-DARPP-32 by opposing its dephosphorylation by PP2B (Snyder, Fisone & Greengard, 1994) whilst 5-HT directly increases the phosphorylation of Thr³⁴-DARPP-32 via the 5-HT₄ and 5-HT₆ receptors (Svenningsson Tzavara, Liu, et al., 2002).

1.8.4 Dopamine and glutamate mediated dephosphorylation of Thr³⁴-DARPP-32

As mentioned above, the dephosphorylation of Thr³⁴-DARPP-32 occurs most potently by PP2B. However, PP2A also makes a minor contribution toward Thr³⁴-DARPP-32 dephosphorylation. Ionotropic, fast excitatory AMPA and NMDA receptors (Nishi et al., 2002) and also D₂ receptors (Nishi et al., 1997) contribute to the dephosphorylation of Thr³⁴-DARPP-32 by elevating intracellular calcium levels which, in turn, activates PP2B. D₂ receptor activation additionally contributes to the dephosphorylation of Thr³⁴-DARPP-32 by directly opposing cAMP formation (Nishi et al., 1997). Indeed, D₂ receptors are negatively coupled to cAMP in the indirect striatopallidal pathway and their activation therefore opposes PKA.

1.8.5 Thr³⁴-DARPP-32 phosphorylation is fine-tuned by activity at serine residues

Activity at Thr³⁴-DARPP-32 is fine-tuned by Ser⁹⁷ and Ser¹³⁰ phosphorylation. Ser⁹⁷ phosphorylation increases the potency by which PKA can phosphorylate Thr³⁴-DARPP-32 (Girault et al., 1989) and Ser¹³⁰ assists in maintaining DARPP-32 in its Thr³⁴ phosphorylated form by opposing the dephosphorylation of Thr³⁴-DARPP-32 by PP2B (Desdouits, Sicilliano, Greengard & Girault, 1995). Ser¹³⁰-DARPP-32 phosphorylation can be stimulated by 5-HT_{2C} receptors and involves phospholipase c (PLC) and CK1 and therefore likely opposes PP2B-mediated dephosphorylation of Thr³⁴-DARPP-32 (Svenningsson, Tzavara, Liu, et al., 2002). In addition to 5-HT_{2C} receptor activation,

mGluR₅ activation increases the phosphorylation of Ser¹³⁰-DARPP-32 via CK1 (Liu et al., 2001).

1.8.6 DARPP-32 mediates nuclear processes

Although DARPP-32 is largely described as a cytoplasmic phosphoprotein, Ser⁹⁷-DARPP-32 phosphorylation regulates the passage of DARPP-32 from the nucleus to the cytoplasm. Whilst Ser⁹⁷-DARPP-32 phosphorylation stimulates the transfer of DARPP-32 from the nucleus to the cytoplasm, D₁/PP2A mediated dephosphorylation of the Ser⁹⁷ residue maintains DARPP-32 in the nucleus, during which DARPP-32 can assist in the phosphorylation of Ser¹⁰-histone H3 by inhibiting PP-1 (Stipanovich et al., 2008).

1.8.7 Thr⁷⁵-DARPP-32 phosphorylation and PKA inhibition

Although many of DARPP-32's phosphorylation residues generate positive feedback signals which either directly enhance Thr³⁴-DARPP-32 phosphorylation or indirectly by preventing the dephosphorylation of this residue, Thr⁷⁵-DARPP-32 exerts an antagonistic influence over the PKA-Thr³⁴-DARPP-32 pathway. Thr⁷⁵-DARPP-32 phosphorylation is stimulated by cdk5 and because this phosphorylated form of DARPP-32 is antagonistic to Thr³⁴-DARPP-32 and the function of PKA, it enhances PP-1 activity and likely opposes the facilitative actions of Thr³⁴-DARPP-32 on downstream targets such as AMPA, NMDA and GABA receptors, as well as gene expression (Bibb et al., 1999). DARPP-32, therefore, is both a PKA and PP-1 inhibitor (Bibb et al., 1999). This dual-functioning capacity of DARPP-32 can be explained, in part, by the bidirectional effects of DA signalling. For example, whilst D₁ receptor stimulation increases Thr³⁴-DARPP-32 phosphorylation it also stimulates a simultaneous decrease in Thr⁷⁵-DARPP-32 phosphorylation and, in contrast to this, D₂ receptor activation stimulates a concomitant increase in Thr⁷⁵-DARPP-32 and a decrease in Thr³⁴-DARPP-32 phosphorylation (Nishi

et al., 2000). The phosphorylation of Thr⁷⁵-DARPP-32 also involves activity at mGluR₁ and mGluR₅ receptors. The mGluR_{1/5} agonist 3,5 dihydroxyphenylglycine (DHPG) increases Thr⁷⁵-DARPP-32 phosphorylation in striatal slices and this effect is blocked by the cdk5 inhibitor butyrolactone or CK1 inhibitors CK1-7 and IC261, indicating that Thr⁷⁵-DARPP-32 phosphorylation involves mGluR_{1/5} stimulated increases in CK1 which subsequently promote cdk5 stimulated increases in Thr⁷⁵-DARPP-32 (Liu et al., 2001). Conversely, the mGluR₅ antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) reduces tonically elevated levels of Thr⁷⁵-DARPP-32 phosphorylation induced by repeated cocaine administration, suggesting that tonic activity at mGluR₅ receptors assists in maintaining DARPP-32 in its Thr⁷⁵-DARPP-32 phosphorylated form in certain instances (Scheggi, Raone, De Montis, Tagliamonte & Gambarana, 2007). Thr⁷⁵-DARPP-32 dephosphorylation is stimulated by D₁ receptor activation and PKA mediated stimulation of PP2A (Nishi et al., 2000) or by 5-HT_{4/6} mediated activation of the PKA pathway (Svenningsson, Tzavara, Liu, et al., 2002). PKA subsequently stimulates PP2A activation resulting in the dephosphorylation of Thr⁷⁵ (Ahn, McAvoy, Rakhilin, et al., 2007) and, in addition to this, Ca²⁺-dependent activation of PP2A also promotes the dephosphorylation of Thr⁷⁵ (Ahn, Sung, McAvoy, et al., 2007). This latter mechanism most likely involves the NMDA & AMPA receptors, which stimulate increases in intracellular Ca²⁺ levels and the subsequent dephosphorylation of Thr⁷⁵-DARPP-32 by PP2A (Nishi et al., 2002). A_{2A} receptor activation by CGS 21680 also stimulates the dephosphorylation of Thr⁷⁵-DARPP-32 and these increases in the dephosphorylation of the Thr⁷⁵-DARPP-32 substrate involve PP2A (Lindskog et al., 2002).

1.8.8 Summary

Depending on its phosphorylation pattern, DARPP-32 can inhibit either PP-1 or PKA. D₁ receptors promote PKA-dependent increases in Thr³⁴ phosphorylation and the

subsequent inhibition of PP-1. In contrast, cdk5-mediated Thr⁷⁵-DARPP-32 phosphorylation inhibits PKA. The Ser⁹⁷ and Ser¹³⁰ residues assist in Thr³⁴-DARPP-32 phosphorylation. Finally, the effect of glutamatergic activity on these processes is complex.

1.9 DARPP-32 is a molecular regulator of MSN receptor phosphorylation and electrophysiology

The net effect of DARPP-32 phosphorylation is the regulation of ion channels and receptor subunit (AMPA GluR₁, NMDA NR₁, GABA_A) phosphorylation and nuclear events such as gene and immediate early gene (IEG) expression or histone phosphorylation which are associated with neuronal excitability (Dell'Anno, Pallottino & Fisone, 2013; Flores-Hernandez et al., 2000; Flores-Hernandez et al., 2002; Håkansson, Galdi, Hendrick, Snyder, Greengard & Fisone, 2006; Snyder, Fienberg, Huganir & Greengard, 1998; Stipanovich et al., 2008; Svenningsson et al., 2000/2004; Yan et al., 1999). Many of these processes are directly affected by PKA or PP-1, thus DARPP-32 has a pivotal role as a mediator of many key neuronal processes.

1.9.1 DARPP-32 and AMPA receptor phosphorylation

Striatal AMPA ser⁸⁴⁵ GluR₁ receptor subunits are dephosphorylated by activation of AMPA and NMDA receptors involving a calcineurin-dependent mechanism which occurs independently of DARPP-32 and PP-1 (Snyder et al., 2003). However, *in vivo* phosphorylation of the AMPA GluR₁ Ser⁸⁴⁵ subunit occurs via PKA and requires DARPP-32 to maintain normal functioning. Acute psychostimulant mediated increases in striatal Ser⁸⁴⁵ GluR₁ phosphorylation are significantly lower in DARPP-32 KO mice (Snyder et al., 2000). Ser⁸⁴⁵ GluR₁ phosphorylation occurs equally between wildtype (WT) & DARPP-32 knockout (KO) mice in response to the PP2A/2B inhibitor okadaic

acid, thus suggesting that the D₁-PKA-Thr³⁴-DARPP-32 pathway modifies AMPA subunit phosphorylation by opposing PP-1 (Snyder et al., 2000). In contrast to the effects of D₁ receptor stimulation on Ser⁸⁴⁵GluR₁ phosphorylation, D₂ receptor activation decreases Ser⁸⁴⁵-GluR₁ phosphorylation, whereas the D₂ receptor antagonist haloperidol increases Ser⁸⁴⁵-GluR₁ phosphorylation (Håkansson, Galdi, Hendrick, Snyder, Greengard & Fisone, 2006). This latter effect requires the co-activation of A_{2A} receptors, and either global deletion of DARPP-32 or replacement of Thr³⁴-DARPP-32 with a non-phosphorylatable alanine residue abolishes this effect. mGluR₅ activation was recently shown to contribute to increased Ser⁸⁴⁵ GluR₁ phosphorylation and this effect also depended upon coincident activation of A_{2A} receptors in the striatopallidal pathway (Dell'Anno, Pallottino & Fisone, 2013). In the same study, the ability of the mGluR_{1/5} agonist DHPG to increase Ser⁸⁴⁵-GluR₁ phosphorylation is blocked by either the mGluR₅ antagonist MPEP or by deletion of striatopallidal DARPP-32. Thus, in D₁-expressing MSNs, D₁ receptor activation stimulates increases in Ser⁸⁴⁵-GluR₁ whereas, the phosphorylation of this subunit in the D₂-expressing indirect pathway is attenuated by activity at D₂ receptors but increased by the co-incident activation of mGluR₅ and A_{2A} receptors.

1.9.1.1 DARPP-32 modulates AMPA channel currents

In addition to the regulation of AMPA subunit phosphorylation, AMPA channel currents are enhanced in vitro by the application of D₁ receptor agonist SKF 81297 or a phosphorylated form of DARPP-32, p-D32. For example, application of either SKF 81297 or p-D32 prevents AMPA channel run down in striatal slices (Yan et al., 1999). However, SKF 81297 mediated increases in AMPA channel currents are severely diminished when applied to striatal slices extracted from DARPP-32 KO mice (Yan et al., 1999). Spinophilin, a PP-1 binding peptide, anchors PP-1 in close proximity to

AMPA receptors and interference with this protein by application of a spinophilin competitive peptide significantly reduces the binding of spinophilin to PP-1 and prevents the rundown of AMPA channel currents (Yan et al., 1999).

1.9.2 DARPP-32 and NMDA receptor phosphorylation

NMDA NR₁ receptor phosphorylation at a Ser⁸⁹⁷ residue is similarly stimulated in vitro by PKA and its dephosphorylation is also regulated by the D₁-PKA-DARPP-32-PP-1 pathway (Fienberg et al., 1998; Snyder et al., 1998). DA plus the DA reuptake inhibitor nomifensine, or D₁ agonists (SKF 81297 or SKF 85256), stimulate NMDA NR₁ phosphorylation. Co-application of the D₂ agonist quinpirole blocks the ability of SKF 81297 to increase Ser⁸⁹⁷ NR₁ phosphorylation (Snyder et al., 1998). Directly inhibiting PP-1/2A with calyculin A increases NR₁ phosphorylation, and inhibiting calcineurin, blocks the ability of D₂ receptor agonists to reduce D₁ increases in NR₁ phosphorylation. Finally, NR₁ phosphorylation following the application of DA plus nomifensine or the adenylyl cyclase activator forskolin is severely attenuated in DARPP-32 KO mice (Fienberg & Greengard, 2000).

1.9.2.1 DARPP-32 modulates NMDA channel currents

Striatal DA activity also exerts a significant influence over NMDA currents. NMDA currents are increased in MSNs by the application of SKF 81297. Co-application of SKF 83566, a D₁ receptor antagonist, dampens these electrophysiological effects (Flores-Hernandez et al., 2002). Co-application of the D₂ agonist quinpirole also reduces SKF 81297-stimulated increases in NMDA currents, again indicating that D₁ vs D₂ receptors differentially affect the electrophysiological properties of MSNs (Flores-Hernandez et al., 2002). SKF 81297-mediated NMDA currents are lower in DARPP-32 KO mice than in WT mice (Flores-Hernandez et al., 2002). SKF 81297 mediated increases in NMDA

currents are normalised in DARPP-32 KO mice when it is co-applied with PP-1 inhibitor okadaic acid (Flores-Hernandez et al., 2002). These findings suggest that similar mechanisms (e.g. D₁-PKA-DARPP-32-PP-1) regulate the phosphorylation and dephosphorylation of both AMPA GluR₁ and NMDA NR₁ subunits and the electrophysiological properties associated with these receptors in DARPP-32 expressing regions.

1.9.3 DARPP-32 and GABA_A receptors

β1/β3 subunits of the GABA_A receptor are phosphorylated by PKA (McDonald et al., 1998) and this event requires DARPP-32 to occur normally (Flores-Hernandez et al., 2000). D₁ agonist (SKF 81297) induced phosphorylation of β1/β3 subunits is severely affected in DARPP-32 KO mice and disturbances in this function have important consequences for the electrophysiological properties of GABA_A currents. For example, D₁ receptor stimulation dampens GABA_A currents and this effect is similarly disturbed in DARPP-32 KO mice (Flores-Hernandez et al., 2000).

1.9.4 DARPP-32 and ion channel regulation

In addition to the direct regulation of AMPA GluR₁, NMDA NR₁ and GABA_A subunit phosphorylation, DARPP-32 influences the activity of other key determinants of neuronal excitability such as the Na⁺, K⁺ ATPase ion pump and voltage-dependent N/P-Q-type Ca²⁺ channels and Na⁺ channels. The Na⁺, K⁺ ATPase pump maintains the potential gradient and influences the hyperpolarisation of neurons. D₁ receptor activation by SKF 82526 reduces Na⁺, K⁺ ATPase activity and this effect is abolished in DARPP-32 KO mice (Fienberg et al., 1998). N/P-type Ca²⁺ channels currents are also reduced by a D₁-PKA dependent pathway following the application of SKF 81297 and this effect is significantly reduced in DARPP-32 KO mice (Fienberg et al., 1998). D₁ receptor

activation reduces sodium channels current via a PKA-mediated mechanism (Schiffmann, Lledo & Vincent, 1995). This process also involves the inhibition of PP-1 by DARPP-32 (Schiffmann et al., 1998) and DARPP-32 KO mice display impairments in D₁ receptor mediated changes in Na⁺ currents (Fienberg et al., 1998).

1.9.5 DARPP-32 is a critical mediator of striatal synaptic plasticity

DARPP-32 is not only an effector of discrete ion channel currents but also a critical determinant of long-term experience-dependent changes in the electrophysiological properties of MSNs. Stimulation of corticostriatal glutamate fibres can induce 2 distinct forms of synaptic plasticity, LTD and LTP depending on whether magnesium is present or absent in the solution respectively. Both of these forms of synaptic plasticity are lost in striatal neurons of DARPP-32 KO mice, however, inhibition of PP-1 returned both LTD and LTP in DARPP-32 KO mice (Calabresi et al., 2000). Moreover, both LTD and LTP were blocked by the D₁ antagonist SCH 23390 but the intracellular mechanisms by which this occurred appear to differ. For example, LTD but not LTP was blocked by the NO donor SNAP or the cGMP inhibitor zaprinast whereas LTP but not LTD was blocked by the PKA inhibitor H89.

Whilst D₁ receptors are involved in the induction of LTP and D₂ receptors in the induction of LTD, LTP and LTD can be induced in both D₁-expressing striatonigral and D₂-expressing striatopallidal pathways. Moreover, local deletion of DARPP-32 in either the striatonigral or striatopallidal pathways prevents the induction of LTP (Bateup et al., 2010). One possible explanation for this apparent contradiction in the induction of LTP in both striatonigral and striatopallidal pathways may relate to the presence of adenosine A_{2A} receptors on striatopallidal MSNs. As mentioned previously, A_{2A} receptors like D₁ receptors, are positively coupled to PKA and A_{2A} blockade prevents the induction of LTP (Shen, Flajolet, Greengard & Surmeier, 2008). Therefore, whilst D₁ and NMDA

activation induce LTP in striatonigral MSNs, A_{2A} and NMDA activation induces LTP in striatopallidal neurons. The authors of this study also showed that LTD in the striatopallidal pathway is induced by the activation of D₂ and mGluR₅ receptors and Cav 1.3 Ca²⁺ channels whereas, in the striatonigral pathway, LTD is dependent on the activation of mGluR₅ and Cav 1.3 Ca²⁺ channels. The well-defined role DARPP-32 has in mediating striatal synaptic plasticity most plausibly explains, at least in part, the relationship it has in mediating the behavioural effects of drugs of abuse - see below - and it also suggests that it might have important functional consequences for reward-related instrumental and associative learning.

1.9.6 DARPP-32 is located in MSN spine heads and necks

A recent study using a combined cell culture and stimulated emission depletion microscopy (STED) approach precisely outlined the spatial distribution of DARPP-32 in dendritic spines of MSNs and, in doing so, provided insight into the possible spatial role DARPP-32 has in regulating the synaptic properties of these neurons (Blom et al., 2013). Clusters of DARPP-32 were reported in MSN spine heads with additional groups present in the spine neck. The authors suggest that the position of DARPP-32 within MSN spine heads bequeaths DARPP-32 with the molecular responsibility of modulating synaptic properties and the secondary clusters in the spine neck may confer DARPP-32 with the ability to modulate other functions such as ion dynamics and other intracellular signalling proteins.

1.9.7 Conclusion

Thus far we have seen how midbrain DA and cortical and amygdala glutamate signalling converging at MSNs is post-synaptically integrated, in part, by DARPP-32. The major consequence of these integrative effects is the fine-grained tuning of MSN

electrophysiology which is partially mediated by the inhibition of PP-1 or PKA, depending on the phosphorylation profile of DARPP-32 and the DA receptor type expressed on MSNs. The net effect of these processes involves changes in the phosphorylation of NMDA and AMPA glutamate receptor subunits and also of GABA receptor subunits, as well as the phosphorylation of Na⁺ and Ca²⁺ ion channels and the Na⁺/K⁺ ATPase pump. In addition to this, glutamate and DA signalling converging at DARPP-32 can dynamically stimulate changes in AMPA and NMDA currents, and also long-term changes in the electrophysiology of MSNs (e.g. LTD and LTP). DARPP-32 also regulates the expression of a number of important neuronal proteins including c-fos, delta fosB, arc, enkephalin and dynorphin which we will discuss below.

1.10 DARPP-32 a common striatal substrate for drugs of abuse: behavioural and molecular insights from acute and repeatedly administered drugs of abuse

The striatum is a common neural pathway for drugs of abuse. Psychoactive substances increase striatal DA release and DARPP-32 is a well-studied post-synaptic integrator of drug-stimulated striatal neurotransmission (see Borgkvist & Fisone, 2007; Nairn et al., 2004; & Svenningsson, Nairn & Greengard, 2005 for reviews). Acutely administered drugs of abuse, including amphetamine (Svenningsson et al., 2003), methamphetamine (Chen & Chen, 2005), cocaine (Nishi et al., 2000), morphine (Borgkvist, Usiello, Greengard & Fisone, 2007), cannabis (Borgkvist, Marcellino, Fuxe, Greengard, & Fisone, 2008) and nicotine (Hamada, Higashi, Nairn, Greengard & Nishi, 2004) increase Thr³⁴-DARPP-32 phosphorylation. Acute amphetamine treatment also increases Ser¹³⁰-DARPP-32 phosphorylation (Svenningsson et al., 2003) and nicotine increases Ser⁹⁷-DARPP-32 phosphorylation and Ser¹³⁰-DARPP-32 phosphorylation, whilst decreasing Thr⁷⁵-DARPP-32 phosphorylation (Hamada et al., 2005). Caffeine increases Thr⁷⁵-DARPP-32 phosphorylation (Lindskog et al., 2002) whilst amphetamine (Svenningsson

et al., 2003) and cocaine (Nishi et al., 2000) cause a decrease in the phosphorylation of this residue. The effects of acute cocaine on DARPP-32 are sensitive to the activation of 5-HT_{2C} receptors in the NAcC. Co-administration of the 5-HT_{2C} receptor antagonist Ro 60-0175 attenuates Thr³⁴-DARPP-32 phosphorylation in NAcC, suggesting that DARPP-32 requires co-incident activation of 5-HT_{2C} receptors to undergo D₁ receptor-induced increases in Thr³⁴-DARPP-32 phosphorylation (Cathala et al., 2014). Thus, DARPP-32 is affected by acutely administered psychoactive drugs mainly via their ability to influence Thr³⁴-DARPP-32 and/or Thr⁷⁵-DARPP-32 phosphorylation.

Numerous well-studied behavioural and molecular effects of drugs of abuse involve DARPP-32, and systematic investigations recruiting mice with either global deletion of DARPP-32, or mice with non-phosphorylatable alanine knockin replacements at specific phosphorylation residues have characterised the pathways by which DARPP-32 mediates these effects. The locomotor activating effects of various psychoactive drugs is disturbed in DARPP-32 mutant mice. For instance, the locomotor activating effects of acutely administered cocaine (Fienberg et al., 1998), caffeine (Lindskog et al., 2002), amphetamine (as cited in Fienberg et al., 1998) and morphine (Borgkvist et al., 2007) are attenuated in DARPP-32 KO mice. In contrast, locomotor activity to an acute dose of ethanol is enhanced in DARPP-32 KO mice (Risinger, Freeman, Greengard & Fienberg, 2001). Using mice with bacterial artificial chromosomes which selectively tag DARPP-32 in the D₁-expressing striatonigral vs D₂-expressing striatopallidal pathways with distinct fluorescence proteins, Bateup and colleagues revealed that cocaine differentially alters the phosphorylation of DARPP-32 in striatonigral and striatopallidal neurons, promoting increases in Thr³⁴-DARPP-32 and decreases in Thr⁷⁵-DARPP-32 phosphorylation in striatonigral MSNs, but promoting decreases in Thr³⁴-DARPP-32 and increases in Thr⁷⁵-DARPP-32 phosphorylation in striatopallidal MSNs. The D₂ receptor

antagonist haloperidol selectively affects the phosphorylation of Thr³⁴-DARPP-32 in the striatopallidal pathway but does not affect Thr⁷⁵-DARPP-32 phosphorylation in either pathway. (Bateup et al., 2008). In a similar study, Bateup and colleagues showed that targeted genetic disruption of DARPP-32 in striatonigral MSNs reduced the acute locomotor activating effects of cocaine whilst targeted disruption of striatopallidal DARPP-32 reduced the cataleptic liability of haloperidol (Bateup et al., 2010). Thus striatonigral D₁ and striatopallidal D₂ receptors differentially modulate DARPP-32 and the motor effects of distinct DAergic compounds.

1.10.1 DARPP-32 and psychomotor sensitisation

In contrast to acutely administered drugs of abuse, cocaine or methamphetamine, when repeatedly administered, stimulate an inverse phosphorylation profile which involves reductions in Thr³⁴-DARPP-32 and increases in Thr⁷⁵-DARPP-32 (Chen & Chen, 2005; Scheggi et al., 2007). Repeated drug administration also induces a profound sensitisation of locomotor activity and this behaviour is disturbed in DARPP-32 KO mice or in knockin mutant mice with a targeted genetic interference of either the Thr³⁴-DARPP-32 or Thr⁷⁵-DARPP-32 substrates. For example, DARPP-32 KO mice display increased psychomotor sensitisation to repeated cocaine administration (Hiroi et al., 1999) and this effect is mirrored in Thr³⁴-DARPP-32 knockin mutants (Zachariou et al., 2006). In contrast to Thr³⁴-DARPP-32 knockin mutants, Thr⁷⁵-DARPP-32 knockin mutants do not acquire psychomotor sensitisation to cocaine (Zachariou et al., 2006). Unlike cocaine, however, psychomotor sensitisation is undisturbed to repeated morphine administration (Borgkvist et al., 2007). The injection protocol recruited in a given study might also be an important consideration when examining the acquisition of psychomotor sensitisation in DARPP-32 mutant mice. When employing the so-called two-injection sensitisation protocol,

Valjent and colleagues reported that both cocaine (Valjent et al., 2005) and morphine sensitisation (Valjent et al., 2010) are blocked in Thr³⁴-DARPP-32 mutant mice.

1.10.2 DARPP-32 is a critical mediator of drug-induced gene transcription

Drugs of abuse such as cocaine and amphetamine, and DA agonists which alter the phosphorylation of the aforementioned GluR₁ and NR₁ glutamate receptor subunits also stimulate the expression of genes and IEGs in striatal neurons. For example, the D₁ agonist SKF 82958 increases the expression of the opioid peptides substance P and prodynorphin, the IEG zif268 and the neuronal activity marker c-fos. The expression of these neuropeptides and IEGs is disturbed in DARPP-32 KO mice (Svenningsson et al., 2000). Drugs of abuse like amphetamine and cocaine similarly stimulate the expression of genes and IEGs such as arc, c-fos and deltaFosB. Psychostimulant induced expression of these genes is severely diminished in DARPP-32 KO mice or in mice with a targeted interference of the Thr³⁴-DARPP-32 substrate (Fienberg et al., 1998; Hiroi et al., 1999; Zachariou et al. 2006). Thus, DARPP-32 is an important effector of striatal gene expression, in addition to its role as an effector of striatal electrophysiology.

1.10.3 DARPP-32 and drug reinforced learning

The repeated pairing of a drug in a discrete context promotes conditioned contextual associations between the drug and its administration environment with subjects preferring to reside in a drug-paired context more than a non-drug paired context when tested under extinction; this behaviour is known as CPP. Cocaine CPP simultaneously increases accumbal Thr³⁴-DARPP-32 and reduces Thr⁷⁵-DARPP-32 (Tropea, Kosofsky & Rajadhyaksha, 2008). In addition to this, cocaine (Zachariou et al., 2002) and ethanol (Risinger et al., 2001) CPP are disturbed in DARPP-32 mutant mice. DARPP-32 also mediates the reinforcing properties of psychostimulant drugs. Drug self-administration

paradigms assess the abuse liability of a given substance by correlating its reinforcing properties with the number of drug infusions subjects earn. Mice with targeted interference of either the Thr³⁴ or the Ser¹³⁰ residue recorded significantly more cocaine infusions than WT controls (Zhang et al., 2006). In contrast, ethanol self-administration is significantly lower in DARPP-32 KO mice (Risinger et al., 2001).

1.11 DARPP-32 and natural reward

Although the vast majority of behavioural work relating to DARPP-32 has been published in the domain of drug reward, DARPP-32's behavioural effects are not limited to psychoactive substances. In similar fashion to drugs of abuse, novel food reinforcers initially promote increases in Thr³⁴-DARPP-32, reductions in Thr⁷⁵-DARPP-32, and increases in GluR₁ and NR₁ phosphorylation (Rauggi et al., 2005). This effect was apparent between 30 & 45 min after receipt of the reinforcer but these values had returned to baseline after approximately 60 min. However, reduced Thr³⁴-DARPP-32 and increased Thr⁷⁵-DARPP-32 were observed 2-3 hrs post reinforcer receipt. The D₁ receptor antagonist SCH 23390 blocked all of these effects whereas the delayed increase in Thr⁷⁵-DARPP-32 was prevented by the mGluR₅ antagonist MPEP. The phosphorylation profile of DARPP-32 in the NAcSh, though not the NAcC, habituates in response to repeated palatable food exposure in non-food-deprived but not food-deprived subjects (Danielli et al., 2010; Scheggi, Secci, Marchese, De Montis & Gambarana, 2013). Nonnutritive sweeteners (e.g. saccharin) promote similar changes in DARPP-32 phosphorylation to palatable foods (Scheggi et al., 2013). However, DARPP-32 phosphorylation in the NAcSh of food-deprived subjects does habituate to nonnutritive sweetener, suggesting that caloric demand prevents the habituation of DARPP-32 phosphorylation in response to motivationally relevant goals. Thus, as with drugs of abuse, food reinforcers promote changes in DARPP-32 phosphorylation that involve time-dependent shifts in

phosphorylation. However, subtle differences in the ability of food vs drug to modify DARPP-32 phosphorylation exist, with motivational state being of particular relevance to food reinforcers.

1.11.1 DARPP-32: instrumental learning and behavioural flexibility

Accumulating evidence has identified instrumentally induced changes in DARPP-32 phosphorylation in the nucleus accumbens and CPu during high effort tasks. Thr³⁴-DARPP-32 phosphorylation in the nucleus accumbens core is associated with the magnitude of responding in a concurrent PR free-feeding choice task (Randall et al., 2012) and the topographical profile of DARPP-32 phosphorylation undergoes the classical ventral-dorsal shift as a function of the duration of training in a fixed ratio-5 (FR-5) schedule of reinforcement procedure (Segovia, Correa, Lenington, Conover & Salamone, 2012). DARPP-32's role in mediating effortful behaviours does not appear to be a mere correlational phenomenon. For instance, evidence suggests that mice with a Ser⁹⁷-DARPP-32 alanine knockin mutation are impaired during food reinforced progressive ratio schedules of reinforcement, though these results were limited to a single session test for PR (Stipanovich et al., 2008). In addition to the role of DARPP-32 in free operant responding, mice lacking the DARPP-32 gene display impairments during a simple instrumental reversal learning task (Heyser, Fienberg, Greengard & Gold, 2000). Finally, DARPP-32 KO mice lack novel object recognition, with KO mice exploring novel and familiar objects equally (Heyser, McNaughton, Vishnevetsky & Fienberg, 2013). The authors of this study suggest that this impairment may reflect disturbances in behavioural flexibility such that the DARPP-32 KO mice may either be less responsive to environmental changes or have deficient attention. Novel object recognition was rescued in DARPP-32 KO mice in this study by administering methylphenidate (MPH) which suggests that behavioural disturbances might be rescued in these mice by

increasing DA transmission. Thus DARPP-32 has important consequences for instrumental learning processes and behavioural flexibility in rodents which require subjects to update/modify their behaviour.

1.12 DARPP-32 and human reinforcement learning

Perhaps in line with the above findings from preclinical (rodent) models, DARPP-32 has been studied in a few human reward learning experiments. For example, performance during a probabilistic reinforcement learning task was predicted by the presence of a single nucleotide polymorphism (SNP) of the DARPP-32 gene with AA homozygote carriers of this rs907094 polymorphism faring better at the task (Frank, Moustafa, Haughey, Curran & Hutchison, 2007). This task requires subjects to learn about the probability that pairs of stimuli have of being correct and to modify their performance as they learn the probabilistic relations between the stimuli and their likelihood of being correct. It has also been suggested that DARPP-32 is involved in the updating of outcome-expectancies in humans. In an electroencephalography (EEG) study, AA homozygote carriers of the same rs907094 SNP of the DARPP-32 gene displayed greater P200 amplitudes whilst performing the same probabilistic reinforcement task utilised by Frank and co-workers (Hämmerer et al., 2013). The task provides subjects with feedback about their performance as they select one of 2 probabilistically constrained stimuli (e.g. 80% correct vs 20% correct, 60% correct vs 40% correct etc.), so as subjects progress through the task they are required to update outcome expectancies in order to maximise gains. It has been suggested that the amplitude of the P200 wave “reflects updating in changing environments” (Hämmerer et al., 2013). In addition to this, T carriers of the rs907094 polymorphism display improved positive reinforcement learning during a task in which subjects were more likely to be rewarded with points if they responded quickly (Frank, Doll, Oas-Tepstra & Moreno, 2009). DARPP-32 therefore has important behavioural

consequences for reinforcement learning and decision-making processes in human subjects.

1.13 The DARPP-32 knockout mouse

The DARPP-32 knockout (KO) mouse is a fairly well-characterised mouse construct which has proved instructive for investigating incentive motivational processes. In these mutant mice, the DARPP-32 gene is disrupted by replacing a 400bp fragment of the DARPP-32 gene with a neomycin gene (Fienberg & Greengard, 2000). The DARPP-32 KO mouse does not show any baseline deficits in locomotion or any abnormalities in gait (Fienberg & Greengard, 2000). As mentioned above, these mice show selective dose-dependent behavioural deficits to acute and repeatedly administered drugs of abuse, disturbances in ethanol and cocaine self-administration, impairments in novel object recognition and in reversal learning. In addition to this, these mice display abnormalities in striatal synaptic plasticity, AMPA subunit phosphorylation, AMPA channel currents, NMDA subunit phosphorylation and NMDA channel currents, as well as disturbances in ion channel properties and also to drug-stimulated gene transcription. There are few, if any, published studies in existence which have examined the relationship between DARPP-32 and complex conditioned reward behaviours, such as PIT or CRf. There are, however, some existing unpublished data (Crombag et al., 2008) which suggest that, whilst DARPP-32 KO mice acquire conditioned approach and variable interval schedules of reinforcement, they are impaired during the general PIT test. In contrast, DARPP-32 KO mice display CRf that is indistinguishable to WTs.

In light of the existing data in animals indicating that DARPP-32 is an important component of instrumental processes and conditioned behaviours, and in light of the insights from human studies indicating that DARPP-32 contributes to cost-benefits computations, it was felt that this mouse represented an important and interesting tool for

addressing the role of DARPP-32 in mediating incentive motivational behaviours that directly involve reward-value relations.

1.14 Thesis aims

Whilst the above evidence exposes DARPP-32 as a major player in the effects of drugs, natural rewards and the ability of organisms to behaviourally adapt (i.e. learn) in their presence, many question remain as to the precise mechanisms by which DARPP-32 does so. As noted at the beginning of this introduction, incentive learning involves complex mechanisms by which animals learn about the relations between rewards and their investments costs. Adaptive reward related behaviours also involve associative and instrumental mechanisms by which animals can learn about the relations between rewards and reward-predictive stimuli and the interaction such stimuli have with instrumentally focussed behaviour. To this end, the following specific aims were explored:

- 1) Whilst DARPP-32 localisation in the mouse has been published, a more thorough anatomical analysis of DARPP-32 expression patterns was conducted in the circuitries involved in incentive learning, focussed on the accumbens and amygdala with the aim of more precisely elaborating the pattern of DARPP-32 in the mouse forebrain.
- 2) Although DARPP-32 is expressed in brain regions (e.g. PFC and NAc) known to underpin delay-based instrumental cost-benefits computations and, even though such computations are sensitive to interference with the DA and glutamate systems, there are no existing studies to have directly addressed whether DARPP-32 is a molecular component of delay-based instrumental reinforcement. This question was addressed by comparing the performance of DARPP-32 knockout mice, DARPP-32 heterozygous mice and wildtype mice during an intertemporal discounting task. Mice

were also exposed to 2 reinforcer devaluation manipulations. Because DARPP-32 is also expressed in regions associated with reinforcer devaluation (e.g. OFC & CPu) and habit formation (e.g. CeA and CPu) and, because DARPP-32 mutant mice exhibited impaired instrumental motivation during a progressive ratio of responding (Stipanovich et al., 2008), it was deemed necessary to establish whether differences in instrumental motivation might account for any potential between genotype differences that might arise during this task.

3) In similar fashion, probabilistically constrained cost-benefits decisions are underpinned by brain regions associated with the expression of DARPP-32 (e.g. PFC, NAc) and, again, probabilistic reinforcement choices are sensitive to direct manipulation of either the DA or glutamate systems. In addition to this, human participants with an allelic variant of a DARPP-32 SNP performed more efficiently in a probabilistic task than participants who were carriers of a different allele (Frank et al., 2007). There are no existing studies to have established whether DARPP-32 is an important component of probabilistic cost-benefits decisions in rodents. To directly address whether DARPP-32 is an important molecular component of probabilistic reinforcement, performance was compared between DARPP-32 KO and WT mice during a probability discounting task. Mice were also subjected to 2 reinforcer devaluation manipulations for the reasons outlined in the previous paragraph.

4) In addition to time and risk, effort costs are also incurred during instrumental reward-seeking. Furthermore, because Ser⁹⁷-DARPP-32 knockin mice were impaired on a single session test of progressive ratio testing (Stipanovich et al., 2008), another aim was to establish whether DARPP-32 KO mice were similarly less willing than

wildtype controls to expend effort to achieve reinforcement. To this end, the performance of DARPP-32 KO mice was compared against the performance of WT mice during an extended food-reinforced progressive ratio experiment. Mice were tested during a variety of progressive ratio schedules, each associated with unique effort costs. Mice were also exposed to a number of reinforcer devaluation manipulations with the aim of establishing the sensitivity of the procedure to measure instrumental motivation and to also identify whether any differential sensitivity to shifts in motivational state were evident between genotypes.

5) Prior research has suggested that DARPP-32 KO mice are unable to perform general PIT. In addition to this, certain behavioural disturbances in DARPP-32 KO mice (e.g. acute cocaine-induced hyperlocomotion) (Fienberg et al., 1998) and novel object recognition (Heyser et al., 2013) have been restored by increasing DA transmission. One further aim of this thesis was to establish whether the absence of general PIT in DARPP-32 KO mice could be rescued by increasing DA transmission prior to PIT test. To this end, DARPP-32 KO and WT mice were first provided with instrumental and Pavlovian training and then given 3 PIT tests, once with saline (SAL) on board, and with methylphenidate (2.5 mg/kg, 5 mg/kg) on board during 2 further tests.

6) Whilst previous research has identified an important role for DARPP-32 in the locomotor response to acutely administered amphetamine and also the acquisition of cocaine psychomotor sensitisation, no data are known to exist which have addressed whether DARPP-32 is an important component of amphetamine psychomotor sensitisation. The final aim of this thesis was to address this question. DARPP-32 KO

and wildtype animals were repeatedly administered saline or amphetamine over 11 sessions, before undergoing a 7 day washout period, which was followed by an escalating dose-response test to identify whether there were any between-genotype differences in the expression of amphetamine psychomotor sensitisation.

Fluorescence immunohistochemistry

2.1 Introduction

DARPP-32 has been extensively mapped and quantified in a host of phylogenetically related and unrelated species including frog (Hemmings & Greengard, 1986), cow (Hemmings & Greengard, 1986), turtle (Smeets, Lopez & Gonzalez, 2003), gecko (Smeets, Lopez & Gonzalez, 2001), rabbit (Hemmings & Greengard, 1986), rat (Hemmings & Greengard, 1986; Ouimet et al., 1984; Ouimet, Langley-Guillon & Greengard, 1998; Walaas & Greengard, 1984), mouse (Perez & Lewis, 1992), human (Brene et al., 1994) and non-human primates (Berger, Febvret, Greengard & Goldman-Rakic, 1990; Ouimet, Lamantia, Goldman-Rakic, Rakic & Greengard, 1992). DARPP-32 is most abundantly enriched in the mammalian brain in the striatum - in both its ventral and dorsal portions - the substantia nigra, the pallidum and the olfactory tubercle (Hemmings & Greengard, 1986). Striatal MSNs and their projections are highly enriched with DARPP-32, with immunolabelling occurring in approximately 96% of MSNs in the rat caudate putamen (Ouimet et al., 1998). DARPP-32 has additionally been observed in the frontal cortex, the amygdala, the ventral tegmental area, the cerebellum, the thalamus and the hippocampus, as well as the olfactory bulb and the septum of the rat and mouse (Ouimet et al., 1984; Perez & Lewis, 1992).

DARPP-32 is expressed throughout the dendrites, spines, cytoplasm and the nucleus of striatal MSNs in the rat brain. (Ouimet et al., 1984; Ouimet & Greengard, 1990; Walaas & Greengard, 1984). In layer II of the rat brain, sparse DARPP-32 enrichment has been observed throughout the cytoplasm, nucleus and apical dendrites of pyramidal cells (Ouimet et al., 1984). Intense – moderate amygdaloid immunolabelling for DARPP-32 has been observed in both the central and basolateral amygdala nuclei of the rat (Ouimet

et al., 1984) whereas DARPP-32 mRNA was highly expressed in the mouse CeA but absent in the BLA (Perez & Lewis, 1992). Pallidal labelling has been observed in axons, rather than the cell body or dendrites of pallidal neurons, presumably in the terminals of striatal MSN projection neurons forming synapses with pallidal cells (Ouimet et al., 1984). In-situ hybridisation performed in mouse tissue has identified a considerable cellular and regional overlap between the expression of DARPP-32 mRNA in the mouse and the expression of the DARPP-32 protein in rats (Perez & Lewis, 1992).

Immunofluorescence was first used to outline the topographical and cellular distribution of DARPP-32 in the rat brain approximately 30 years ago (Ouimet et al., 1984). However, no such data are known to exist for the adult mouse. Therefore, the primary objective of this study was to use fluorescence immunohistochemical detection (fIHC) to more precisely delineate the topographical distribution of DARPP-32 in the mouse brain by focussing on 3 dopaminoceptive areas that are known to play a role in incentive motivational behaviours (e.g. PFC, striatum and amygdala). In particular, and in light of reported differences in the expression profile of DARPP-32 protein in the rat amygdala, and the DARPP-32 mRNA profile in the mouse amygdala (Ouimet et al., 1984; Perez & Lewis, 1992), a second specific aim was to delineate the precise anatomical distribution of DARPP-32 expression within the amygdala compartments (central versus basolateral compartments). To this end, slices were double labelled for DARPP-32 and the calcium binding protein calbindin D-28k which is expressed preferentially in the basolateral, but not the central nucleus of the rodent amygdala (McDonald, 1997). In light of previous anatomical evidence, as well as evidence from behavioural tasks (e.g. general PIT and CRf) recruiting distinct amygdala sub-divisions (Crombag et al., 2008), it was predicted that fIHC detection of DARPP-32 would be high in the central, but not in the basolateral compartment of the amygdala. Finally, other anatomical regions implicated in

incentive motivational tasks were also explored, including the NAc, CPu, ACC, mPFC and CPu.

2.2 Methods

2.2.1. Genotyping

DARPP-32 status was identified by polymerase chain reaction (PCR) gel electrophoresis. Tissue was first collected from individual mouse ears and then immersed in a 20µl solution containing 1 mg/ml proteinase K (Roche Products LTD, UK) in lysis buffer solution consisting of 10mM EDTA (Sigma-Aldrich, Dorset, UK) and 20 mM Tris HCl (Sigma-Aldrich, Dorset, UK). Each sample was then overlaid with 2 drops of PCR grade mineral oil (Sigma-Aldrich, Dorset, UK) before being placed into a PCR machine to undergo tissue digestion.

Once digested, each sample was diluted in 80µl of PCR grade water. The primers identifying the DARPP-32 gene were a forward (AGAGAACTGAATCTTCTTGCG) and a reverse primer (GCGGGATTTTCCTGG). A forward (GCAAGGTGAGATGACAGGAGATC) and a reverse primer (CGCTTGGGTGGAGAGGCTATTC) were used to identify the neomycin replacement gene which was substituted for DARPP-32 in KO mice. Two samples per mouse were analysed, 1 sample to identify the presence or absence of the DARPP-32 gene and 1 sample to identify the presence or absence of the neomycin replacement gene. To do this, 0.5 µl of each primer was placed into 23 µl of Mega Mix Blue (Microzone LTD, Haywards Heath, UK), along with 1µl of DNA. This mixture was then overlaid with 2 drops of PCR grade mineral oil before being subjected to PCR.

After completion of the PCR cycles, samples were subjected to gel electrophoresis. In preparation for this process, a 1.5% agarose gel consisting of 300ml 1 x TAE buffer and

4.5g agarose and containing 10 µl of ethidium bromide was made. The completed gel was then placed into an electrophoresis tank containing 1 X TAE running buffer, each sample was added to the gel and 120v was then applied to the gel for 30 min. DARPP-32 status was then identified by photographing the gel whilst it was exposed to ultraviolet light.

2.2.2 Subjects

Six DARPP-32 KO mice and 6 WT littermates, aged 8-16 weeks old were used for this study. Mice were at least the 8th generation, backcrossed on a C57BL/6J strain and were bred at the University of Sussex through heterozygote parent mating to yield WT and KO offspring. Mice were sacrificed with a terminal dose (200 mg/kg) of sodium pentobarbital (Euthatal, Merial Animal Health LTD, Harlow, UK) delivered intraperitoneally (IP). Brains were fixed via transcardial perfusion performed with 4% paraformaldehyde (PFA). Following dissection, brains were post-fixed for between 2 to 4 hr in 4% PFA and then suspended in a 30% sucrose in 0.1M PBS solution for up to 3 days at 4°C before being stored at -80°C.

2.2.3 Fluorescent immunohistochemical (fIHC) detection of DARPP-32

Free-floating brain sections containing the PFC (containing ACC, OFC and mPFC), striatum (containing the dorsal and ventral striatum, including both the NAcC and NAcSh sub-divisions, and the OT), the ventral pallidum and the amygdaloid complex (containing both CeA and BLA divisions) were identified with guidance from Paxinos and Franklin's Brain Atlas (Academic Press, San Diego, CA, USA). Brain sections 30 microns in thickness were collected from these regions and stored in PBS (0.1M) containing 0.02% sodium Azide at 4°C.

Briefly, the fIHC procedure was as follows: sections from regions of the prefrontal cortex, striatum and amygdala were exposed to 3 x 10 min washes in 1 x Tris-buffered

saline (TBS), followed by a 30 min dual incubation/blocking procedure consisting of bathing the sections in a 10% normal goat serum (NGS) solution in 0.2% TBS-TX to permeabilise the tissue. Sections were then incubated overnight for between 14-16 hrs in DARPP-32 anti-rabbit (1/10000, Cell Signalling Technologies) and Calbindin-D28k anti-mouse (1/8000, Sigma-Aldrich, Dorset, UK) antibodies in a 3% NGS solution in 0.2% TBS-TX at 4°C. After incubation with the primary Ab, the sections were given 3 additional 10 min washes in TBS followed by incubation with Alexa Fluor 488 anti-rabbit and 568 anti-mouse (1/200, Life Technologies, Paisley, UK) secondary Ab for 1 hr in 3% NGS in 0.2 TBS-TX. Slices were given 3 final 10 min washes and were then mounted onto either gelatin coated or Super Frost Plus (Fisher Scientific, Loughborough, UK) histology slides and air-dried prior to coverslipping. Coverslips were mounted to histology slides with PermaFluor mounting medium (Thermo Fisher Scientific, Loughborough, UK).

2.2.4 Imaging

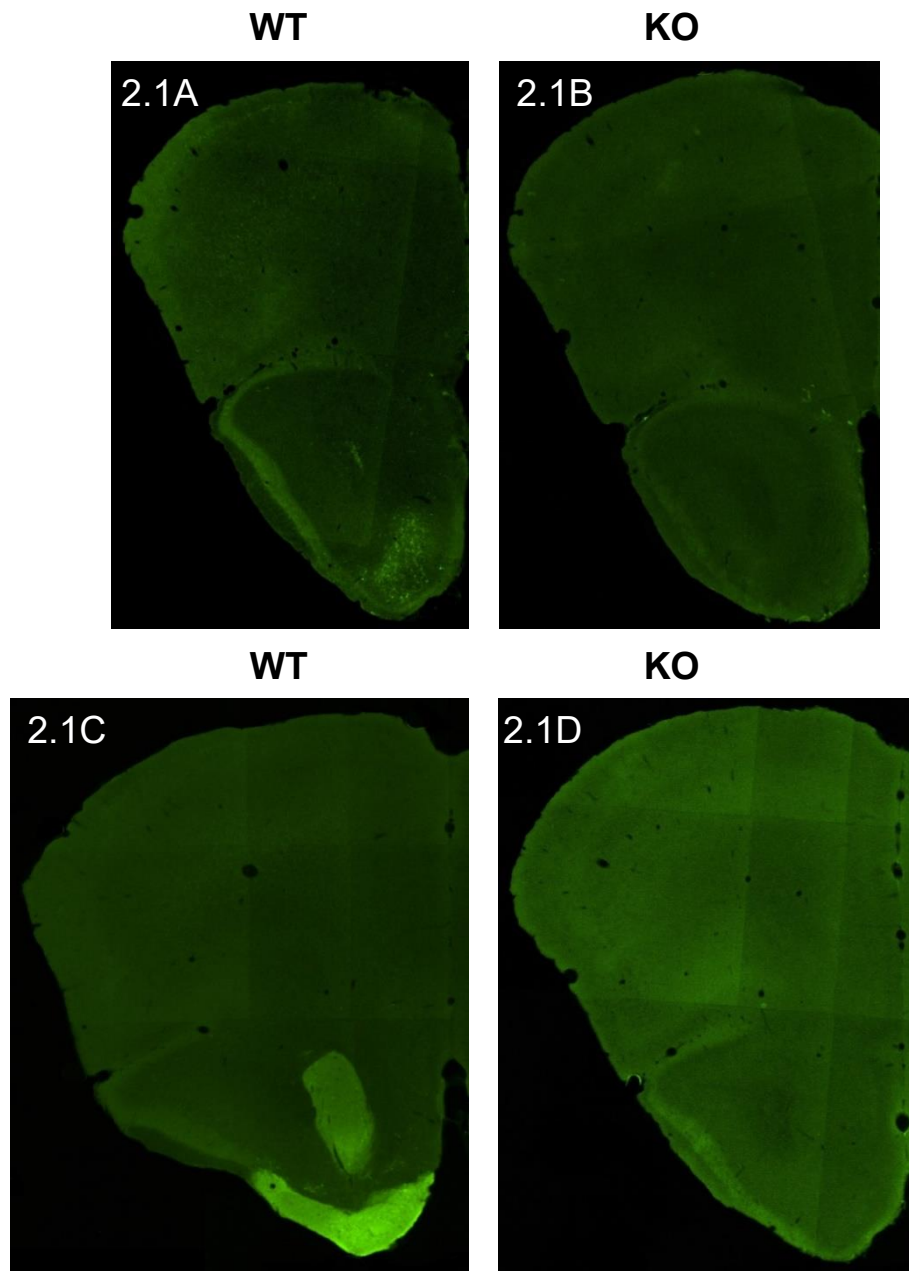
All microscopy was performed using a BX53 Epifluorescent microscope (Olympus, Southend, UK) and all images were captured with a QI Click (Q Imaging, Surrey, BC, Canada) 12-bit fluorescent imaging camera controlled by iVision (Biovision Technologies, Exton, PA, USA) for Mac software. Multiple images taken at 4x magnification (mag) were captured to map an entire hemisphere of each target area for DARPP-32 expression patterns and joined using iVision to create a single composite image of the entire hemisphere. These composite images depicting DARPP-32 expression in an entire hemisphere are compared between WT and KO subjects in target regions in order to provide the reader with evidence of antibody specificity. For finer, within-region and cellular level qualitative analysis of DARPP-32 distribution, additional images were taken at 4x, 10x and 20x magnifications of brain slices from WT mice.

2.3 Results

Table 2.1 Summary of DARPP-32 expression in the mouse forebrain

Region	DARPP-32 expression
Anterior olfactory nucleus	Weak-to-moderate-to-intense
Basolateral nucleus of the amygdala	Null
Caudate-putamen	Intense
Central nucleus of the amygdala	Moderate-to-intense
Layers II/III	Weak
Layer VI	Weak
Medial prefrontal cortex	Null
Nucleus accumbens core	Intense
Nucleus accumbens shell	Intense
Olfactory tubercle	Intense
Orbitofrontal cortex	Weak
Primary motor cortex	Moderate
Primary somatosensory cortex	Moderate
Ventral pallidum	Intense

2.3.1 Telencephalon



Figs. 2.1A & 2.1B. Composite images representing rostral forebrain areas in a WT mouse (**Fig. 2.1A**) (approx. bregma 2.22mm – 2.34 mm) and a comparable section from a DARPP-32 KO mouse (**Fig 2.1B**) (approx. bregma 2.22mm – 2.34mm). **Figs. 2.1C & 2.1D** Comparatively caudal forebrain sections taken from a WT mouse (**Fig. 2.1C**) (approx. bregma 1.98mm - 2.10mm) and a DARPP-32 KO mouse (**Fig. 2.1D**) (approx. bregma 1.98 – 2.10mm).

2.3.2 Prefrontal, motor and somatosensory cortices

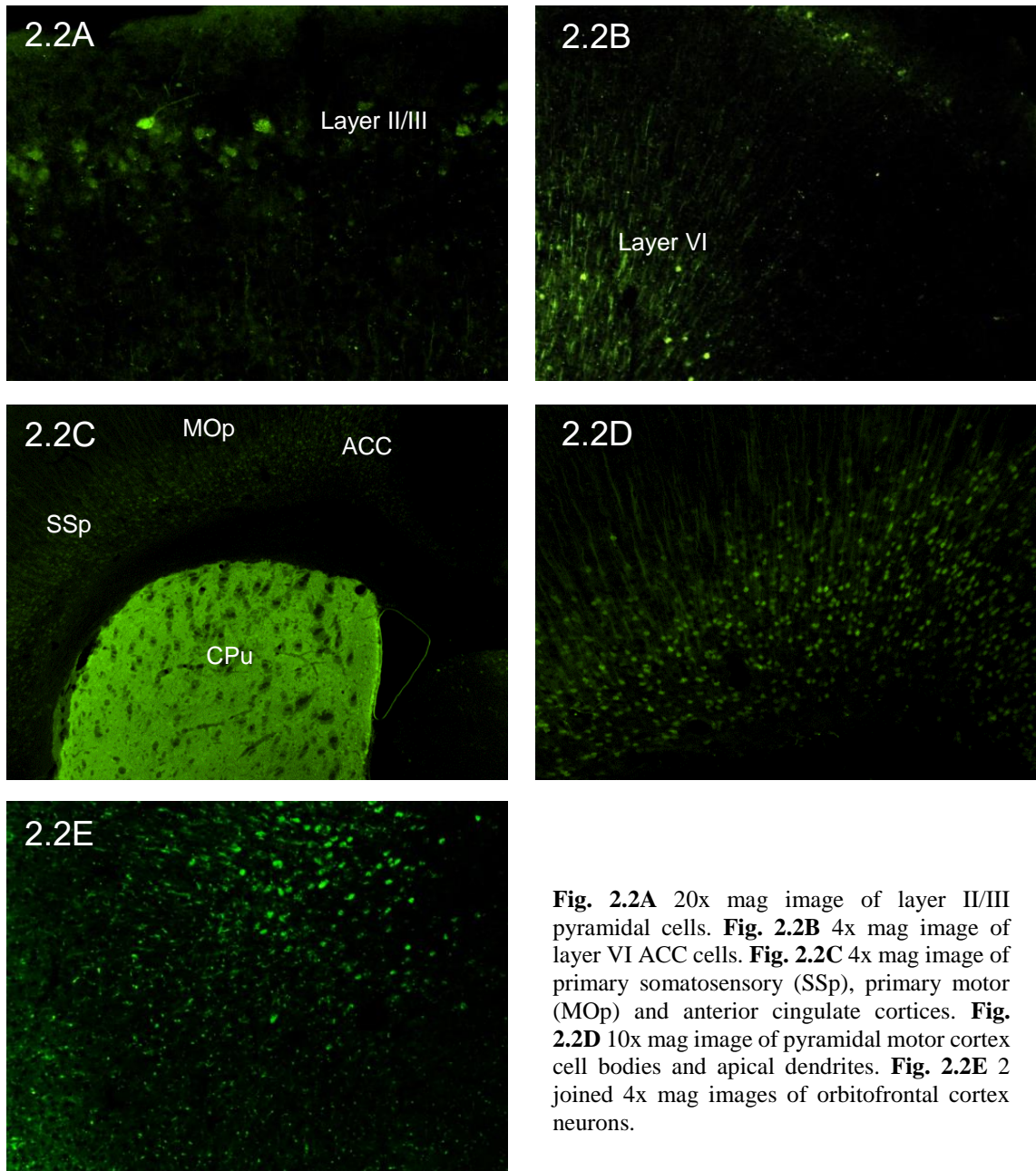
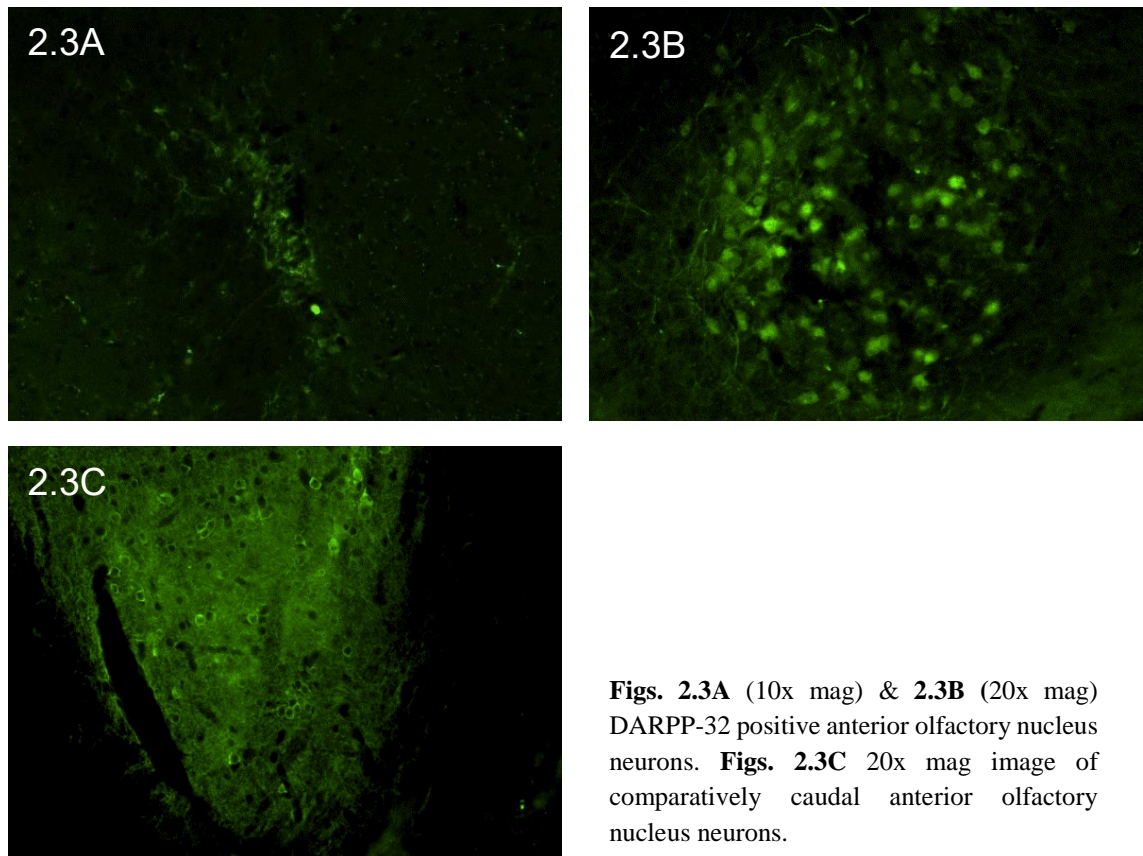


Fig. 2.2A 20x mag image of layer II/III pyramidal cells. **Fig. 2.2B** 4x mag image of layer VI ACC cells. **Fig. 2.2C** 4x mag image of primary somatosensory (SSp), primary motor (MOp) and anterior cingulate cortices. **Fig. 2.2D** 10x mag image of pyramidal motor cortex cell bodies and apical dendrites. **Fig. 2.2E** 2 joined 4x mag images of orbitofrontal cortex neurons.

Immunoreactivity for DARPP-32 was mostly weak and only present in sparse populations of pyramidal neurons in layers II-III and VI of the cerebral cortex (Figs. 2.2A, 2.2B, 2.2C, 2.2D & 2.2E). Weak pyramidal labelling was seen throughout the cell bodies and apical dendrites of a narrow band of neurons in layers II/III throughout most of these cortical layers (Figs. 2.2A & 2.2B). Layer VI pyramidal labelling was similarly seen in cell bodies and apical dendrites and although this expression was more intense than in

layers II-III, it was restricted to sparse clusters of neurons in the forebrain resembling the profile seen in Fig. 2.2B. In layer VI, DARPP-32 expression was seen with greater intensity in comparatively caudal forebrain slices in the somatosensory, cingulate and motor cortices (Figs. 2.2C & 2.2D). However, this caudal labelling was still restricted to sparse networks of pyramidal cells. Sparse pyramidal labelling was also evident in the orbitofrontal cortex (Fig. 2.2E). The medial PFC (prelimbic and infralimbic cortices) appeared to be almost entirely devoid of DARPP-32 labelling (images not shown).

2.3.3 Anterior olfactory nucleus (AON)



Figs. 2.3A (10x mag) & **2.3B** (20x mag) DARPP-32 positive anterior olfactory nucleus neurons. **Figs. 2.3C** 20x mag image of comparatively caudal anterior olfactory nucleus neurons.

Weak-to-moderate-to-intense labelling was present in AON cells. Visual comparison of immunoreactivity between rostral and caudal forebrain regions indicated a general rostral-caudal gradient in the intensity of DARPP-32 expression in these regions, with caudal forebrain sections fluorescing more intensely. For example, relatively sparse

populations of moderate labelling were seen in the nuclei, cytoplasm and dendrites of cells of rostral forebrain sections (Figs. 2.3A & 2.3B). In comparison, a comparatively dense network of intense DARPP-32 immunolabelling was evident in the nuclei, cytoplasm and dendrites of caudal AON neurons (Fig. 2.3C).

2.3.4 Amygdala

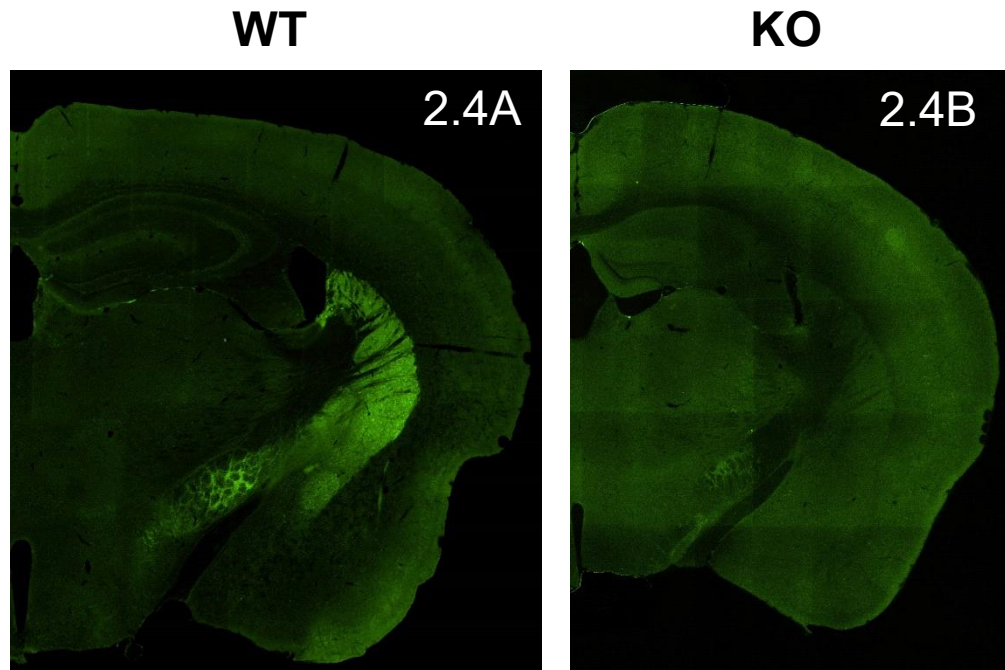


Fig. 2.4 Composite images of a full brain hemisphere taken from a WT mouse (**Fig. 2.4A**) (approx. bregma -1.46mm - -1.58mm) and a DARPP-32 KO mouse (**Fig. 2.4B**) (approx. bregma -1.22mm - -1.34mm) mice determined to contain the CeA and BLA.

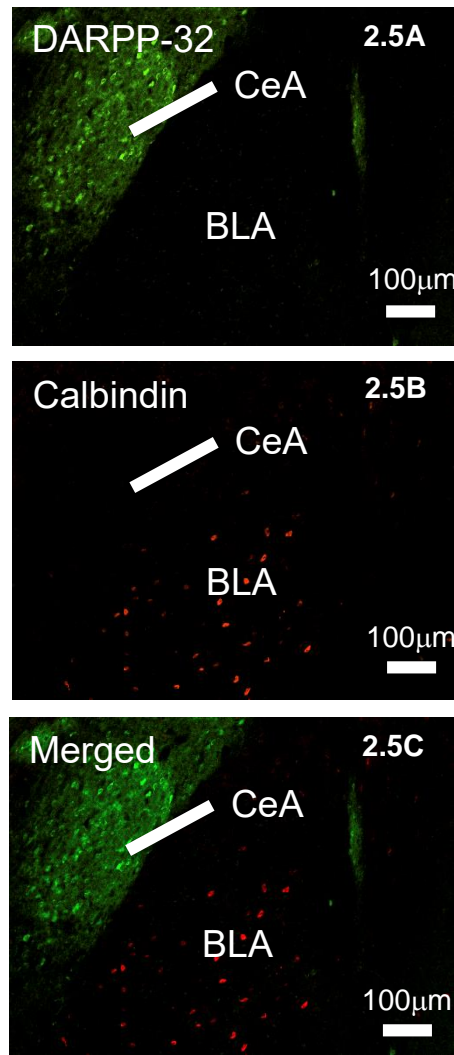


Fig. 2.5 DARPP-32 positive CeA and DARPP-32 negative BLA (**Fig. 2.5A**), calbindin positive BLA and calbindin negative CeA (**Fig. 2.5B**), and Figs. 2.5A & 2.5B merged (**Fig. 2.5C**).

CeA neurons were moderately-to-intensely immunoreactive for DARPP-32 (Fig. 2.5A). In contrast, the basolateral portion of the amygdala was entirely devoid of DARPP-32 labelling (Fig. 2.5A). This anatomical dissociation in the expression of DARPP-32 between amygdala CeA and BLA sub-compartments was complemented by images of tissue double-labelled for DARPP-32 and calbindin-D28k taken at 10x magnification. Calbindin expression was seen only in the BLA (Fig. 2.5B) and no overlap in expression of DARPP-32 and calbindin-D28k is seen in Fig. 2.5C. Thus, DARPP-32 expression was restricted to the CeA and calbindin-D28K immunoreactivity was isolated to the BLA.

2.3.5 Basal ganglia

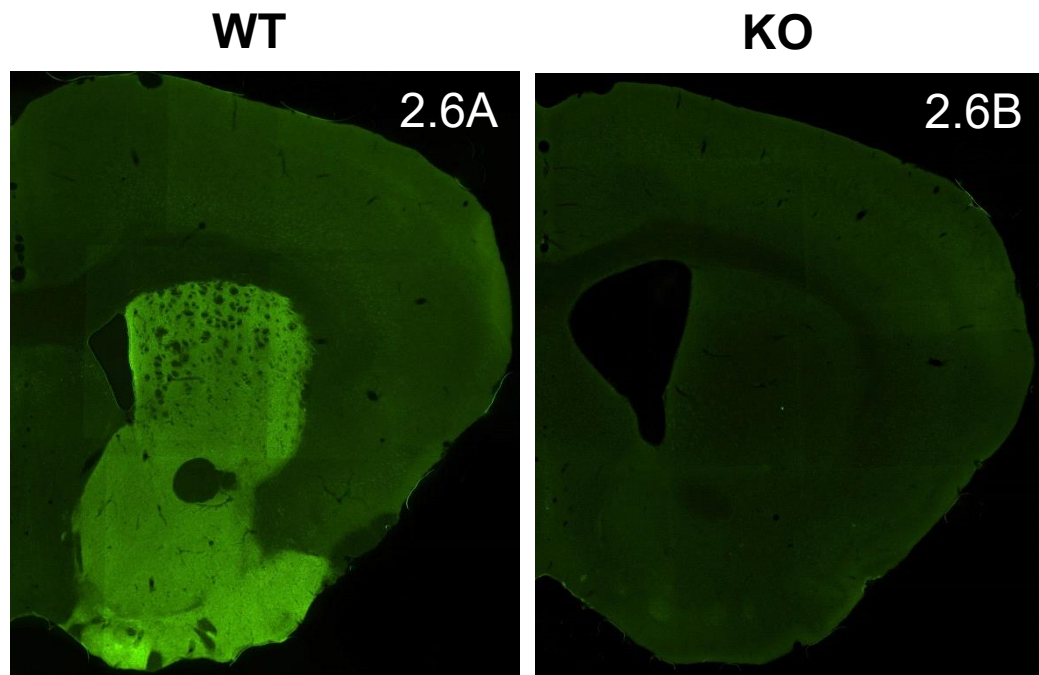


Fig. 2.6 Images representing a full hemisphere of comparable striatal sections taken from DARPP-32 WT (**Fig. 2.6A**) (approx. bregma 1.44mm - 1.54 mm) and DARPP-32 KO (**Fig. 2.6B**) (approx. bregma 1.34mm - 1.42mm) mice.

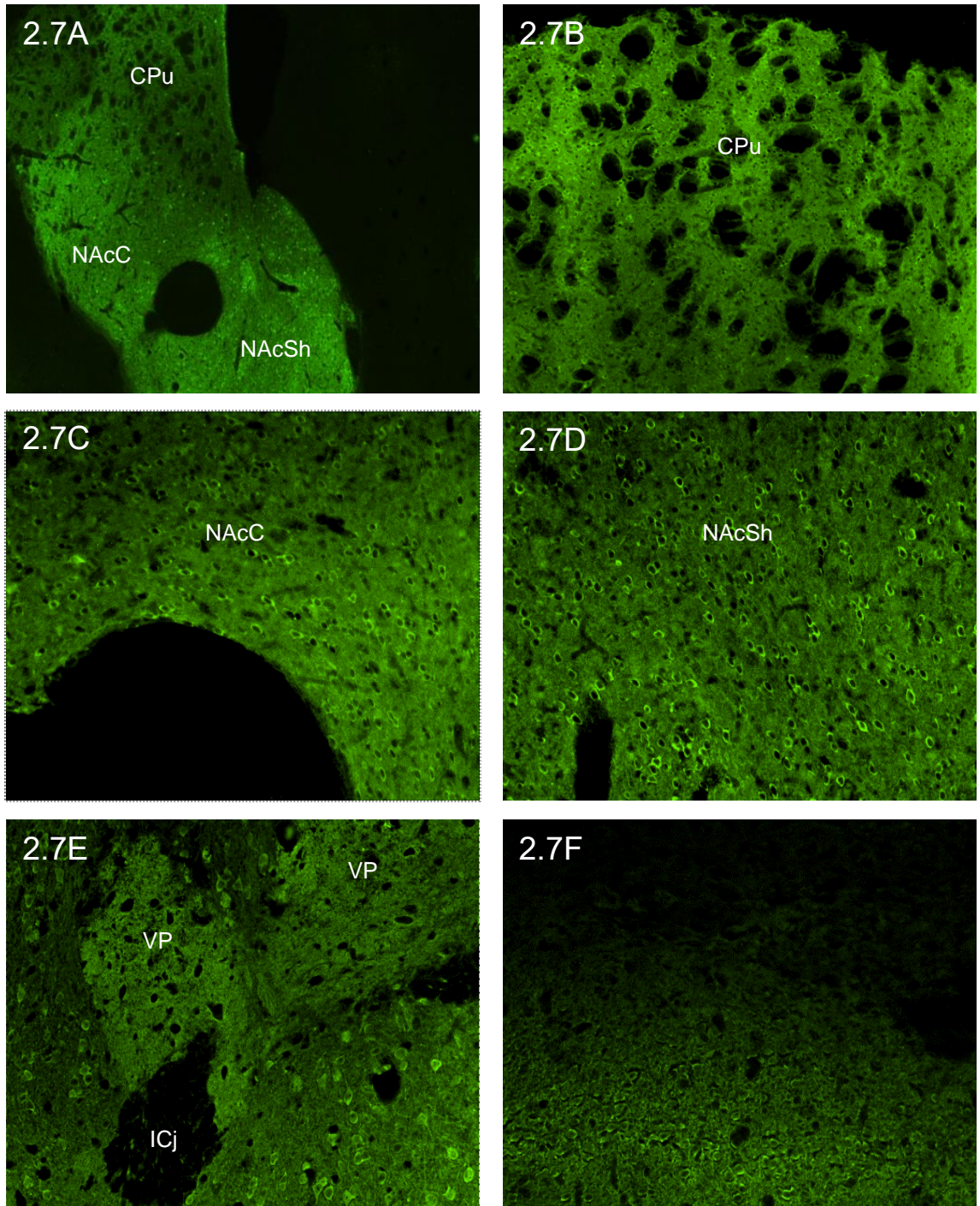


Fig. 2.7A 4x mag image of the dorsal and ventral striatum. **Fig. 2.7B** 10x mag image of the CPu. **Fig. 2.7C** 20x mag image of the NAcC. **Fig. 2.7D** 20x mag image of the NAcSh. **Fig. 2.7E** 20x mag image of the VP and the islands of calleja (ICj). **Fig. 2.7F** 20x mag image of the olfactory tubercle.

Consistent with earlier reports, intense DARPP-32 enrichment was observed in the CPu (Figs. 2.7A & 2.7B), the NAcC (Figs. 2.7A & 2.7C), the NAcSh (Figs. 2.7A & 2.7D), the VP (Fig. 2.7E) and the olfactory tubercle (Fig. 2.7F). Although intense patches of

immunolabelling were evident in the striatum - as shown in Fig. 2.6A - there was no ('to-the-eye') obvious distinction in the levels of immunoreactivity seen between striatal regions. DARPP-32 enrichment was evident in CPu and NAcC and NAcSh MSN nuclei but its label was most intense in the cytosol and dendrites in these areas.

Unlike the pattern of immunolabelling seen in the CPu and the NAc, DARPP-32 expression in the VP was observed predominantly in areas outside of the cell body in a profile resembling the VP expression in the rat and monkey brain (Ouimet et al., 1984; Ouimet et al. 1994). For example, VP expression was observed in patches of unlabelled cells encircled by bundles of immunolabelled fibres, presumed to be the axons of striatal MSNs rather than the cell bodies of VP neurons (Ouimet et al., 1984) (Fig. 2.7E).

DARPP-32 was highly enriched throughout the nucleus, cytosol and dendrites of olfactory tubercle neurons. Such was the extent of DARPP-32 labelling, DARPP-32 positive OT cells appeared to be embedded in a fabric-like matrix of fluorescent dendritic fibres (Fig. 2.7F). Whilst there were examples of unlabelled nuclei in these neurons, DARPP-32 immunoreactivity was present in almost (to-the-eye) equal intensities in the cytosol and nucleus in the majority of these neurons.

2.4 Discussion

Unlike slices extracted from KO mice, which were entirely devoid of DARPP-32 immunolabelling (Figs. 2.1B, 2.1D, 2.4B & 2.6B), slices extracted from WT mice displayed DARPP-32 immunolabelling in varying intensities in discrete regions of the forebrain. This absence of DARPP-32 immunoreactivity in KO mice and the regionally specific expression of DARPP-32 in WT mice establishes the specificity of the antibodies used in this experiment. Moreover, the findings presented in this chapter gathered using fIHC detection of DARPP-32 in the mouse closely, but not completely, resemble those of the rat (Ouimet et al., 1984) and demonstrate intense DARPP-32 labelling throughout

DA innervated brain regions that make up the basal forebrain and associated regions. Thus, in MSNs neurons of the striatum, immunoreactivity levels in the dorsal and ventral portions were largely indistinguishable and immunolabelling intensity was also indistinguishable within the ventral striatum between the shell and core subdivisions of the nucleus accumbens. Additional consilience between mice and other species (including rat) was evident elsewhere. For example, sparse populations of pyramidal cortical neurons were reportedly immunoreactive for DARPP-32 in layer III in the rat (Ouimet et al., 1984) and layer II-III in the rhesus monkey (Berger et al., 1990). A similar pattern of expression was seen in the mouse, with layers II-III exhibiting weak-moderate levels of fluorescence. Cortical layer II-III DARPP-32 mRNA has previously been identified in the mouse (Perez and Lewis, 1992). In addition, the observation that layer VI was moderately fluorescent in pyramidal cells and apical dendrites throughout the motor and somatosensory cortex resembled the fluorescence profile described in the rat (Ouimet et al., 1984), the mRNA distribution characterised in the mouse (Perez and Lewis, 1992) and immunohistochemistry findings captured from rhesus monkey tissue (Berger et al., 1990). Layer VI immunoreactivity was evident in the ventral portion of the anterior cingulate cortex and in the caudal portion of the orbitofrontal cortex. DARPP-32 expression was moderately expressed in sparse networks of pyramidal neurons in layer VI of the cingulate cortex. Intense DARPP-32 immunoreactivity was present in the ventral pallidum. Although this expression was seen predominantly outside of the cell body, it resembled the regional profile described in the rat and rhesus monkey VP (Ouimet et al., 1984; Ouimet et al., 1992). In addition, accumbal MSNs project to the ventral pallidum and it has previously been suggested that the pronounced level of DARPP-32 expression in the VP resides in the axons arriving from MSN projections, giving rise to the dark, unlabelled clusters of pallidal neurons seen in Fig. 2.7E (Ouimet

et al., 1984). As also shown in Fig. 2.7E, DARPP-32 immunoreactivity was absent in the islands of Calleja. This observation is also consistent with earlier findings described in the rodent and non-human primate literature which have also noted the absence of DARPP-32 immunolabelling and mRNA in the islands of Calleja in the rat (Ouimet et al., 1984), mouse (Perez & Lewis, 1992) and rhesus monkey (Ouimet et al., 1992) respectively.

Although pockets of DARPP-32 negative cells were observed in the CeA, the majority of CeA neurons displayed moderate to vivid fluorescence whereas BLA neurons appeared devoid of DARPP-32 labelling. These findings extend those reported by Perez and Lewis (1992) who identified pronounced DARPP-32 mRNA in the CeA but little to no DARPP-32 mRNA in the BLA. DARPP-32 has been reported in other amygdala nuclei in the mouse; however, the analysis in this chapter did not extend to the caudal amygdala regions where this mRNA expression reportedly occurs. Taken together, the report in this chapter of the topographical profile of DARPP-32 protein expression in the mouse amygdala, and the DARPP-32 mRNA expression reported in the mouse amygdala (Perez & Lewis, 1992), provides substantive evidence of a subtle species difference in the regional expression of DARPP-32 in the subdivisions of the amygdala. Fluorescent labelling was evident in both the rat CeA and BLA (Ouimet et al. 1984). Despite earlier observations that DARPP-32 mRNA was devoid in the mouse BLA, fluorescence in-situ hybridisation solely detects nucleic messenger RNA. Perez & Lewis (1992) noted that DARPP-32 mRNA cannot be detected in areas such as the globus pallidus where DARPP-32 expression has been identified solely in cellular compartments distal to the cell body such as axons. However, DARPP-32 was reportedly present in the cell bodies of BLA neurons in the rat, rather than axon terminals synapsing onto these neurons and, as such, it is unlikely that the different methods employed in these studies is a plausible

explanation for the observed differences in amygdaloid DARPP-32 expression between these sub-species of rodent (Ouimet et al., 1984).

In addition to the regions that were inspected in the current study, DARPP-32 immunolabelling or mRNA has been reported in the globus pallidus (Ouimet et al., 1984), hippocampus (Ouimet et al., 1984; Perez & Lewis, 1992), cerebellum (Ouimet et al., 1984; Perez & Lewis, 1992), hypothalamus (Liedtke et al., 2011; Meister et al., 1988; Ouimet et al., 1984), the bed nucleus of the stria terminalis, and the piriform and entorhinal cortices (Ouimet et al., 1984; Perez & Lewis, 1992) in rodents. Furthermore, little, if any, DARPP-32 was observed in the prelimbic and infralimbic cortices in this study whereas others have identified sparse labelling in these regions (Trantham-Davidson, Kroner & Seamans, 2008). Because the analysis in this chapter was cross-sectional, it could be that DARPP-32 positive mPFC cells are present in different sections along the rostral-caudal plane to those examined in this study. For a fuller account of the rodent expression profile of DARPP-32, see Ouimet et al (1984) and Perez & Lewis (1992). In general, the expression profile of DARPP-32 in the mouse brain also resembles the profile reported in humans, where low-moderate labelling was reported in the cortex and intense labelling was reported throughout the divisions of the striatum (Brene et al., 1994). Furthermore, DARPP-32 expression is also seen in the amygdala and the hippocampus in both humans (Brene et al., 1994), rats (Ouimet et al., 1984) and mice (Perez & Lewis, 1992) suggesting that there is a significant degree of translational relevance to studying DARPP-32 in rodents. Although we have briefly discussed some potential cross-species differences and their implications, for the most part, there is a considerable degree of cross-species consilience in the topographical, morphological and cellular expression of DARPP-32, highlighting the potential translation relevance of this complicated phosphoprotein.

DARPP-32 is expressed in regions associated with instrumental learning, instrumental cost-benefits computations, Pavlovian learning and incentive motivation. In light of this expression profile and the well-defined role this molecule has in mediating experience-dependent changes (e.g. LTP and LTD), DARPP-32 might have important functional (behavioural) consequences for reward-related Pavlovian and instrumental processes. In considering this and the existing behavioural data, it was predicted that DARPP-32 KO mice would display impairments in complex reward-based decision-making tasks (e.g. intertemporal and probability discounting), as well as impairments in food-reinforced progressive ratio of reinforcement testing and incentive salience (e.g. general PIT).

Chapter 3

Intertemporal discounting**3.1 Introduction**

Time is a finite resource which must be flexibly and efficiently invested to promote survival and achieve effective social integration. Accordingly, choices concerning the temporal costs of positive and negative reinforcement are important investment decisions. Choosing to wait for an extended period could, in some instances, reduce the likelihood of reinforcement and of receiving alternative rewards. Conversely, electing for instant gratification could conceivably reduce one's profitability by biasing one towards less profitable but immediately available rewards and to discounting propitious but delayed alternatives. Such decisions can have important consequences. For example, an enduring and impulsive preference for instant gratification is a neuropsychological hallmark of many psychiatric disorders, including addiction (Petry, 2001), pathological gambling (Alessi and Petry, 2003), attention deficit hyperactivity disorder (ADHD) (Scheres, Lee & Sumiya, 2008), bipolar disorder and schizophrenia (Ahn et al., 2011).

The intertemporal/delay discounting task is routinely used in laboratory settings as a measure of choice impulsivity. During this task, subjects can choose between a small, immediately available reward and a large but delayed reward. When the delay associated with the large reward is brief, subjects will typically prefer to choose that reward. However, as the delay associated with the large reward is increased across blocks of trials, subjects come to choose the large reward less and less, instead opting for the small but immediately available alternative. Extending the delay to reinforcement, therefore, devalues the incentive value of the large reward.

Neurobiological investigations have established a necessary role for DA and glutamate innervated fronto-striatal-amygdala regions in the provision of intertemporal choices, many of which are associated with DARPP-32 expression. These intertemporal choice facilitating regions include the OFC (Mar, Walker, Theobald, Eagle & Robbins, 2011; Winstanley et al., 2004), mPFC (Churchwell, Morris, Heurtelou & Kesner, 2009) BLA (Winstanley et al., 2004), ventral hippocampus (Abela & Chudasama, 2013) and NAc (Acheson et al., 2006; Cardinal et al., 2001).

Findings from pharmacological studies have been mixed but broadly support a DAergic, glutamatergic and serotonergic influence in the facilitation of delayed reinforcement choices. For example, in some studies, amphetamine reduced impulsive choices (Cardinal et al., 2000; Floresco et al., 2008; Winstanley, 2003) but enhanced them in others (Cardinal et al., 2000; Helms, Reeves & Mitchell, 2006). Amphetamine has also been shown to bi-directionally modify intertemporal choices, enhancing the preference for delayed gratification in a group of subjects where a cue bridged the delay to reinforcement but conversely increasing instant gratification in a group trained without a cue (Cardinal et al., 2000).

Despite these DAergic effects, reducing NAc DA levels by 70%-75%, by performing NAc 6-hydroxydopamine (6-OHDA) lesions, does not impair amphetamine's ability to alter discounting. For example, when amphetamine was administered to 6-OHDA lesioned rats, it modestly but temporarily enhanced the delay tolerating effects of amphetamine. Yet when amphetamine was co-administered with the 5-HT_{1A} agonist 8-OH-DPAT, its capacity to affect intertemporal choices was blocked in sham-lesioned but only attenuated in 6-OHDA lesioned animals, indicating that the DA and 5-HT systems interact in the nucleus accumbens to mediate intertemporal choices (Winstanley, Theobald, Dalley & Robbins, 2005).

A variety of other compounds with an affinity for the DA and 5-HT systems have been shown to affect intertemporal choices: these include the D₂ receptor antagonist haloperidol (Denk et al., 2005), the D₁ receptor antagonist SCH 23390 (Koffarnus et al., 2011), the mixed D₁-D₂ antagonist alpha-flupenthixol (Cardinal et al., 2000), the selective DA reuptake inhibitor GBR 12909 (Baarendse & Vanderschuren, 2012), and the mixed 5-HT_{2A/C} agonist 2,5-dimethoxy-4-iodoamphetamine (DOI) (Wischhof, Hollensteiner, & Koch, 2011).

In addition to DA and 5-HT, glutamate transmission has also been identified as a significant effector of intertemporal choices. For example, the NMDA receptor antagonist ketamine was shown to increase delay discounting (Floresco et al., 2008). In contrast to this report, Yates et al (2014) reported that ketamine altered baseline preference for the large but delayed reward (i.e. when there was no delay associated with the large reward) but had no effect on choices during blocks where a delay to reinforcement was associated with the delayed reward lever. This group did, however, report that the NMDA receptor antagonist MK 801 significantly reduced impulsive choices.

Despite the pharmacological basis of impulsive choice being fairly well elaborated, little is known about the molecular mechanisms residing downstream from receptors in brain regions implicated in the provision of such choices. Therefore, the purpose of the current experiment was to investigate whether DARPP-32, a target of DA, 5-HT and glutamate signal transduction in mPFC, OFC & NAc makes a significant contribution to the facilitation of intertemporal choices.

3.2 Methods

3.2.1 Subjects

Eight DARPP-32 knockout mice (mean weight = 22.14 g), 8 heterozygous (HET) DARPP-32 mice (mean weight = 21.68 g) and 8 wildtype littermates (mean weight = 23.40 g) aged 7-8 to 14 weeks old were used for this study. DARPP-32 status was identified prior to study commencement using the genotyping protocol described in chapter 2. Each group consisted of 5 male and 3 female mice. Subjects were at least the 5th generation of backcrossed mice bred from heterozygous mating pairs to yield WT, HET & KO pups at the University of Sussex from a C57BL/6J background. Mice were housed in the conditions listed previously. Mice were food restricted to approximately 85% of their free-feeding weight and provided with *ad libitum* access to tap water in their home cages. Mice were handled for 5 min per day for 3 consecutive days prior to study commencement to limit handling stress.

3.2.2 Apparatus

All testing was conducted in 8 identical conditioning chambers (22.5 x 18 x 13 cm) (Med Associates, Georgia, VT, USA), each housed in a sound-attenuating cabinet. The front and rear walls of the chambers were constructed from removable steel plates and the lateral walls consisted of transparent acrylic panels. Each cabinet was fitted with an extractor fan to conceal extraneous environmental noise and provide continuous ventilation. Each conditioning chamber contained 2 highly sensitive operant levers, positioned equidistant from a centrally located reward delivery magazine. Reward magazines were fitted with an infrared head entry detector configured to detect the number of beam breaks. The operant chambers were also fitted with a houselight. A 10% liquid sucrose solution, contained in a 20ml Plastipak syringe and delivered by a single

speed syringe pump (Med Associates, Georgia, VT, USA), served as the reinforcer in all operant chamber components of the experiment.

3.2.3 Procedure

3.2.3.1 Magazine training

Mice were first provided with 3 sessions, 1 per day, of magazine training during which sucrose reward was delivered on average of 60 s (range 30 s - 90 s) using a random interval schedule (RI-60) to familiarise them with the location of reward receipt. A session commenced when the mouse made its first magazine head entry and continued until it had received 20 reinforcements; each reinforcement consisted of a 1.5 s pump activation, calculated to deliver 46.4 μ l of sucrose.

3.2.3.2 Lever training

Mice were next given daily sessions, 1 lever per day for 6 days in total, of fixed ratio-1 (FR-1) lever training to encourage reliable and comparable responding on both operanda. Thus each lever response was reinforced on an FR-1 schedule with 46.4 μ l of sucrose.

3.2.3.3 Trial initiation training

Subjects were then given 8 sessions of trial initiation training. These sessions consisted of 5 blocks of 12 trials with each trial lasting 80 s. All trials were forced choice trials, thus only 1 lever was present per trial and each lever was presented 6 times per block (i.e. a total of 12 trials per block). Trials began in darkness and their start was indicated by the illumination of the houselight. Mice were given 20 s to make a head entry into the reward delivery receptacle following the illumination of the houselight. Failure to respond within

this period resulted in the houselight being extinguished, the trial being recorded as an omission and the subject being forced into a timeout period for the remaining duration of the trial. If mice made a magazine response within 20 s from the start of the trial, 1 lever, selected pseudorandomly with respect to position, was extended into the chamber. Failure to respond on the lever within 20 s of its introduction resulted in the lever being retracted, the houselight extinguishing, the trial being recorded as an omission, and the subject being forced into a timeout period for the remainder of the trial. Lever responses during the 20 s period resulted in the immediate delivery of 46.4 μ l of sucrose and the simultaneous extinguishing of the light and the lever being retracted into the chamber wall. Trial length was held constant regardless of whether the animal achieved reinforcement or recorded a lever or magazine omission.

3.2.3.4 Delay discounting

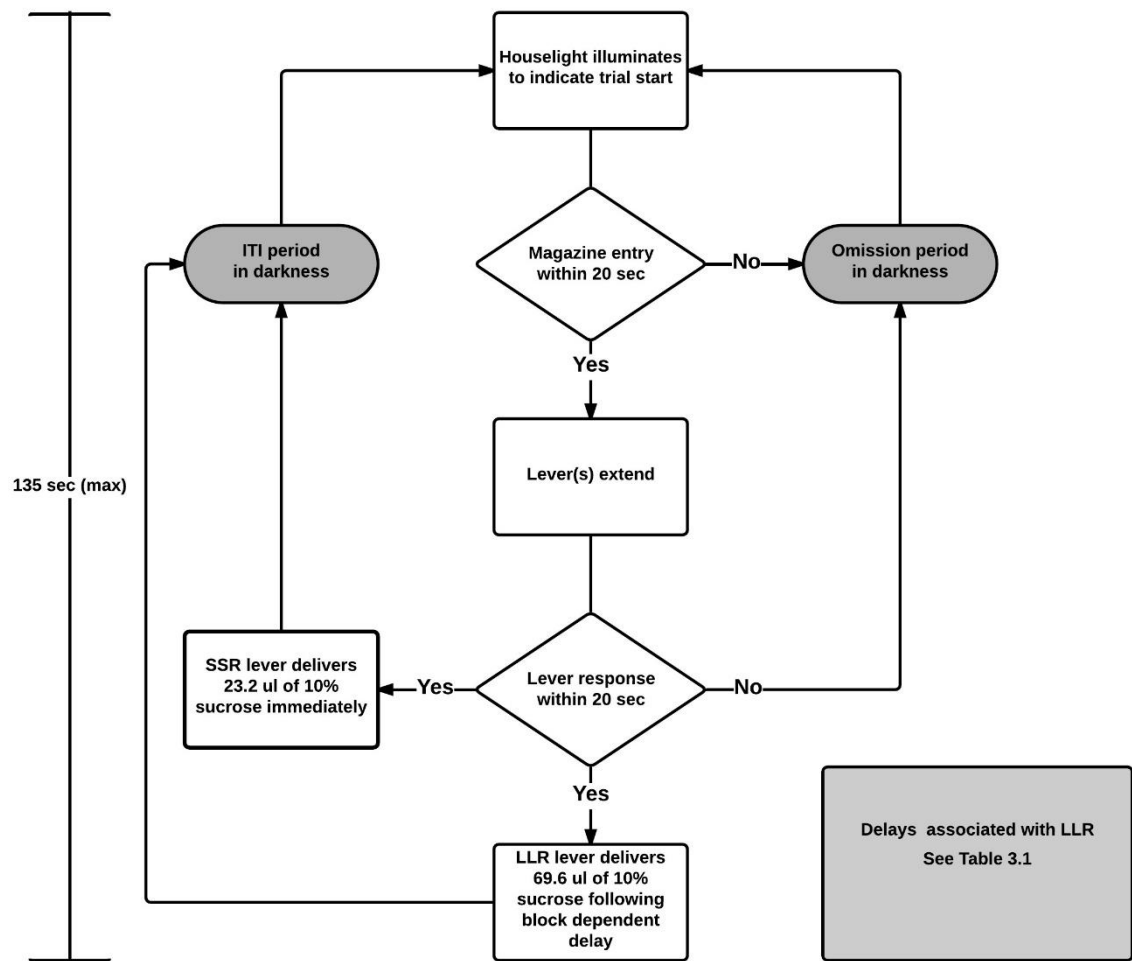


Fig. 3.1 Simplified schematic diagram of the delay discounting procedure

Table 3.1 Delay discounting session parameters

Sessions	Delays (s)	Trial length (s)	Delay pattern
1-7	0, 4, 8, 16, 32	90	Ascending
8-12	0, 8, 16, 32	90	Ascending
13-37	0, 8, 16, 32	90	Descending
38-51	0, 16, 32, 48	105	Descending
52-68	0, 16, 32, 48	105	Ascending
69-70	0, 16, 32, 64	125	Ascending
71-94	0, 25, 50, 75	135	Ascending

Discounting sessions consisted of either 5 blocks of 12 trials or 4 blocks of 12 trials (see Table 3.1). The first 4 trials of each block were always forced choice trials to ensure that subjects experienced the programmed consequences of each lever at the start of each ‘delay block’. The remaining 8 trials were choice trials during which mice were freely able to select which of the 2 levers to press. As before, discounting sessions began with the illumination of the houselight. If mice made an entry into the reward magazine within 20 s of the trial start, either 1 lever during forced choice trials, selected pseudorandomly with regards to lever position, was inserted into the chamber, or both levers were inserted during choice trials. As before, failure to make a head entry into the reward delivery magazine or to respond on the lever within 20 s of the trial start or lever insertion, respectively, resulted in the houselight being extinguished and mice entering a timeout period for the remainder of the trial. The 2 levers were designated as either the delayed-large/larger later (LLR) or immediate-small/smaller sooner (SSR): counterbalanced across genotypes with respect to the physical position of the lever. LLR responses resulted in the delivery of 69.6 μ l of sucrose solution delivered in 3 consecutive 0.75 s activations of the syringe pump (23.2 μ l per activation) after a progressively increasing (by block)

delay (See Table 3.1 for delays associated with the LLR lever at each block). SSR responses always immediately delivered 23.2 μ l of sucrose in a single 0.75 s activation of the syringe pump. Responses on either lever resulted in the immediate retraction of both levers and the houselight being extinguished. Trial length was held constant irrespective of whether subjects completed the trial successfully or entered an omission period (see Table 3.1). Mice were exposed to a variety of delays presented in an ascending and/or descending pattern (see Table 3.1).

3.2.3.5 Reinforcer devaluation

To assess the potential relationship between motivational state and DARPP-32 in the attribution of intertemporal choices, subjects undertook the following 2 types of reinforcer devaluation manipulations: a sensory-specific and a general satiety (pre-feeding) manipulation were conducted to validate whether within-session shifts in LLR choices could alternatively be explained by a) either satiation of a sucrose appetite or b) a significant shift in the general motivational state of the mice.

The first reinforcer devaluation manipulation, a sensory-specific intervention, involved pre-feeding half of all animals with 10% sucrose for 2 hrs on day 1. During these pre-feeding sessions, home cage water bottles were replaced with bottles containing 10% sucrose. Pre-feeding was counterbalanced with respect to genotype and lever position. All animals, including those that were not sucrose pre-fed, were then immediately subjected to a temporal discounting test session. On day 2, the remaining half of animals that had not undergone sucrose pre-feeding were given a 2 hr sucrose pre-feeding session which was followed with all animals conducting a temporal discounting session.

The second reinforcer devaluation procedure was a general motivation intervention. These sessions, conducted over 2 days, were procedurally similar to the sucrose pre-

feeding sessions, except animals were pre-fed with their dietary maintenance chow for 2 hrs prior to the discounting sessions.

3.2.4 Data analysis

Lever response rates for WT, HET & KO mice during the final session of lever training were compared by conducting a genotype (WT vs HET vs KO) by lever (1 vs 2) repeated measures analysis of variance (ANOVA) to ensure that performance was equal on both levers within- and between genotypes.

A mixed genotype (WT vs HET vs KO) by lever (1 vs 2) by block (1 vs 2 vs 3 vs 4 vs 5) ANOVA of the final trial initiation training session was conducted to ensure that performance was not biased between genotypes, levers or blocks.

Magazine and lever omissions recorded during the final trial initiation training session were each analysed separately and compared between genotypes by conducting one-way ANOVAs.

The percentage of total choices in each block that were LLR choices was the dependent variable. This was calculated by dividing total LLR responses by the total number of lever responses for each block and multiplying by 100 (i.e., $(\text{LLR choices} / (\text{SSR choices} + \text{LLR choices})) * 100$). Responses from each delay block were averaged over 5 sessions and these values were used to calculate the rate of discounting as a function of delay, except during the devaluation sessions (79-80 & 93-94), which were analysed separately, and those sessions containing a maximum delay of 75 s which were averaged over 20 sessions. The values were compared between genotypes at each stage of the experiment by subjecting % LLR choices to a genotype (WT vs HET vs KO) by delay (4 levels) repeated measures ANOVA. The area under the discounting curve (AUC) was also calculated for each subject using the method reported by Myerson and colleagues (Myerson, Green & Warusawitharana, 2001). AUC provides an atheoretical approach to analysing

discounting data by avoiding many of the problems associated with hyperbolic or exponential discounting models. AUCs were compared between groups with one-way ANOVA except when stated otherwise.

The percent LLR choices during devaluation sessions were analysed with genotype (WT vs HET vs KO) by motivational state (food-deprived vs pre-fed) by delay (0 s vs 25 s vs 50 s vs 75 s) mixed model ANOVA.

AUCs corresponding to the devaluation sessions were analysed by conducting genotype (WT vs HET vs KO) by motivational state (food-deprived vs pre-fed) repeated measures ANOVA.

Trial initiation and choice latencies were analysed separately by conducting genotype (WT vs HET vs KO) by delay (4 levels) repeated measures ANOVA except during the devaluation sessions which were analysed with genotype (WT vs HET vs KO) by motivational state (food-deprived vs pre-fed) by delay (0 s vs 25 s vs 50 s vs 75 s) mixed model ANOVAs.

Forced choice latencies were analysed by conducting lever (SSR vs LLR) by genotype (WT vs HET vs KO) by delay (4 levels) mixed model ANOVAs.

Although choice latencies were inspected by collapsing latencies across levers, lever was included as a within-subjects factor for forced choice latencies to identify whether there were any between-genotype differences in the time taken to respond when only presented with one lever.

Total magazine (i.e. trial initiation) and total lever omissions were analysed separately for each stage of the experiment and compared between genotypes using one-way ANOVA, except during the devaluation sessions where these values were treated to a genotype (WT vs HET vs KO) by motivational state (food-deprived vs pre-fed) repeated measures ANOVA.

Mice failing to discount their LLR preference in the final block by less than 10% of the maximum objective value relative to their % LLR choice value at baseline were excluded from statistical analysis on the basis that these mice were considered to be delay insensitive (e.g. a mouse recording a % LLR choice of 90 in block one and 81 in the final block would be excluded). This criteria was based on Johnson and Bickel's (2008) algorithm for identifying non-systematic data in discounting studies.

Mice with AUC scores more than 2 standard deviations from their group mean were also excluded from the analysis.

Data were not presented from a significant number of sessions. These data were omitted from the analysis because, having adjusted the task parameters, it took subjects a significant number of sessions to respond appropriately to the changes and significant numbers of mice were producing delay insensitive/inflexible response patterns.

3.3 Results

3.3.1 Lever training

Response rates during the final lever training session were equal as a function of lever designation (would-be) and genotype, either in main or interaction effect ($F_s = < .76$, $P_s = > .48$).

3.3.2 Trial initiation training

Trial initiation training performance was consistent across levers, genotypes and blocks, either as main effects or interaction effects during the final trial initiation training session ($F_s = < 2.38$, $P_s = > .07$). Magazine and lever omissions were also consistent between genotypes ($F_s = < 1.46$, $P_s = > .26$).

3.3.3 Delay discounting

Data from the first 7 sessions were not analysed on the basis that mice had not yet exhibited block-dependent discounting for 5 consecutive sessions.

3.3.3.1 Sessions 8-12 (ascending delays of 0 s, 8 s, 16 s & 32 s)

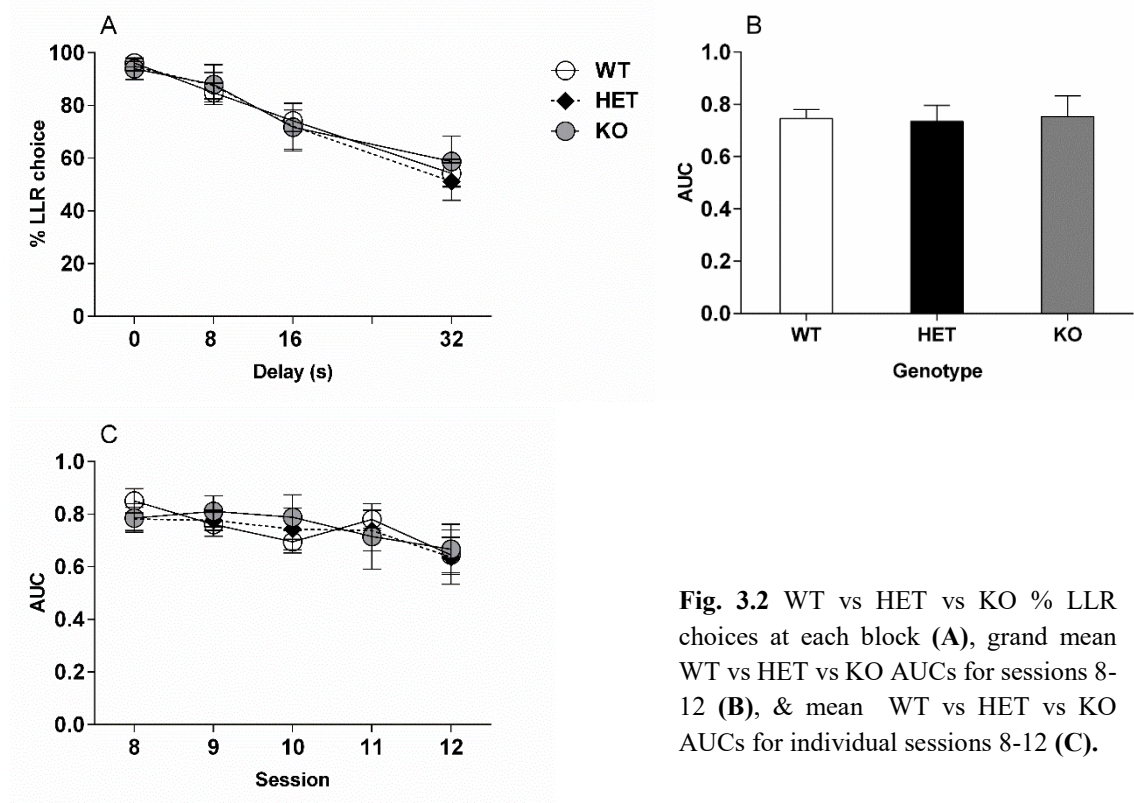


Fig. 3.2 WT vs HET vs KO % LLR choices at each block (A), grand mean WT vs HET vs KO AUCs for sessions 8-12 (B), & mean WT vs HET vs KO AUCs for individual sessions 8-12 (C).

At this stage of the experiment, 8 WT, 7 HET and 6 KO mice met the inclusion criteria. There was a significant main effect of delay ($F_{(1.92, 34.46)} = 78.27$, $p = .001$) (Fig. 3.2A), with subjects recording a smaller percentage of LLR choices as the delay increased. However, the prediction that differential discounting would emerge between DARPP-32 KO mice and their WT and HET littermates was not supported during discounting sessions constrained by the parameters listed above; the main effect of genotype and the delay by genotype interaction were not significant ($F_s = < .47$, $P_s = > .75$). Similarly, there were no between genotype differences in AUCs ($F_{(2,18)} = .02$, $p = .98$) (Fig 3.2B). Thus, DARPP-32 deletion had no effect on both measures of intertemporal discounting at this point in the experiment. Both magazine and lever omissions were not significantly

different between genotypes ($F_s = < .70$, $P_s = > .51$). Although trial initiation ($F_{(1.56, 28.08)} = 47.11$, $p = .001$) and choice ($F_{(1.26, 22.63)} = 13.30$, $p = .001$) latencies were significantly slower as the delay to reinforcement increased, there were no significant main effects of genotype or significant delay by genotype interactions for either of these performance measures ($F_s = < 2.09$, $P_s = > .12$). Mice made significantly faster forced choices when presented with the LLR lever (main effect of lever ($F_{(1, 18)} = 34.24$, $p = .001$)). There were, however, no other significant main or interaction effects when inspecting forced choice latencies ($F_s = < 2.10$, $P_s = > .10$).

3.3.3.2 Sessions 33-37 (descending delays of 32 s, 16 s, 8 s & 0 s)

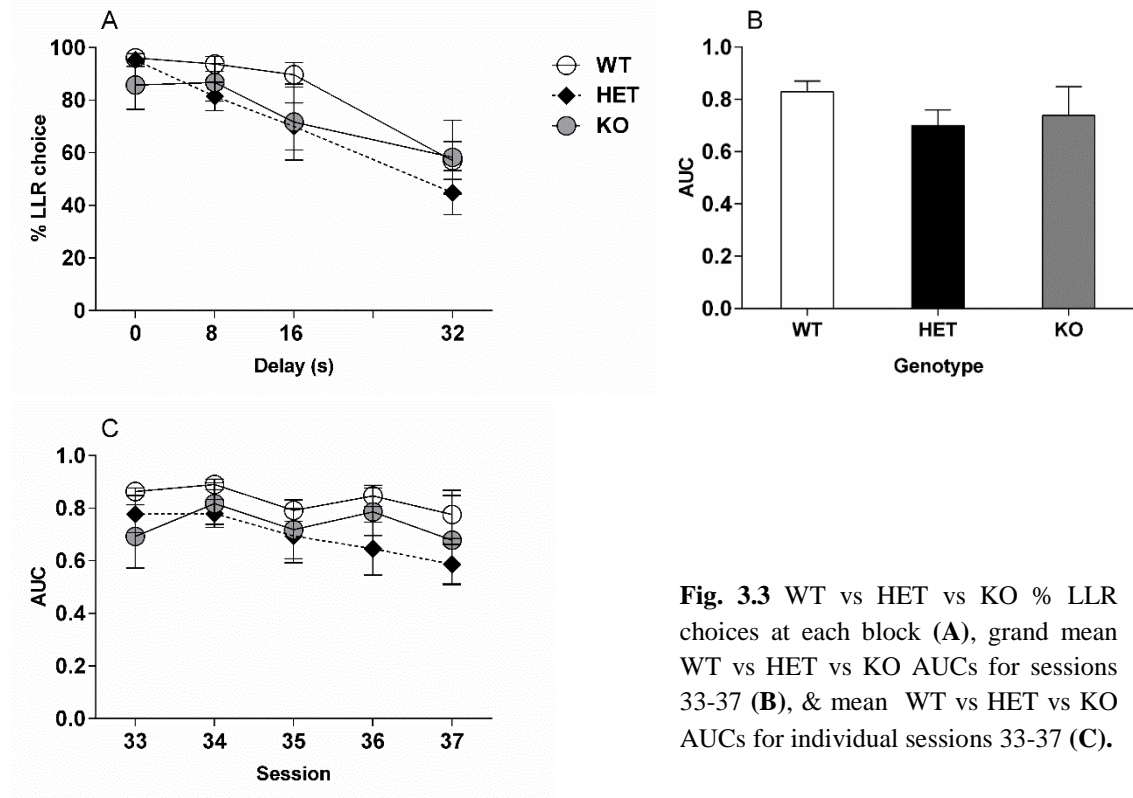
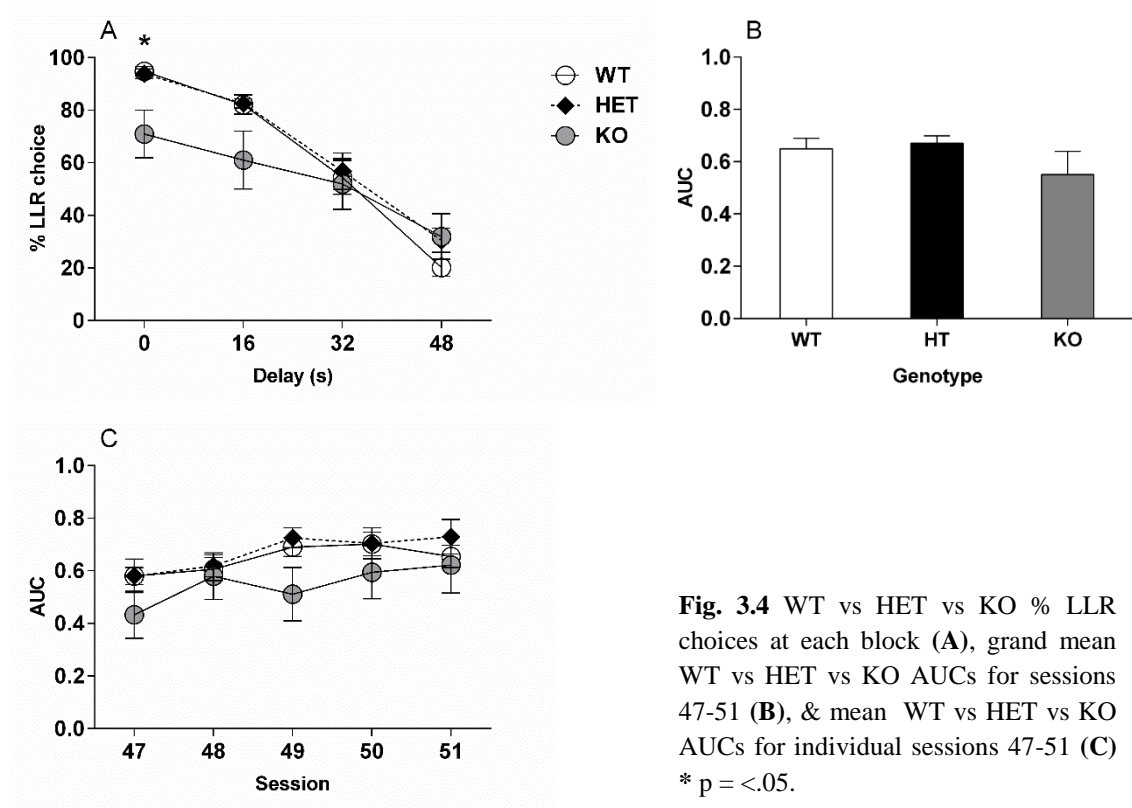


Fig. 3.3 WT vs HET vs KO % LLR choices at each block (A), grand mean WT vs HET vs KO AUCs for sessions 33-37 (B), & mean WT vs HET vs KO AUCs for individual sessions 33-37 (C).

Because subjects took a significant number of sessions to adjust to the reversal of the delay order, data captured during the intervening sessions (i.e. sessions 13-32) were not analysed. Only 3 knockout subjects, compared to 6 WT and 5 HET subjects met the inclusion criteria which suggests that KO mice were impaired in reconfiguring intertemporal choices in response to changes in the task parameters. Mice made a smaller

percentage of LLR choices as the delay to reinforcement increased ($F_{(2.0, 21.97)} = 34.58$, $p = .001$) Fig. 3.3A). However, the prediction that DARPP-32 deletion would differentially alter intertemporal discounting performance was again unsupported at this stage of the experiment, as the main effect of genotype and the delay by genotype interaction were not significant ($F_s = < 1.42$, $P_s = > .27$). Likewise, AUCs were not significantly different between genotypes ($F_{(2, 11)} = 1.53$, $p = .26$) Fig. 3.3B). Magazine and lever omissions were also not significantly different between genotypes ($F_s = < 1.08$, $P_s = > .37$) and there was also no significant main effect of delay and no significant delay by genotype interactions for either trial initiation or choice latencies ($F_s = < 2.11$, $P_s = > .16$). Mice continued to execute significantly faster forced choices when presented with the LLR lever ($F_{(1, 11)} = 13.63$, $p = .004$) but there were no significant main effects of genotype or delay in this measure ($F_s = < .72$, $P_s = > .55$). Whilst there was a significant lever by delay interaction ($F_{(3, 33)} = 4.59$, $p = .009$), there were no other significant interaction effects when inspecting forced choice latencies ($F_s = < 1.91$, $P_s = > .20$).

3.3.3.3 Sessions 47-51 (descending delays of 48 s, 32 s, 16 s and 0 s)



Eight KO, 8 WT & 7 HET mice met the inclusion criteria at this stage of the experiment. Mice recorded a smaller percentage of LLR choices as the delay to reinforcement increased ($F_{(1.67, 33.45)} = 146.96$, $p = .001$) (Fig. 3.4A). Whilst there was no significant main effect of genotype ($F_{(2, 20)} = 1.10$, $p = .35$), there was a significant delay by genotype interaction ($F_{(3.35, 33.45)} = 5.72$, $p = .002$) and post hoc one-way ANOVAs revealed that KO mice recorded a significantly smaller percentage of LLR choices than WT or HET mice during the 0 s delay block ($F_{(2, 20)} = 5.77$, $p = .01$) (Bonferroni post hoc tests $P_s = < .05$) but not at any other delays ($F_s = < 3.12$ $P_s = > .07$). Despite the abovementioned effects, there were no significant differences in AUCs between genotypes ($F_{(2, 20)} = 1.10$, $p = .35$) (Fig. 3.4B). Overall, then, DARPP-32 deletion modestly altered baseline choices at this stage of the experiment but did not appear to have any overall effect on the rate of intertemporal discounting. Thus, the experimental predictions continued to be unsupported. Magazine and lever omissions were also not different between genotypes ($F_s = < 1.88$, $P_s = > .18$). Trial initiation latencies were

significantly different across blocks ($F_{(1.52, 30.41)} = 3.82, p = .04$) with mice taking longer to initiate trials as the sessions progressed. KO mice also took significantly longer than WT but not HET mice to initiate trials ($F_{(2, 20)} = 4.41, p = .03$) (Bonferroni post hoc tests $P_s = .03$ & $.11$ respectively). The delay by genotype interaction was not significant ($F_{(3.04, 30.41)} = .74, p = .54$). Choice latencies were not significantly different across delay blocks ($F_{(3, 60)} = 2.41, p = .08$) but there was a significant main effect of genotype ($F_{(2, 20)} = 5.53, p = .01$), with KO mice taking significantly longer than HET ($p = .02$) but not WT ($p = .06$) mice to execute choices. The delay by genotype interaction was not significant ($F_{(6, 60)} = .20, p = .98$). Mice recorded similar forced choice latencies for both levers ($F_{(1, 20)} = 1.79, p = .20$). There was a significant main effect of genotype ($F_{(2, 20)} = 7.82, p = .003$) with HET mice making faster forced choices than WT and KO mice ($P_s = < .03$). Forced choice latencies were similar between WT and KO subjects. There was a significant main effect of delay ($F_{(2.21, 44.16)} = 3.48, p = .04$) and a significant lever by delay interaction ($F_{(2.29, 45.89)} = 7.30, p = .001$). None of the other interactions were significant, however ($F_s = < 1.39, P_s = > .24$).

3.3.3.4 Sessions 64-68 (ascending delays of 0 s, 16 s, 32 s 48 s)

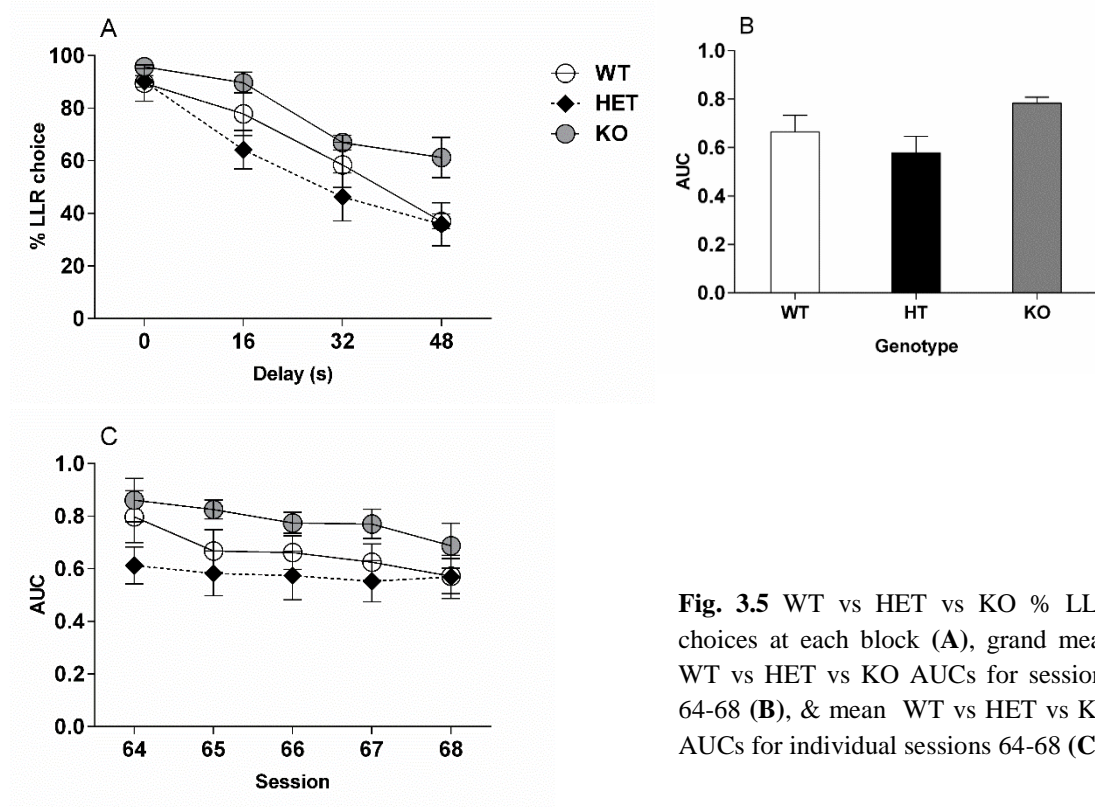


Fig. 3.5 WT vs HET vs KO % LLR choices at each block (A), grand mean WT vs HET vs KO AUCs for sessions 64-68 (B), & mean WT vs HET vs KO AUCs for individual sessions 64-68 (C).

Once again, after reversing the delays, only 3 KO mice, compared to 5 WT & 7 HET mice met the inclusion criteria at this stage of the experiment. Whilst mice continued to discount the LLR lever in a delay sensitive fashion ($F_{(3, 36)} = 60.19$, $p = .001$) (Fig. 3.5A), there was no significant main effect of genotype and no significant delay by genotype interaction ($F_s = < 1.95$, $P_s = > .18$). There were also no differences between genotypes in AUCs ($F_{(2, 12)} = 1.88$, $p = .20$) (Fig. 3.5B). Magazine ($F_{(2, 12)} = 2.37$, $p = .14$) and lever omissions ($F_{(2, 12)} = 2.69$, $p = .11$) were also similar between genotypes. Trial initiation latencies slowed as sessions progressed ($F_{(1.09, 13.03)} = 14.04$, $p = .002$) and whilst the significant main effect of delay indicated that choice latencies also differed across delay blocks ($F_{(1.66, 19.94)} = 3.63$, $p = .05$) none of the Bonferroni post hoc tests were significant ($p = > .14$). There were no significant main effects of genotype or significant delay by genotypes interactions in either of these performance measures ($F_s = < 3.11$, $P_s = > .08$). Mice made significantly faster forced choices when presented with the LLR lever ($F_{(1, 12)}$

= 10.91, $p = .006$) but there were no significant main effects of genotype or delay ($F_s = < 1.88$, $P_s = > .19$). The lever by delay interaction ($F_{(3, 36)} = 3.32$, $p = .06$) approached significance. However, none of the other interaction effects were significant ($F_s = < 1.62$, $P_s = > .20$).

3.3.3.5 Sessions 69-70 (ascending delays of 0 s, 16 s, 32 s, 64 s)

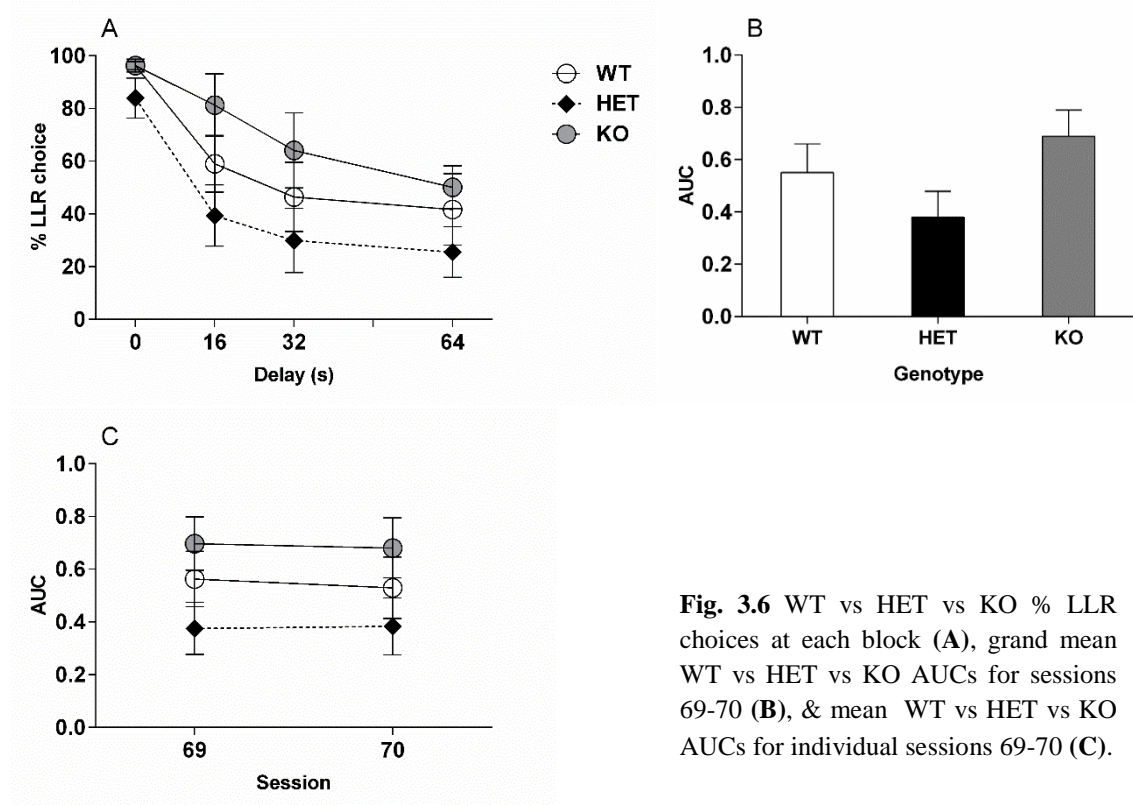


Fig. 3.6 WT vs HET vs KO % LLR choices at each block (A), grand mean WT vs HET vs KO AUCs for sessions 69-70 (B), & mean WT vs HET vs KO AUCs for individual sessions 69-70 (C).

Five KO, 7 WT and 7 HET mice were eligible for inclusion. Whilst mice recorded a significantly smaller percentage of LLR choices as the delay to reinforcement increased ($F_{(1.70, 27.24)} = 42.87$, $p = .001$) (Fig. 3.6A), there were no between genotype differences, either as a main effect or as an interaction between delay and genotype ($F_s = < 2.20$, $P_s = > .14$). AUCs were also not significantly different between genotypes ($F_{(2, 16)} = 2.02$, $p = .17$) (Fig. 3.6B). Likewise, magazine and lever omissions were similar between genotypes ($F_s = < .11$, $P_s = > .90$). Whilst trial initiation latencies slowed as sessions progressed ($F_{(1.76, 28.09)} = 27.33$, $p = .001$), there were no between genotype differences or significant interactions in this measure and there were no significant effects or

interactions involving choice latencies ($F_s = < 2.01$, $P_s = > .13$). LLR forced choices were executed significantly faster than SSR forced choices ($F_{(1, 16)} = 5.42$, $p = .03$), however, there were no other significant main or interaction effects ($F_s = < 2.64$, $P_s = > .09$).

3.3.3.6 Sessions 71-78 and 81-92 (ascending delays 0 s, 25 s, 50 s, 75 s)

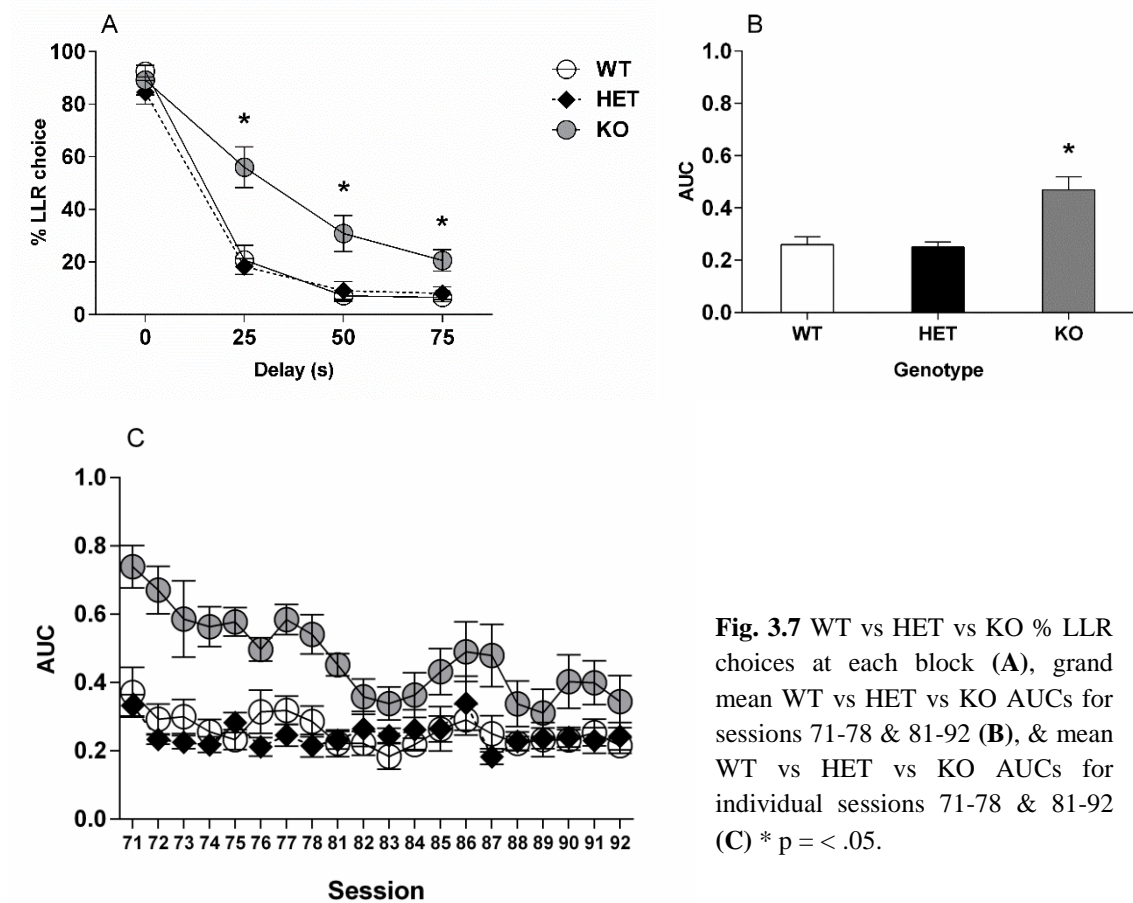


Fig. 3.7 WT vs HET vs KO % LLR choices at each block (A), grand mean WT vs HET vs KO AUCs for sessions 71-78 & 81-92 (B), & mean WT vs HET vs KO AUCs for individual sessions 71-78 & 81-92 (C) * $p < .05$.

Six KO, 6 WT and 7 HET mice met the inclusion criteria at this stage of the experiment. Mice made a smaller percentage of LLR choices as the delay to reinforcement increased ($F_{(1.61, 25.68)} = 230.54$, $p = .001$) (Fig. 3.7A). Notably, there was a significant main effect of genotype ($F_{(2, 16)} = 15.09$, $p = .001$) with KO mice recording a higher percentage of LLR choices than WT and HET mice (Bonferroni post hoc tests $P_s = < .01$). There was also a significant delay by genotype interaction ($F_{(3.21, 25.68)} = 4.80$, $p = .008$) with post hoc one-way ANOVAs revealing that KO mice recorded a higher percentage of LLR choices than both WT and HET mice at the 25 s, 50 s and 75 s blocks (F_s

= > 7.09, $P_s = < .006$) but not the zero s delay block ($F_{(2, 16)} = .86$, $p = .44$). AUCs were also significantly different between genotypes ($F_{(2, 16)} = 14.75$, $p = .001$) (Fig. 3.7B) with DARPP-32 KO mice producing significantly larger AUCs than WT and HET mice (Bonferroni post hoc tests $P_s = < .001$). There were no significant differences between WT and HET animals in AUCs. Magazine and lever omissions were not significantly different between genotypes ($F_s = < .04$, $P_s = > .96$). Trial initiation latencies slowed as sessions progressed ($F_{(1.57, 25.05)} = 37.55$, $p = .001$) but they were not significantly different in any way between genotypes, and choice latencies were not different between delay blocks, genotypes or when these variables were analysed in interaction ($F_s = < 2.32$, $P_s = > .13$). Forced choices were significantly different between levers ($F_{(1, 16)} = 12.56$, $p = .003$), however, unlike previous phases of the experiment, latencies were faster when mice were presented with the SSR lever ($F_{(1, 16)} = 12.56$, $p = .003$). The main effect of genotype was not significant ($F_{(2, 16)} = .31$, $p = .74$). There was a significant main effect of delay ($F_{(3, 48)} = 21.04$, $p = .001$) with mice tending to make slower forced choices as the session progressed (i.e. at the longest delays). There was also a significant lever by delay interaction ($F_{(1.89, 30.23)} = 44.78$, $p = .001$). Post hoc tests examining the effect of delay separately for each lever revealed that SSR forced choices were executed significantly faster as the delay to reinforcement increased ($F_{(3, 54)} = 11.10$, $p = .001$) whereas LLR choices were executed significantly slower as the delay increased ($F_{(3, 54)} = 38.88$, $p = .001$). There were no other significant interaction effects ($F_s = < 2.89$, $P_s = > .09$).

3.3.3.7 Sessions 71-75 vs sessions 88-92 stability analysis

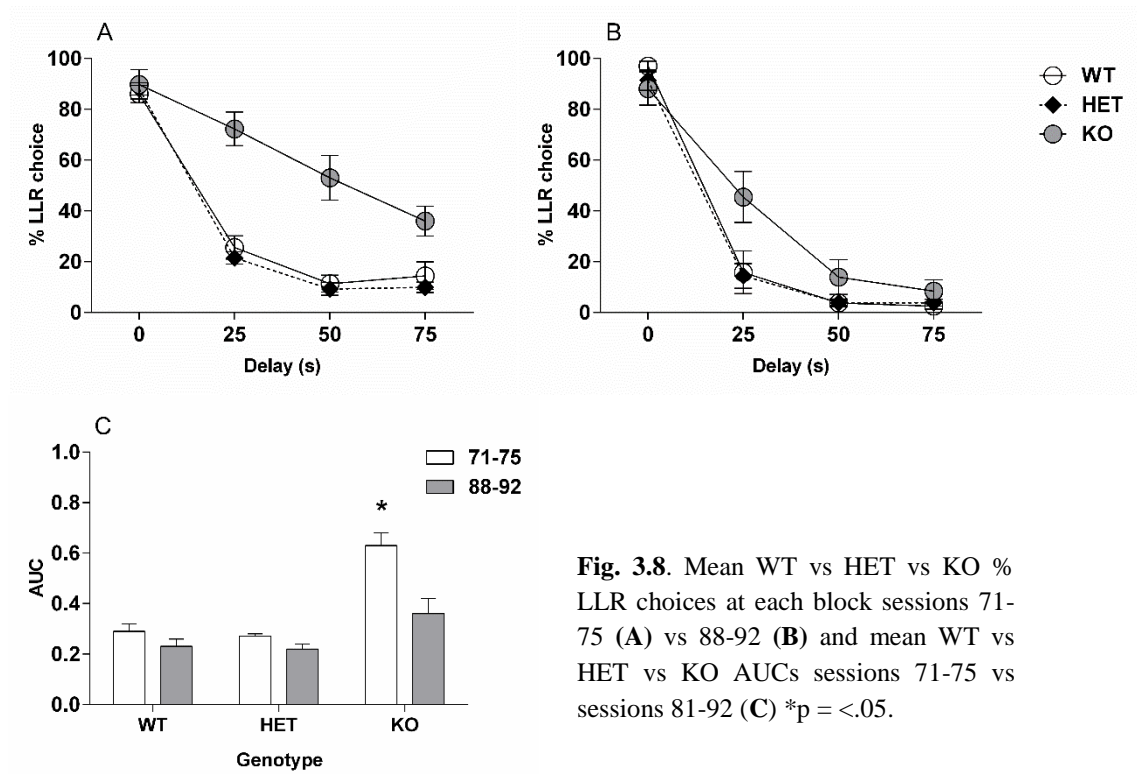


Fig. 3.8. Mean WT vs HET vs KO % LLR choices at each block sessions 71-75 (A) vs 88-92 (B) and mean WT vs HET vs KO AUCs sessions 71-75 vs sessions 81-92 (C) * $p < .05$.

In light of the aforementioned transient effects during sessions 47-51 and the relatively inflexible profile of DARPP-32 KO mice in response to changes in task parameters, AUCs captured during the first 5 sessions with a maximum 75 s delay (i.e. sessions 71-75) were compared with AUCs captured during the final 5 (non-feeding) sessions with a maximum 75 s delay (i.e. sessions 88-92). There was a significant main effect of session ($F_{(1, 16)} = 26.36$, $p = .001$) (Fig. 3.8C) with mice producing smaller AUCs in sessions 88-92 compared to sessions 71-75. A significant session by genotype interaction ($F_{(2, 16)} = 11.12$, $p = .001$) followed up with post hoc paired t-tests indicated that KO mice produced significantly smaller AUCs during the final 5 sessions of testing with a maximum delay of 75 s compared to the first 5 sessions at these delays ($t_{(5)} = 5.51$, $p = .003$) whereas WT ($t_{(5)} = 1.61$, $p = .17$) & HET ($t_{(6)} = .71$, $p = .51$) mice both produced similar AUCs between sessions 71-75 and sessions 88-92. When comparing AUCs between genotypes in the first sessions with a maximum delay of 75 s and the final 5 session at these parameters separately, AUCs were only significantly different between genotypes in the first 5

sessions ($F_{(2, 16)} = 32.48$, $p = .001$) but not the final 5 sessions ($F_{(2, 16)} = 3.01$, $p = .08$), indicating that by the end of the experiment, discounting performance was equivalent between genotypes. These findings suggest that with extended training, DARPP-32 KO mice achieved comparable performance to their WT and HT counterparts.

3.3.3.8 Sensory-specific reinforcer devaluation (sessions 79-80)

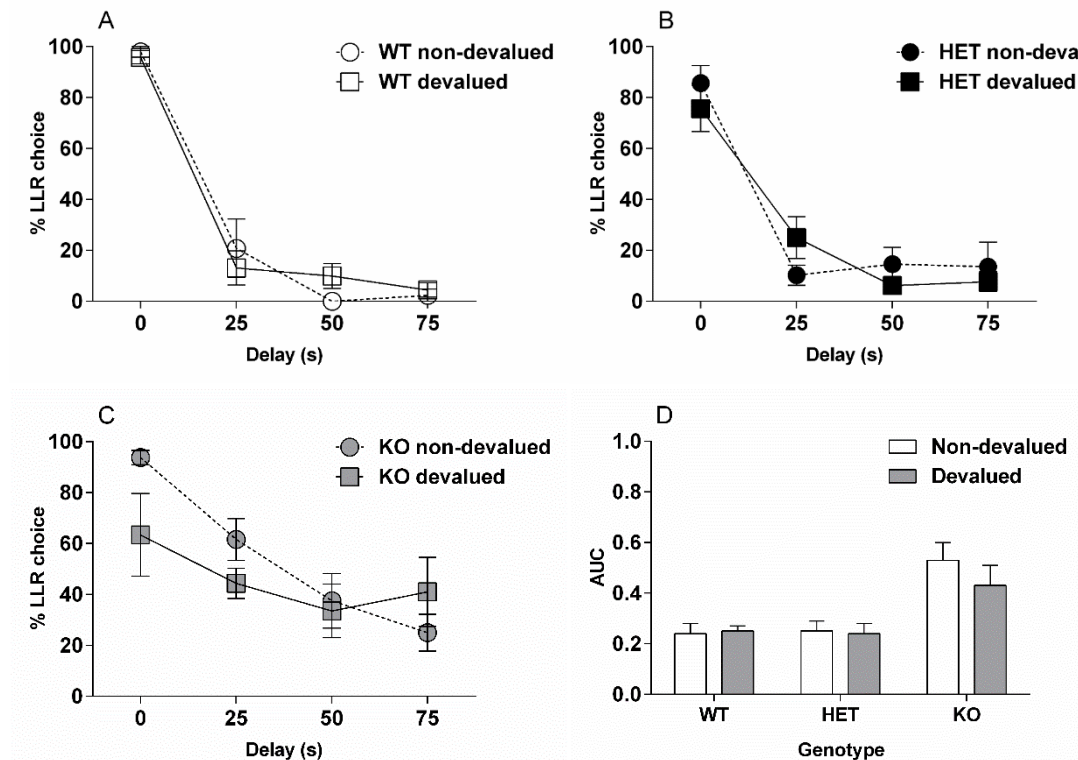


Fig. 3.9 Non-devalued vs devalued % LLR choices for WT (A), HET (B) & KO (C) mice and non-devalued vs devalued AUCs for WT, HET & KO mice (D).

Table 3.2 Mean (\pm SEM) magazine omissions during the sucrose pre-feeding devaluation

Session	WT	HET	KO
Non-devalued	.50 (.22)	2.43 (1.81)	.33 (.21)
Devalued	3.17 (1.51)	4.14 (2.06)	5.33 (2.45)

Table 3.3 Mean (\pm SEM) lever omissions during the sucrose pre-feeding devaluation

Session	WT	HET	KO
Non-devalued	1.00 (.63)	.29 (.18)	1.67 (.92)
Devalued	.83 (.65)	.29 (.29)	.17 (.17)

Pre-feeding mice with sucrose generally had no effect on % LLR scores. The main effect of motivational state and all associated interaction terms were not significant ($F_s = < 2.02$, $P_s = > .08$) (Figs. 3.9A, 3.9B & 3.9C). There was a significant main effect of delay ($F_{(1.81, 28.96)} = 147.50$, $p = .001$), with mice continuing to discount the LLR lever as the delay to reinforcement increased, and a significant main effect of genotype ($F_{(2, 16)} = 7.12$, $p = .006$), with KO mice recording a significantly higher percentage of LLR choices than WT and HET mice (Bonferroni post hoc comparisons $P_s = < .018$). There was also a significant delay by genotype interaction ($F_{(3.62, 28.96)} = 7.29$, $p = .001$). Pre-feeding subjects with sucrose similarly had no effect on AUCs when inspected as either a main effect of motivational state or as a motivational state by genotype interaction ($F_s = < 1.21$, $P_s = > .30$) (Fig. 3.9D). However, AUCs were significantly different between genotypes, collapsing across motivational state ($F_{(2, 16)} = 9.28$, $p = .002$), with KO subjects producing significantly larger AUCs than both WT and HET subjects ($P_s = .005$). Pre-feeding increased the number of magazine omissions ($F_{(1, 16)} = 12.08$, $p = .003$) but did not increase lever omissions ($F_{(1, 16)} = 3.08$, $p = .10$) (Tabs. 3.2 & 3.3). However, there were no significant main effects of genotype and no significant interactions between genotype and motivational state in either of these measures ($F_s = < 2.22$, $P_s = > .14$). Whilst pre-feeding slowed trial initiation latencies ($F_{(1, 16)} = 18.64$, $p = .001$) and initiation latencies also slowed as sessions progressed ($F_{(1.85, 29.60)} = 10.62$, $p = .001$), choice latencies were not affected by pre-feeding and nor did they change across delay blocks ($F_s = < 1.04$, $P_s = > .32$). There were no between genotype differences or any significant interactions between genotype, delay and motivational state in any combination in either of these response measures ($F_s = < 2.48$, $P_s = > .12$). Forced choice latencies were similarly not affected by pre-feeding ($F_{(1, 16)} = .21$, $p = .65$). The main effect of genotype was not significant ($F_{(2, 16)} = .13$, $p = .88$). There was a significant main effect of lever ($F_{(1, 16)} =$

24.23, $p = .001$) with mice continuing to record faster response latencies on the SSR lever and a significant main effect of delay ($F_{(3, 48)} = 7.83$, $p = .001$) with mice recording significantly slower latencies as the session progressed. There was also a significant lever by delay interaction ($F_{(3, 48)} = 17.78$, $p = .001$). None of the other interaction terms were significant ($F_s = < 2.86$, $P_s = > .09$).

3.3.3.9 General motivational reinforcer devaluation

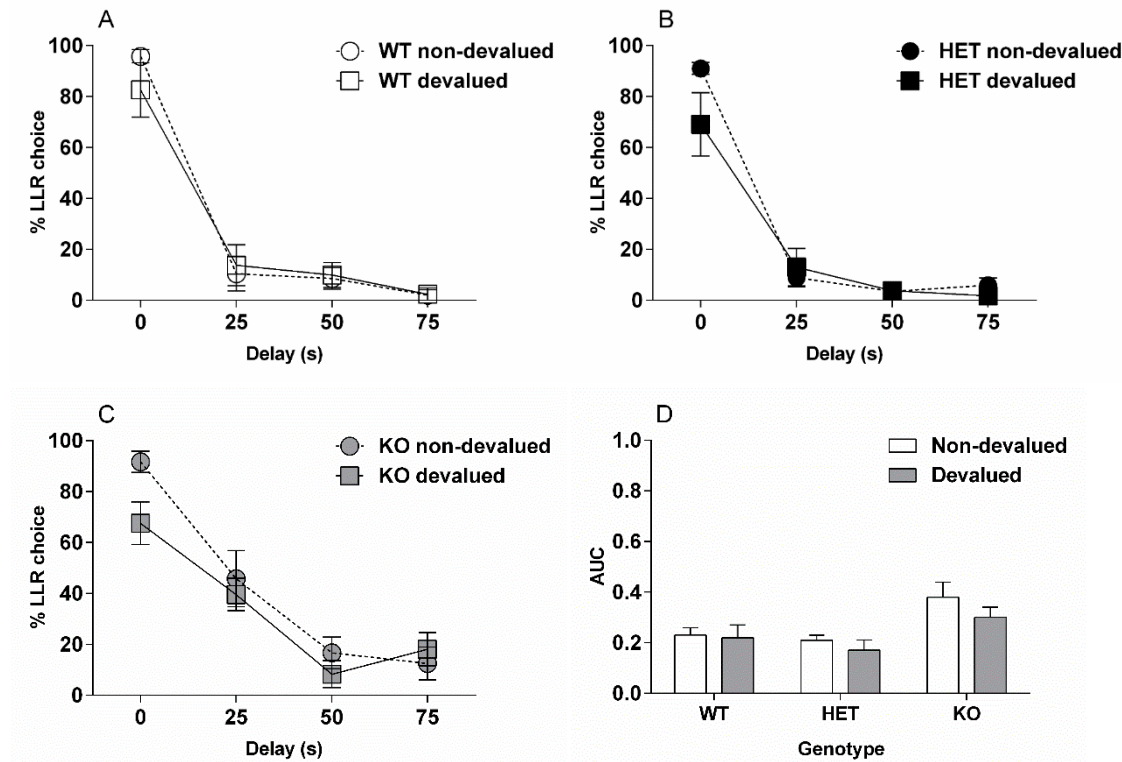


Fig. 3.10. Non-devalued vs devalued % LLR choices for WT (A), HET (B) & KO (C) mice and non-devalued vs devalued AUCs for WT, HET & KO mice (D).

Table 3.4 Mean (\pm SEM) magazine omissions during the chow pre-feeding devaluation

Session	WT	HET	KO
Non-devalued	1.17 (.65)	1.43 (1.27)	.17 (.17)
Devalued	7.17 (1.49)	3.71 (1.80)	4.17 (1.19)

Table 3.5 Mean (\pm SEM) lever omissions during the chow pre-feeding manipulation

Session	WT	HET	KO
Non-devalued	.83 (.54)	.14 (.14)	.50 (.34)
Devalued	.83 (.40)	.14 (.14)	.83 (.48)

Pre-feeding mice with their maintenance diet produced a trend toward a significant main effect of motivational state, with mice tending to record a smaller percentage of LLR choices in the devalued vs non-devalued session ($F_{(1, 16)} = 3.75$, $p = .07$) (Figs. 3.10A, 3.10B & 3.10C). There was a significant motivational state by delay interaction ($F_{(1.85, 28.54)} = 5.18$, $p = .01$). Post hoc paired t-tests revealed that the percentage of LLR choices recorded during the 0 s block of the devalued session was significantly smaller than the percentage of LLR choices recorded during the non-devalued session ($t_{(18)} = 2.97$, $p = .008$). However, % LLR choices were not different during any other delay blocks ($P_s = > .48$). There were no significant interactions between motivational state and genotype or between motivational state, delay and genotype ($F_s = < .49$, $P_s = > .67$). There was a significant main effect of delay ($F_{(2.00, 31.97)} = 224.63$, $p = .001$), with mice discounting the LLR lever as the delay to reinforcement increased. KO mice made a significantly higher percentage of LLR choices than HET but not WT subjects when collapsing across motivational state ($F_{(2, 16)} = 4.59$, $p = .03$) (Bonferroni post hoc tests $p = .03$ and $p = .16$ respectively). Finally, there was also a significant delay by genotype ($F_{(4.00, 31.97)} = 4.64$, $p = .005$) interaction. Pre-feeding mice with chow produced no significant main effect of motivational state and no significant genotype by motivational state interaction when analysing AUCs ($F_s = < 1.86$, $P_s = > .19$) (Fig. 3.10D). However, a significant main effect of genotype ($F_{(2, 16)} = 5.50$, $p = .02$) revealed that KO mice produced significantly larger AUCs than HET ($p = .02$) but not WT ($p = .09$) mice. Pre-feeding increased the number of magazine omissions ($F_{(1, 16)} = 13.16$, $p = .002$) but not the number of lever omissions ($F_{(1, 16)} = .19$, $p = .67$) (Tabs. 3.3 & 3.4). However, there was no significant main effect

of genotype or any significant genotype by motivational state interactions for either of these measures ($F_s = < 1.71$ $P_s = > .21$). In comparison to the non-devalued session, both trial initiation ($F_{(1, 16)} = 42.90$, $p = .001$) and choice latencies ($F_{(1, 16)} = 22.30$, $p = .001$) were slower during the devalued sessions. Whilst initiation latencies slowed as sessions progressed ($F_{(3, 48)} = 12.12$, $p = .001$), choice latencies were indifferent across delay blocks ($F_{(2, 08, 33, 33)} = .45$, $p = .65$). There were no other significant main or interaction effects in the time taken to initiate trials ($p = > .34$). There was a significant main effect of genotype for choice latencies ($F_{(2, 16)} = 4.12$, $p = .04$), however Bonferroni post hoc tests were not significant ($p = > .07$). A significant motivational state by genotype interaction ($F_{(2, 16)} = 4.61$, $p = .03$), followed up with one-way ANOVA comparing choice latencies between genotypes separately for each session revealed that KO mice produced significantly slower choice latencies than HET but not WT mice during the devalued session ($F_{(2, 16)} = 4.91$, $p = .02$) (Bonferroni post hoc tests $P_s = .03$ and $.07$ respectively) but not during the non-devalued session ($F_{(2, 16)} = .84$, $p = .45$). The motivational state by delay by genotype interaction was not significant ($F_{(6, 48)} = .85$, $p = .54$). Forced choice latencies were also significantly slower during the pre-fed session ($F_{(1, 16)} = 10.12$, $p = .006$), however, forced choice latencies were not significantly different between genotypes ($F_{(2, 16)} = .25$, $p = .78$). There was a significant main effect of lever ($F_{(1, 16)} = 6.76$, $p = .02$) with mice continuing to execute faster choices when presented with the SSR lever and a significant main effect of delay ($F_{(3, 48)} = 3.48$, $p = .02$), with mice recording slower latencies as the session progressed. There was also a significant lever by delay interaction ($F_{(3, 48)} = 11.61$, $p = .001$). However, none of the other interaction terms were significant ($F_s = < 2.26$, $P_s = > .14$).

3.4 Discussion

KO mice recorded a significantly greater proportion of LLR choices than both WT and HET mice at the 25 s, 50 s and 75 s blocks during the final 20 sessions of training. At first sight, these results imply that DARPP-32 KO mice are significantly less sensitive to the effects of delay to reinforcement than their DARPP-32 positive counterparts when delays to reinforcement are relatively long but not when the delays to reinforcement are comparatively short. Such a proposal that DARPP-32 KO mice are less sensitive to the effects of delay to reinforcement than their DARPP-32 carrying counterparts would be commensurate with our predictions. However, insofar that signalling via DARPP-32 transduction has a simple direct relationship with choice under delay discounting conditions, the finding that the genotype effect only appeared after extensive training seems unexpected. That is, relative to both WT and HET mice, KO mice diverged to become more myopic only after approximately 70 training sessions. Moreover, there was a ‘to-the-eye’ relatively higher level of delay insensitivity/inflexibility in DARPP-32 KO mice when inspecting the discounting curves for individual mice (data not shown). Percent LLR choices for individual KO mice were often characterised by block insensitive choice patterns. And, on the basis of the inclusion criteria, DARPP-32 KO mice also exhibited relatively higher levels of delay insensitivity compared to their WT and HET counterparts with fewer KO mice reaching the inclusion criteria during most stages of the experiment. In addition to this, KO mice appeared to take longer than both WT and HET mice to modify their LLR choices following changes in task parameters, especially after the delays were reversed from an ascending to a descending profile and vice versa. For example, only 3 KO mice were eligible for inclusion compared to 6 WT & 5 HET mice during sessions 33-37. There was also comparatively inflexible responding in DARPP-32 KO mice during sessions 64-68 with only 3 KOs, compared to 5 WTs & 7

HETs meeting the eligibility criteria. Again, this was approximately 13-18 sessions after the delays had been reversed from a descending to an ascending profile. By session 71, however, 6 KO mice met the eligibility criteria. KO mice then discounted less steeply than WT and HET mice during sessions (non-feeding sessions only) conducted with a maximum delay of 75 s. However, when these sessions were broken down statistically and discounting performance was compared between the first 5 and final 5 sessions, performance was only significantly different between genotypes during the first 5 sessions after the maximum delay was increased to 75 s. This suggests that, after extended experience at these parameters, DARPP-32 KO mice achieved comparable performance with their DARPP-32 carrying littermates.

When analysing data from delay discounting tasks, especially in studies recruiting non-human subjects where the contingencies between responses and outcomes are unknown by subjects at the outset of the study, it is crucial that one dissociates differences in the initial learning of the task from differences in performance once the task is fully learned (Cardinal, et al., 2003). It is also crucial, one assumes, to dissociate learning to update action/response-outcome contingencies following adjustment in the task parameters from the performance of the task once those parameters are fully learned. In accordance with this idea, care must be taken when analysing data captured in the intervening sessions following changes in task parameters. Animals must first learn that the contingencies have changed. Thus, any between-group differences which emerge in sessions following changes in the task parameters could reflect either an impairment in detecting such changes and/or a corresponding slowness to accordingly update choices (i.e. learning) or these group differences might reflect a genuine and enduring group difference in intertemporal choices. It is only through extended training following such changes that one can be sure of the underlying nature of these differences. On this basis,

it is possible that the between genotype differences which were observed during sessions with a max 75 s delay reflected difficulties in rapidly modifying choices following alterations in the task parameters.

DARPP-32 is densely enriched in the NAc and lesions to this region impairs both delay-based action-outcome instrumental learning and performance of already learned contingencies of the same nature (Cardinal & Cheung, 2005). Additionally, whilst NAc lesions promote delay aversion when delays are predictable (Cardinal et al., 2001), such lesions also increase delay tolerance when the delays are unpredictable (Acheson et al., 2006). When delays are suddenly increased, as was the case in the current experiment, subjects are clearly incapable of having pre-empted this change, so it is possible that owing to the unpredictable nature of these adjustments, that any subsequent group differences which occurred after the delays were extended might have reflected a relative slowness to detect and/or respond to changes in the delays rather than a true performance difference between genotypes. Again, it is only following extended training that one is able to discern the underlying nature of such differences, and by the completion of the experiment, the between-group differences had disappeared. Even though evidence suggests that the NAc is broadly involved in time-based action-outcome learning and intertemporal discounting, the mPFC (Churchwell et al., 2009) and OFC (Mar et al., 2011) also contribute to intertemporal choices so, of course, one cannot exclude the contribution that the loss of OFC or mPFC DARPP-32 might have had in producing the effects reported above.

At first sight, it appears somewhat peculiar that between groups differences were observed to delays of 25 s and 50 s in the final arrangement of delays (i.e 0 s, 25 s, 50 s 70 s) but not to delays of 32 s or 48 s in earlier delay arrangements. However, an important point warrants discussion when considering performance during within-session

discounting procedures. LLR choices in any given block may be affected by the delay associated with reinforcement in previous blocks within and between sessions. That is, the experience which subjects have with a reinforcement contingency within a delay block is believed to carryover into subsequent blocks and subsequent sessions, such that it impacts intertemporal choices during blocks of trials which are constrained by different temporal contingencies. It has been suggested that these carryover effects might be the underlying reason behind groups of subjects rarely achieving 100% LLR choice in the 0 s delay block, even though LLR choices are reliably reinforced in this block (Madden & Johnson, 2010). It is believed that the encounters subjects have with delayed reinforcement choices in previous sessions carryover into subsequent sessions and devalue the LLR in the no delay block, as well as in other blocks. When seen in this light, it is advisable not to consider delays in sequential within-session discounting paradigms as wholly discrete phenomena which can be compared with similar delays in different portions of a study, or between different studies, when other delays are present in one arrangement but not the other. That is, even though group differences were observed to the 25 s and 50 s delays, but not the 32 s or 48 s delays, one cannot reliably compare performance between these delays because the maximum delay in the arrangement containing the 25 s and 50 s delays was 75 s, whereas the maximum delay subjects experienced during any arrangement containing the 32 s or 48 s delays was 48 s or 64 s respectively. Moreover, aside from the 0 s delay block, subjects encountered a minimum delay of 25 s in the arrangement containing a maximum delay of 75 s. In comparison, subjects experienced a minimum delay of either 8 s or 16 s in the arrangements containing 32 s or 48 s delays. Put simply, delays of 0 s, 25 s, 50 s and 75 s (average delay of 37.5 s) are more stringent than delays of 0 s, 8 s, 16 s and 32 s (average delay of 14 s), or 0 s, 16 s, 32 s and 48 s (average delay of 26 s), or delays of 0 s, 16 s, 32 s and 64 s (average

delay of 28 s). Therefore one might expect carryover effects to exert a more pronounced influence upon the LLR choices of animals in the 0 s, 25 s, 50 s and 75 s arrangement compared to their influence during less stringent delay arrangements. Whilst this point in isolation cannot provide an adequate explanation for the presence of group differences to delays of 25 s and 50 s but not to comparable delays of 32 s or 48 s, when it is considered in conjunction with the proposal that the impairment might reflect either an insensitivity to detect changes in the task and/or a failure to efficiently reorganise behavioural output following such changes, it might have been that KO animals were initially less sensitive to detect and/or respond to the introduction of 3 novel delays (25 s, 50 s, 75 s) and initially less sensitive to the carryover effects one might expect from these much more stringent delays. For example, 3 unfamiliar delays (25 s, 50 s & 75 s) were introduced into the experiment during the period where group differences emerged. In comparison, when delays were extended to include either a 48 s or a 64 s delay, only one delay was changed in the arrangement, and in earlier arrangements containing a maximum delay of 32 s, no changes in delay length were introduced. Indeed, subjects conducted 70 sessions of testing which included a 32 s delay. Thus, the group differences might have been present during the 25 s and 50 s blocks, but not the 32 s and 48 s blocks, because subjects were required to assimilate much more information following the introduction of 3 novel delays (e.g. 25 s, 50 s, 75 s) and also because the delay arrangement differentially impacted the subjective value of the LLR across blocks and sessions (i.e. carryover effects).

Nevertheless, the precise nature of the disturbances that were observed in DARPP-32 KO mice during intertemporal discounting are difficult to disentangle on the basis of the data presented in this chapter. There were 2 general disturbances in DARPP-32 KO mice related to changes in the task parameters. First, and as noted above, DARPP-32 mice took

longer to modify/devalue LLR choices when the delays were extended. Second, DARPP-32 KO mice displayed a relatively inflexible choice pattern following the reversal of the delay order. These 2 disturbances might reflect a disturbance in a single process or they could be distinct. For instance, when the delay order is reversed, mice must learn to reverse their choices in relation to the block order. To elaborate this point, mice had initially experienced the shortest delay in the first block but, following delay order reversal, mice experienced the longest delay in the first block. Mice therefore must learn to no longer select the LLR lever most frequently in the first block and to select the SSR lever most frequently instead. This adaptation requires animals to overcome the previously learned response and to replace it with a novel response strategy. In contrast, when the delays were extended, the 0 s delay block maintained its sequential position as the first block subjects experienced, and mice merely had to detect that the length of the delay had increased within single blocks, as opposed to the delay order having been reversed between blocks. Although the NAc has a role in mediating learning about delays to reinforcement and intertemporal choice, there is reason to believe that the NAc is involved in specific kinds of behavioural flexibility. For example, in a study examining the effects of NAc lesions on behavioural flexibility, lesioned animals were impaired in learning the Morris Water Maze task (Annett, McGregor & Robbins, 1989). However, subjects were eventually able to achieve comparable performance with controls. In the same study, NAc lesions impaired spatial discrimination and reversal learning in a T-maze task. However, rather than simply displaying perseverative responding, the authors deemed that the lesioned animals were slow in learning the new position of the reward following its relocation. In another study, NAc inactivation also impaired set-shifting, not by increasing perseveration but by hindering the learning and performance of a novel strategy (Floresco, Ghods-Sharifi, Vexelman & Magyar, 2006). In exploring the

pharmacological basis of strategy shifting, Haluk & Floresco (2009) reported that NAcC injections of D₁ antagonist SCH 23390 impaired strategy switching not by inducing perseverative errors but by increasing regressive errors which are indicative of difficulties in sustaining novel strategies. In contrast, the D₂ receptor agonist quinpirole increased perseverative errors in the same task and also reversal learning. This is not to suggest that the abovementioned tasks are analogous to the changes introduced in this experiment. Merely I am suggesting that the nucleus accumbens assists in both time-based instrumental learning and in flexible responding to changes in tasks that require subjects to update a previously learned strategy by acquiring a new one and, as noted above, diminished transmission through accumbal D₁ receptors impairs this kind of flexibility whilst accumbal D₂ receptors also contribute to behavioural flexibility (Haluk & Floresco, 2009). Of course, one cannot exclude the role of DA in dorsal striatal (O'Neill & Brown, 2007) or PFC (Floresco, 2013; Winter et al., 2009) regions in mediating behavioural flexibility. Furthermore, DARPP-32 is distributed in regions more intimately associated with behavioural flexibility than the NAc, such as the DMS (Castañé et al., 2010), OFC (Kim & Ragozzino, 2005) and mPFC (Winter et al., 2009). OFC lesions, for example, impair learning when the positions of the SSR and LLR levers are reversed (Mar et al., 2011).

Whilst the precise nature of the disturbances reported in this chapter are unknown, there is reason to believe that DARPP-32 assists in the flexible updating of behaviour. In a human study, subjects with an SNP of the DARPP-32 gene had higher P200 event related potentials during a task which explicitly requires subjects to update outcome-expectancies (Hämmerer et al., 2013). And as noted elsewhere, DARPP-32 KO mice are also generally less flexible/responsive to changes in instrumental contingencies, as determined by instrumental reversal learning (Heyser et al., 2000) and to alterations in

environmental stimuli (e.g. novel object recognition) (Heyser et al., 2013). This evidence, as well as that presented in this chapter, suggests that DARPP-32 assists in adapting to changes in task parameters.

Finally, while selective or general devaluation produced an effect on consummatory magazine approach, it failed to markedly alter responding under delay discounting conditions irrespective of genotype. Although the general motivation devaluation significantly reduced baseline preference for the LLR lever, it did not alter the % LLR choices in any blocks during which a delay was ascribed to the LLR lever and nor did pre-feeding significantly alter AUCs. Importantly, there were no significant interaction effects between motivational state and genotype nor a main effect of genotype during pre-feeding sessions. On this basis, it is unlikely that the between genotype differences in LLR choices can be alternatively accounted for by motivational differences. Whilst there were some small transient differences between KO and HET mice in initiation latencies, there were no clear or enduring differences in the time taken between DARPP-32 KO mice and HET or WT mice to initiate trials or to execute choices during this experiment, thus there does not appear to be any slowing or speeding of responses as a result of deleting DARPP-32.

In summary, there were no differences between KO, HET and WT mice at the beginning of the experiment. However, once the parameters were reversed from an ascending to a descending profile, DARPP-32 KO mice exhibited a relatively inflexible profile with the majority failing to meet the inclusion criteria during sessions 33-37. Then, when the parameters were reversed from a descending to an ascending profile, KO mice again exhibited a relatively inflexible profile. Thus, there were comparatively higher levels of inflexibility in DARPP-32 KO mice following changes in the task parameters. Moreover, there were no clear differences in performance between mice with or without

the DARPP-32 gene for the overwhelming majority of sessions and only when the delays were changed toward the end of the experiment did a genotype difference appear, and only then was it a transient effect. This suggests that rather than being inherently different from DARPP-32 carrying littermates in their performance during intertemporal discounting tasks, that DARPP-32 KO mice are impaired in the ability to flexibly adapt to task changes and/or at updating outcome-expectancies following changes in the task parameters. These were unexpected findings, though given the role of this signalling molecule in facilitating flexible responding in rodents and decision-making processes in humans, it is not altogether unsurprising that DARPP-32 KO mice were comparatively inflexible in this task.

Chapter 4

Probability discounting**4.1 Introduction**

In the previous chapter, we examined the relationship between global DARPP-32 deletion and its effects on intertemporal choices. In addition to dimensions of time, reinforcement choices can be isolated along dimensions of uncertainty/risk and the procedures which capture risky choices are important tools for identifying the biological basis of risk proneness. An exaggerated preference for risky outcomes is associated with life-limiting pathologies such as addiction (Bornovalova, Daughters, Hernandez, Richards & Lejuez, 2005; Brand, Roth-Bauer, Driessen & Markowitsch, 2008), ADHD (Groen, Gaastra, Lewis-Evans & Tucha, 2013), pathological gambling (Kräplin et al., 2014), and also with the side effects of antiparkinson medications (Weintraub et al., 2006). On the other hand, maintaining a normative level of risk is often necessary for the exploitation of ecologically relevant reinforcers and an excessively risk averse predisposition might limit opportune reinforcement.

Research conducted with the probability discounting task has shown that when the probabilities of reinforcement for a small or a large reinforcer are equal and certain ($p = 1$), subjects will preferentially choose the large reinforcer. However, as uncertainty - or risk - is systematically and unevenly introduced (i.e. when the probability of reinforcement associated with the large reinforcer decreases and the probability of reinforcement for the small reinforcer remains constant), subjects will begin to direct their preference away from the large uncertain reinforcer towards the small certain reinforcer (Cardinal & Howes, 2005; Ghods-Sharifi, St Onge & Floresco, 2009; Rachlin, Ranieri & Cross, 1991; Richards, Zhang, Mitchell & de Wit 1999; St Onge & Floresco, 2009; St Onge & Floresco, 2010; Stopper et al., 2013; Stopper, Green & Floresco, 2012). Thus, in

such tasks, risk-prone subjects are identified as those who execute risky decisions in the face of a probabilistically diminishing positive outcome or, conversely, a probabilistically increasing likelihood of a less desirable outcome.

Neurobiological studies have elucidated many structures involved in the provision of probabilistically constrained reinforcement choices. Some of these regions include the mPFC (St Onge & Floresco, 2010), the OFC (Abela & Chudasama, 2013; Stopper et al., 2012), the BLA (Ghods-Sharifi et al., 2009), and the NAcC (Cardinal & Howes, 2005). However, temporary inactivation of the NAcSh but not the NAcC affected probability discounting (Stopper & Floresco, 2011), suggesting that the NAcSh but not the NAcC has a role in mediating probabilistic reinforcement choices. As noted by Stopper & Floresco (2011), the lesions in the Cardinal & Howes (2005) study also damaged parts of the NAcSh. Many of these regions but particularly the NAc are associated with the enrichment of DARPP-32 and studies examining the pharmacological underpinnings of probabilistic reinforcement have identified a role for transmitter systems (e.g. DA & glutamate) that regulate the phosphorylation of DARPP-32.

There is a growing empirical literature exploring how different DA receptor subtypes contribute to probabilistic positive reinforcement choices. For instance, systemic administration of the D₁ receptor agonist SKF 82197 increases risky probability discounting choices at a low (0.3mg/kg) dose. At a higher dose (1.0 mg/kg), the effects on choice were bi-directional, reducing risky choices in a low-risk block ($p = 0.5$) but increasing risky choices in a high-risk block ($p = 0.25$) (St Onge & Floresco, 2009). Conversely, amphetamine or the D₂ receptor agonist bromocriptine also increased risky choices and systemic administration of the D₁ receptor antagonist SCH 23390 or the D₂ receptor antagonist eticlopride both reduced risky choices. Co-administration of amphetamine with either of these latter 2 compounds attenuated the risk promoting effects

of amphetamine, although this effect was most pronounced when amphetamine was co-administered with SCH 23390. The D₃ receptor antagonist nafadotride was inefficacious in affecting choices, however, when it was co-administered with amphetamine, it potentiated the risky-choice enhancing effects of amphetamine. The D₃ receptor agonist PD 128907 sub-optimally reduced choices of a large but uncertain reward by driving down choices in high probability of reinforcement trials.

Intra-mPFC SCH 23390 significantly decreased risky probabilistic choices whereas the D₂ receptor antagonist eticlopride increased risky decisions (St Onge et al., 2011). In a similar study, Stopper, Khayambashi & Floresco (2013) showed by directly targeting NAc DA receptors that NAc D₁ & D₃, but not D₂, receptors are important determinants of probabilistic positive reinforcement choices. In summary, DA is a significant mediator of probabilistic choices but the specific DA receptor sub-types that mediate uncertain reinforcement choices vary on a regional basis, with D₁ receptors being of relevance in the mPFC and NAc, whereas D₂ receptors appear to have relevance to probability discounting in the mPFC but not NAc.

It was recently shown that glutamate transmission is involved in the facilitation of uncertain reinforcement choices, especially via the NMDA receptor. The NMDA receptor antagonist, MK801, but not the AMPA receptor antagonist CNQX, reduces probability discounting (Yates et al., 2014). MK801 abolishes the ability of glutamate to increase Thr³⁴-DARPP-32 phosphorylation in striatal slices (Nishi et al., 2005). To the best of one's knowledge, whilst there are no published studies providing evidence of the precise neural locus of the glutamatergic mediation of probabilistic reinforcement choices it is likely that, given the tightly interwoven relationship between DA and glutamate in regions of the brain that mediate incentive motivational processes and reinforcement

choices, these 2 transmitter systems converge in the striatum to modify probability discounting.

As noted earlier, probabilistic choice procedures recruit DA innervated brain regions, some of which are associated with the profound expression of DARPP-32, in particular the NAc where the D₁ receptor exerts a prominent influence on probabilistic reinforcement choices. As an integrator of accumbal DA and glutamate (originating from e.g. amygdala and prefrontal cortex) transmission, DARPP-32 is well positioned as a credible molecular candidate for the targeted investigation of intracellular mediators of probabilistic choices. Perhaps to date, the most direct evidence implicating DARPP-32 in probabilistic reinforcement is provided by Michael Frank and colleagues who showed that an allelic variant of a DARPP-32 SNP was associated with improved performance during a probabilistic reinforcement learning task in humans (Frank et al., 2007). On the basis of these studies, it was proposed that DARPP-32 deletion would significantly increase probability discounting in knockout mice relative to a cohort of DARPP-32 possessing WT littermates. To test this prediction, a probability discounting paradigm which was a modified version of the procedure described by Cardinal and Howes (2005) was implemented. The effects of reinforcer devaluation on probabilistic choices were examined as such manipulations have been shown to induce risk aversion (St Onge & Floresco, 2009). In light of these reports, and of the expression of DARPP-32 in regions identified as mediating reinforcer devaluation (CPu, OFC,) and habit formation (CPu, CeA, OFC), it was deemed important to establish whether DARPP-32 KO mice are differentially sensitive to the effects of pre-feeding on risky choices compared to WT mice. In addition to this, pre-feeding provides a degree of control for establishing whether differences in motivational state might differentially contribute to instrumental choices between genotypes.

4.2 Methods

4.2.1 Subjects

Eight DARPP-32 KO mice (mean weight = 24.30 g), aged 14 to 24 weeks old and 8 WT littermates (mean weight = 25.60 g), aged 15 to 24 weeks at study commencement were used for this study. Subjects were at least the 7th generation of backcrossed mice bred at the University of Sussex on a C57BL/6J background in the manner described previously. Genotyping was conducted prior to study commencement using the protocol described in chapter 2. Equal numbers of male and female mice were present in each group. Animals were food restricted to 85% of their free-feeding weight for the duration of the experiment, except when stated otherwise, and provided with *ad libitum* access to tap water in their home cages. Mice were housed in the conditions described earlier. Each mouse was handled for 5 min per day on 3 consecutive occasions prior to study commencement to reduce handling stress.

4.2.2 Apparatus

All testing was conducted in the operant chambers described in chapter 3. A 10% liquid sucrose solution was used as the reinforcer in all operant box sessions.

4.2.3 Procedure

4.2.3.1 Magazine training

Mice were first given 2 magazine training sessions, 1 per day, to develop an association with the location of reward receipt. These sessions commenced when subjects made their first magazine entry and continued until a total of 20 rewards had been dispensed. Reinforcements consisted of 46.4 µl of sucrose which were dispensed on a RI-

60 schedule. One mouse responded markedly lower after 2 sessions and was therefore given an additional magazine training session.

4.2.3.2 Lever training

Mice were then given 6 daily sessions of fixed ratio-1 (FR-1) instrumental training, 1 lever per session, to establish reliable and equivalent responding on both operanda. Both levers, when activated, were reinforced with 46.4 μ l of sucrose. Lever training sessions terminated after 60 min and were conducted daily until mice had acquired 50 reinforcements on each lever during 2 consecutive sessions. One poorly performing mouse was given 2 additional lever training sessions to overcome a side bias.

4.2.3.3 Trial initiation training

Subjects were next provided with up to 11 sessions of trial initiation training. These sessions consisted of 4 blocks of 22 trials with each trial lasting 70 s. All trials were forced choice trials, such that only 1 lever was presented per trial but each lever was presented an equal number of times per block. Trials began in darkness and their commencement was indicated by the illumination of the houselight. Mice were given 20 s to nosepoke into the reward magazine following the illumination of the houselight. Failure to respond within this period resulted in the houselight being extinguished, the trial being recorded as an omission, and being forced into a timeout period that lasted the duration of the trial. If mice made a magazine response within 20 s of the light illuminating, 1 lever, selected pseudorandomly with respect to position, was extended into the chamber. In turn, failure to respond on the lever within 20 s of its presentation resulted in the lever being retracted, the houselight being extinguished, the trial being recorded as an omission and the subject experiencing a timeout period for the duration of the trial. Responding on the lever prior to the 20 s timeout period resulted in the immediate delivery of 24.7 μ l of sucrose and the

simultaneous extinguishing of the light and retraction of the lever. Trial length was held constant at 70 s regardless of whether the animal achieved reinforcement or recorded omissions.

4.2.3.4 Probability discounting

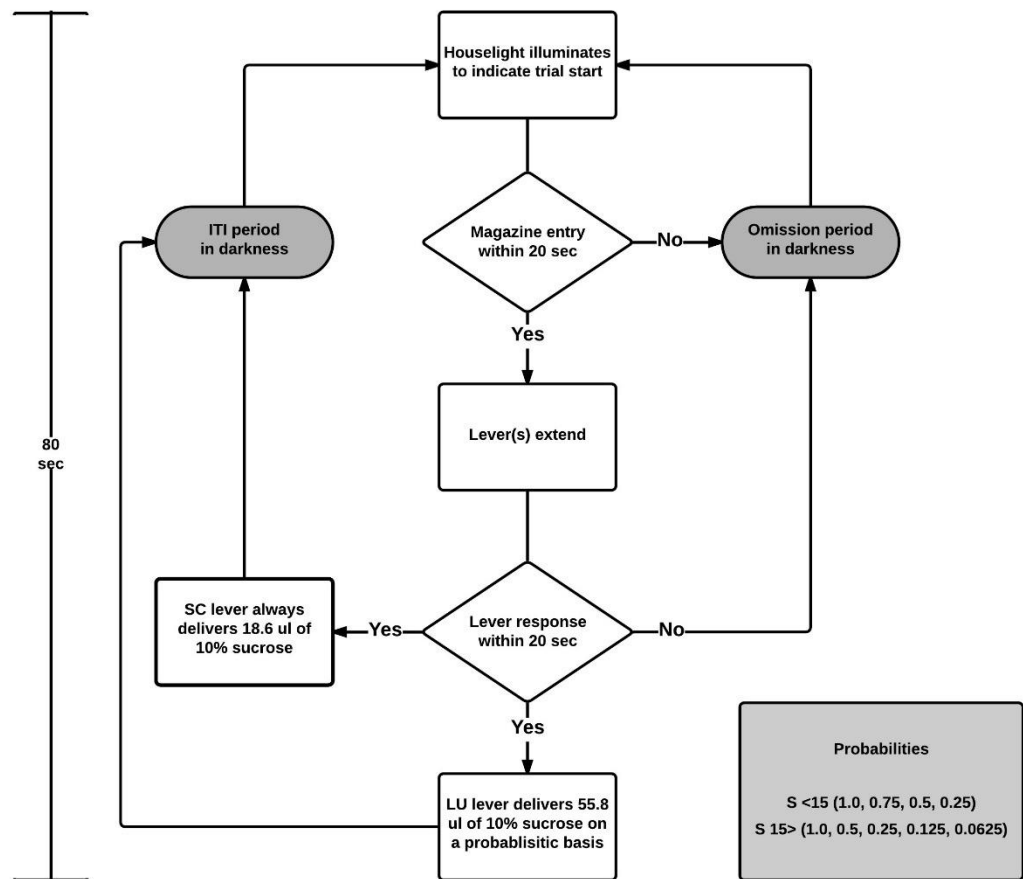


Fig. 4.1 Simplified schematic diagram of the probability discounting procedure.

The probability discounting procedure was similar in many ways to the one described in the delay discounting chapter. Between sessions 1 and 14, the first 10 trials of each block were forced choice trials, involving the extension of only 1 lever per trial, and the remaining 12 were choice trials involving the presentation of both levers. Discounting sessions began with the illumination of the houselight. If mice made a head entry into the reward magazine within 20 s of the trial start, either 1 lever, selected pseudorandomly

with regards to lever position, was inserted into the chamber during forced choice trials, or both levers were inserted during choice trials. Failure to make a head entry into the reward delivery magazine or to respond on the lever within 20 s of the trial start or lever insertion, respectively, resulted in the houselight being extinguished and mice entering a timeout period for the remainder of the trial. Levers were presented an equal number of times during the forced choice component of each block of trials to ensure that mice experienced the programmed consequences of both levers at the start of each 'probability block'. Lever responses were reinforced on a probabilistic basis in relation to the lever selected, with 1 lever designated as the small certain (SC) lever and the other lever designated as the large uncertain (LU) lever: counterbalanced across genotypes with respect to the physical position of the lever. During the first 14 sessions, responses on the SC lever were always reinforced with 23.2 μ l of sucrose following a single 0.75 s reward pump activation and LU lever responses were reinforced with 3 deliveries of 23.2 μ l of sucrose (i.e. 69.9 μ l of sucrose) dispensed over 3 consecutive 0.75 s activations of the reward pump. LU rewards were delivered on a probabilistic basis which progressively diminished across consecutive blocks (e.g. 1.0, 0.75, 0.5, and 0.25). Therefore, during choice trials mice were provided with the opportunity to select either a small certain reinforcer or a large uncertain reinforcer.

Because neither WT nor KO groups were discounting by session 14, the procedure was altered as follows: first, by increasing the number of blocks to 5, with each consisting of 20 trials, 10 of which were forced choice trials. Second, the probabilities of delivery of the large reward were adjusted so that $p = 1.0, 0.5, 0.25, 0.125, \text{ or } 0.0625$, depending on the block to accelerate the acquisition of block-dependent discounting. Third, the reward magnitude was reduced to compensate for the increased number of trials, so that

responses on the SC lever yielded 18.6 μ l of sucrose and responses on the LU lever yielded 3 deliveries of 18.6 μ l of sucrose dispensed via consecutive 0.75 s activations of the reward pump.

4.2.3.5 Reinforcer devaluation

Mice were subjected to 2 different reinforcer devaluation procedures on 2 separate occasions. The first reinforcer devaluation procedure was a sensory-specific intervention which involved pre-feeding half of all animals with 10% sucrose for 2 hrs on day 1. During these pre-feeding sessions, home cage water bottles were replaced with bottles containing 10% sucrose. Pre-feeding was counterbalanced with respect to genotype and lever position. All animals, including those that were not sucrose pre-fed, were then immediately subjected to a probability discounting session. On day 2, the remaining half of animals that had not undergone sucrose pre-feeding were given a 2 hr sucrose pre-feeding session which was followed with all animals conducting a probability discounting session.

The second reinforcer devaluation procedure was a general motivation intervention. These sessions, conducted over 2 days, were identical to the sensory-specific devaluation session except that during the 2 hr pre-feeding sessions, mice were pre-fed with regular laboratory chow prior to the discounting sessions.

4.2.4 Data analysis

The response rates of WT & KO mice on each lever during the final session of lever training were compared by conducting a genotype (WT vs KO) by lever (1 vs 2) repeated measures ANOVA to ensure that performance was equal on both levers within and between genotypes.

A mixed genotype (WT vs KO) by lever (1 vs 2) by block (1 vs 2 vs 3 vs 4) ANOVA of the final trial initiation training session was conducted to ensure that performance was equivalent between genotypes, levers or blocks.

Magazine and lever omissions recorded during the final trial initiation training session were each analysed separately and compared between genotypes by conducting independent t-tests.

The percentage of total choices made in each block that were LU choices during discounting sessions was the dependent variable (i.e. $\text{LU choices}/(\text{SC choices} + \text{LU choices}) \times 100$). Responses from each block (e.g. $p = 1.0$, $p = 0.5$, $p = 0.25$, $p = 0.125$, $p = 0.0625$) for each animal were averaged over 5 sessions. These values were then used to calculate the overall rate of discounting as determined by AUC (see chapter 3 for details). To this end, the probability of reinforcement was converted to the odds against reinforcement using the formula $(1 - p)/p$ and then normalised as a proportion of the largest odds against reinforcement (see Myerson et al., 2001 for a more detailed description). Figures report the probabilities of reinforcement rather than the odds against. To compare discounting performance between genotypes, a genotype (WT vs KO) by probability ($p = 1.0$ vs $p = 0.5$ vs $p = 0.25$ vs $p = 0.125$ vs $p = 0.0625$) repeated measures ANOVA of % LU choices at each block corresponding to the 5 session average was conducted except when stated otherwise. Independent t-tests were conducted to compare AUCs between genotypes except when stated otherwise.

% LU choices during the devaluation sessions were compared by conducting genotype (WT vs KO) by motivational state (food-deprived vs pre-fed) by probability ($p = 1$ vs $p = 0.5$ vs $p = 0.25$ vs $p = 0.125$ vs $p = 0.0625$) mixed model ANOVAs.

AUCs from the 2 reinforcer devaluation manipulations were subjected to genotype (WT vs KO) by motivational state (food-deprived vs pre-fed) repeated measures ANOVAs.

Magazine and lever omissions were similarly averaged over the corresponding 5 sessions. Each omission type was analysed separately and compared between genotypes by independent t-test.

Magazine and lever omissions recorded during the devaluation manipulation sessions were analysed separately by conducting genotype (WT vs KO) by motivational state (food-deprived vs pre-fed) repeated measures ANOVAs for each variable of interest.

A post hoc stability analysis was conducted by comparing the AUCs from sessions 30-34 with the AUCs from sessions 40-44 by conducting a genotype (WT vs HET vs KO) by session (30-34 vs 40-44) repeated measures ANOVA.

Trial initiation and choice latencies were analysed separately by conducting genotype (WT vs KO) by probability ($p = 1$ vs $p = 0.5$ vs $p = 0.25$ vs $p = 0.125$ vs $p = 0.0625$) repeated measures ANOVA except during the devaluation sessions which were analysed with genotype (WT vs KO) by motivational state (food-deprived vs pre-fed) by probability ($p = 1$ vs $p = 0.5$ vs $p = 0.25$ vs $p = 0.125$ vs $p = 0.0625$) mixed model ANOVAs.

Forced choice latencies were analysed by conducting lever (SC vs LU) by genotype (WT vs KO) by probability ($p = 1$ vs $p = 0.5$ vs $p = 0.25$ vs $p = 0.125$ vs $p = 0.0625$) mixed model ANOVAs except during the devaluation manipulations when an additional within-subjects variable of motivational state (food-deprived vs pre-fed) was introduced to the ANOVA.

4.3 Results

4.3.1 Lever training

Response rates on both levers were similar for both genotypes during the final session of lever training ($F_s = < 2.94$, $P_s = > .11$).

4.3.2 Trial initiation training

Trial initiation training performance was consistent between levers, probability blocks and genotypes ($F_s = < 2.95$, $P_s = > .11$). Magazine ($t_{(14)} = -.36$, $p = .73$) and lever ($t_{(14)} = .86$, $p = .41$) omissions were not significantly different between genotypes.

4.3.3 Probability discounting

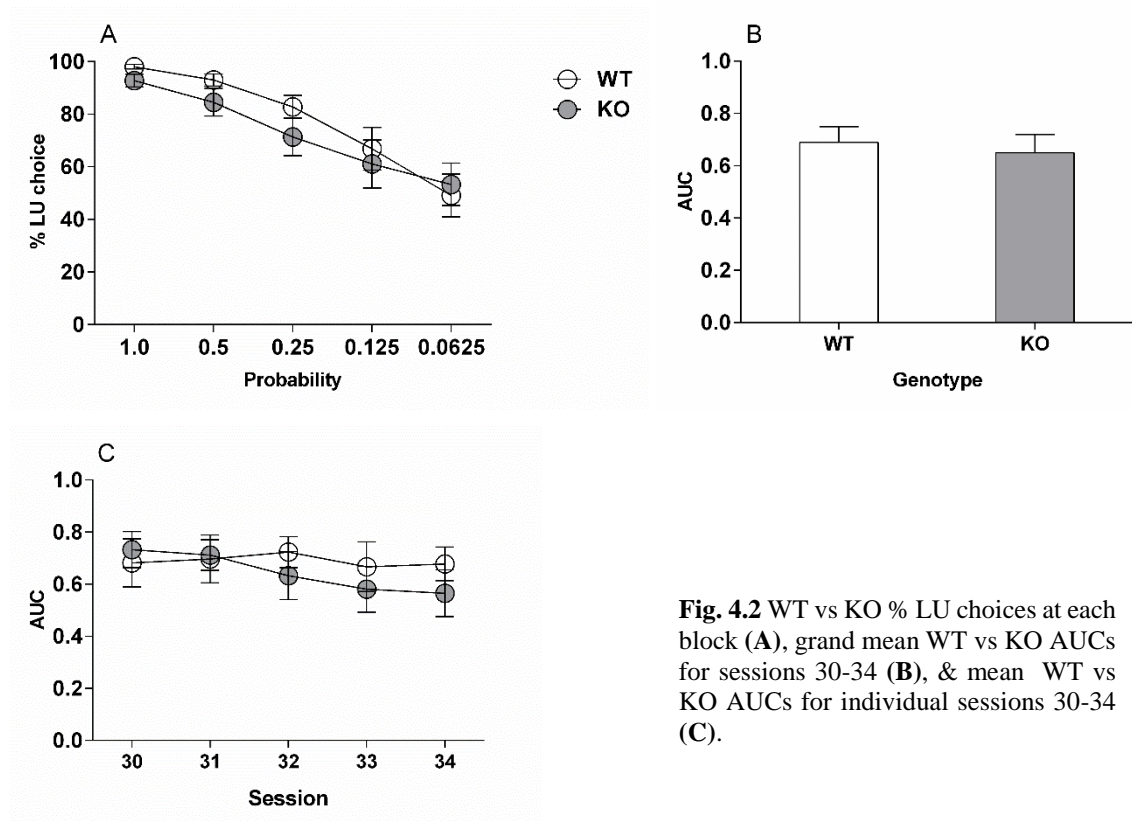
No data are presented from the first 14 sessions because animals were persistently selecting the LU lever in a block-insensitive fashion (i.e. the discounting curves were flat in both groups).

Table 4.1 Mean (\pm SEM) magazine omissions

Session	WT omissions	KO omissions
30-34	1.16 (.32)	1.03 (.33)
35-39	.90 (.18)	1.80 (.93)
40-44	1.35 (.82)	1.35 (.33)
Sucrose non-devalued	1.25 (.62)	2.00 (1.12)
Sucrose devalued	7.13 (2.22)	5.50 (3.00)
Chow non-devalued	13.63 (4.43)	10.88 (7.00)
Chow devalued	36.88 (5.36)	23.63 (5.27)

Table 4.2 Mean (\pm SEM) lever omissions

Session	WT	KO
30-34	1.45 (.78)	1.05 (.35)
35-39	.65 (.46)	1.19 (.59)
40-44	.20 (.17)	.40 (.15)
Sucrose non-devalued	.25 (.25)	.00 (.00)
Sucrose devalued	1.50 (1.22)	.63 (.50)
Chow non-devalued	.88 (.61)	.13 (.13)
Chow devalued	1.38 (.84)	.63 (.18)

4.3.3.1 Sessions 30-34**Fig. 4.2** WT vs KO % LU choices at each block (A), grand mean WT vs KO AUCs for sessions 30-34 (B), & mean WT vs KO AUCs for individual sessions 30-34 (C).

Mice discounted the LU lever as the probability of reinforcement decreased across blocks ($F_{(1.52, 21.34)} = 42.26$, $p = .001$) but there was no significant main effect of genotype and no significant probability by genotype interaction ($F_s = < .76$, $P_s > .45$) (Fig. 4.2A). Average AUCs were also similar between genotypes during sessions 30-34 ($t_{(14)} = .46$, $p = .65$) (Fig. 4.2B). Likewise, magazine ($t_{(14)} = .30$, $p = .77$) and lever omissions ($t_{(14)} =$

.47, $p = .65$) were not significantly different between genotypes (Tabs. 4.1 & 4.2). Trial initiation latencies slowed as sessions progressed ($F_{(2.11, 29.57)} = 5.55$, $p = .008$). However, there were no differences between genotypes in this measure, either as a main effect or genotype by probability interaction and there were no significant main effects or interactions relating to choice latencies ($F_s = < 3.22$, $P_s = > .10$). Mice were quicker to press the LU lever during forced choice trials when collapsing across genotypes ($F_{(1, 14)} = 58.99$, $p = .001$). KO mice were significantly slower to respond during forced choice trials than WT mice ($F_{(1, 14)} = 5.88$, $p = .03$). KO mice were also significantly slower at depressing the SC lever but not the LU lever than WT mice during forced choice trials (lever by genotype interaction ($F_{(1, 14)} = 10.08$, $p = .007$); post hoc independent t-tests comparing SU lever latencies ($t_{(10.39)} = -3.14$, $p = .01$) and LU lever latencies ($t_{(14)} = -1.03$, $p = .32$) for each lever between genotypes). There was also a significant lever by probability interaction ($F_{(2.33, 32.55)} = 7.05$, $p = .002$). There were no other significant effects involving forced choice latencies ($F_s = < 1.17$, $P_s = > .33$).

4.3.3.2 Sessions 35-39

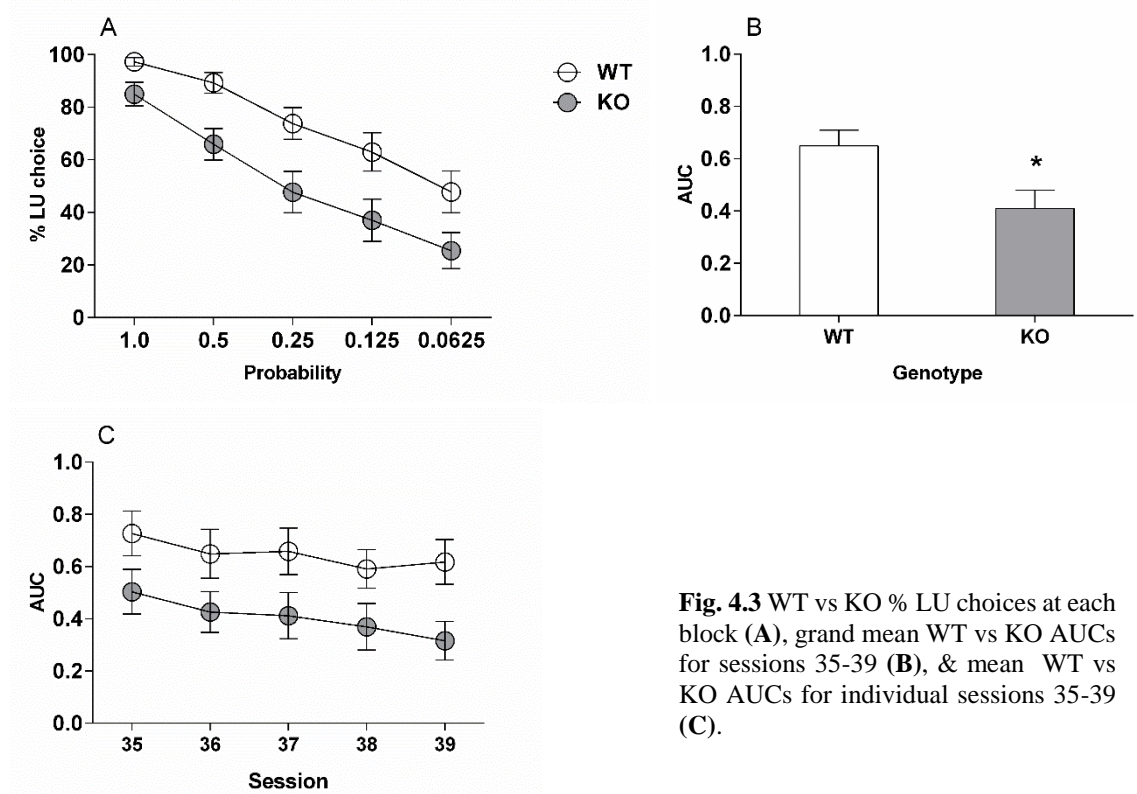


Fig. 4.3 WT vs KO % LU choices at each block (A), grand mean WT vs KO AUCs for sessions 35-39 (B), & mean WT vs KO AUCs for individual sessions 35-39 (C).

As training progressed beyond session 33, a distinct genotype difference emerged. Whilst all mice significantly reduced the proportion of LU choices they made across probability blocks ($F_{(2.24, 31.37)} = 85.17$, $p = .001$) (Fig. 4.3A) - i.e. discounted the larger but progressively uncertain reward option - DARPP-32 KO mice made a significantly smaller percentage of LU choices than WT mice ($F_{(1, 14)} = 7.74$, $p = .02$). The probability by genotype interaction was not significant ($F_{(2.24, 31.37)} = 1.46$, $p = .25$). AUCs were also significantly different between genotypes ($t_{(14)} = 2.58$, $p = .02$), with KO mice producing significantly smaller AUCs than WT mice (Fig. 4.3B). Neither magazine omissions ($t_{(7.53)} = -.95$, $p = .37$) or lever omissions were significantly different between genotypes ($t_{(14)} = -.73$, $p = .48$) (Tabs 4.1 & 4.2). Although trial initiation latencies continued to slow as sessions progressed ($F_{(1.79, 25.08)} = 7.81$, $p = .001$), there were no significant differences between genotypes in the time taken to initiate trials either as main effects or as an interaction between probability and genotype ($F_s = < 1.26$, $P_s = > .28$). Although choice latencies were similar across blocks ($F_{(4, 56)} = .51$, $p = .73$), KO mice took significantly

longer than WT mice ($F_{(1, 14)} = 6.66$, $p = .02$) to execute their choices. The probability by genotype interaction was not significant ($F_{(4, 56)} = .35$, $p = .84$). Forced choice latencies were similar between levers ($F_{(1, 14)} = .04$, $p = .86$) and across probability blocks ($F_{(2.58, 36.10)} = .81$, $p = .48$). However, KO mice continued to record significantly slower forced choice latencies than WT mice ($F_{(1, 14)} = 5.13$, $p = .04$). There was a significant lever by probability interaction ($F_{(4, 56)} = 6.45$, $p = .001$). However, there were no other significant interaction effects relating to forced choice latencies ($F_s = < 2.06$, $P_s = > .10$).

4.3.3.3 Sessions 40-44

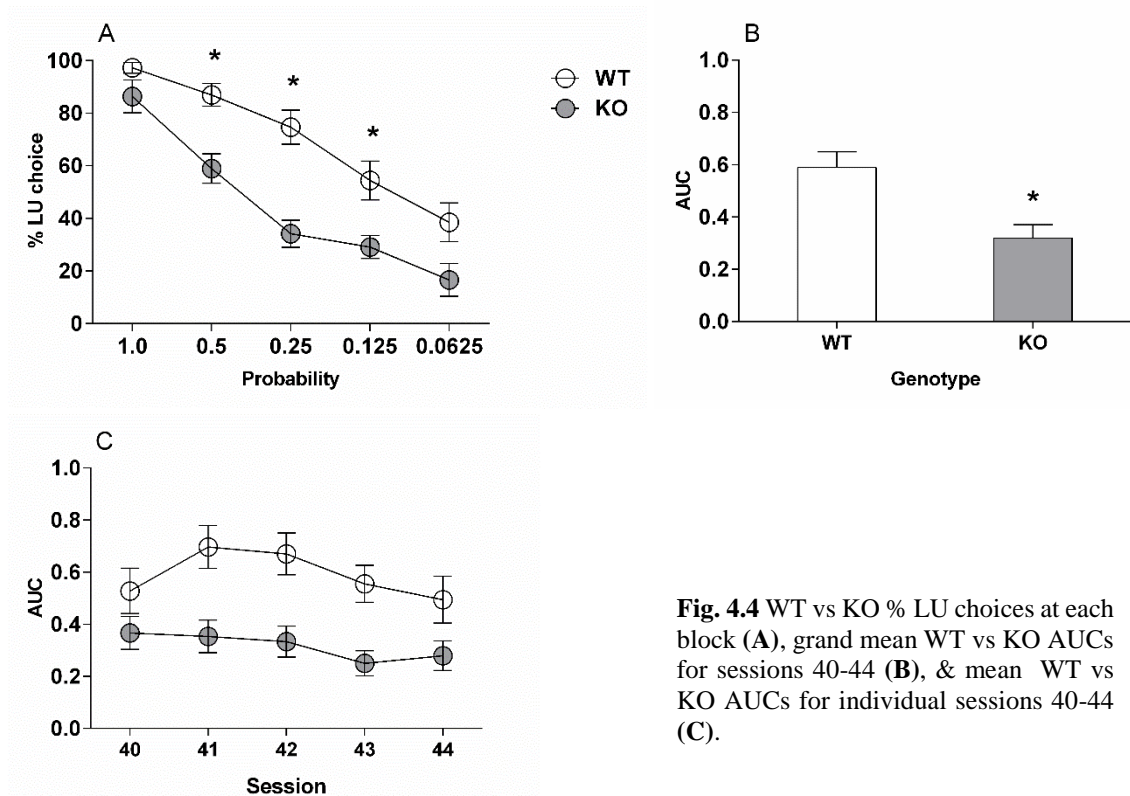


Fig. 4.4 WT vs KO % LU choices at each block (A), grand mean WT vs KO AUCs for sessions 40-44 (B), & mean WT vs KO AUCs for individual sessions 40-44 (C).

With additional sessions, mice dramatically reduced the proportion of LU choices they made as the probability of reinforcement decreased across blocks ($F_{(1.71, 23.89)} = 148.04$, $p = .001$) (Fig. 4.4A). LU choice patterns were significantly different between genotypes ($F_{(1, 14)} = 12.41$, $p = .003$) and there was also a significant probability by genotype interaction ($F_{(1.71, 23.89)} = 6.60$, $p = .007$), suggesting that DARPP-32 KO mice were discounting the LU lever significantly more steeply than WT mice. Post hoc independent

t-tests revealed that KO mice recorded a significantly smaller percentage of LU choices during the $p = 0.5$ ($t_{(14)} = 3.97$, $p = .001$), the $p = 0.25$ ($t_{(14)} = 4.89$, $p = .001$) and the $p = 0.125$ ($t_{(14)} = 2.96$, $p = .01$) blocks, but not the $p = 1$ or $p = 0.0625$ blocks when correcting the significance value for multiple comparisons ($P_s = > .04$, $\text{sig} = .01$). These results suggest that DARPP-32 KO mice executed sub-optimal choices during the 0.5 probability block, where selecting the LU lever is the most efficient path to reinforcement but, as the probability of reinforcement diminished, they executed choice patterns that were better optimised than WT mice. The AUC analysis also showed that KO mice continued to discount more steeply than WT mice ($t_{(14)} = 3.37$, $p = .005$) (Fig. 4.4B). Both magazine ($t_{(14)} = .001$, $p = .99$) and ($t_{(14)} = -.88$, $p = .39$) lever omissions were similar between genotypes (Tabs 4.1 & 4.2). Trial initiation latencies slowed as trials progressed ($F_{(4, 56)} = 9.50$, $p = .001$) but there were no significant differences between genotypes in this measure, either as a main effect or an interaction between probability and genotype ($F_s = < 2.08$, $P_s = > .15$). In contrast, choice ($F_{(2.36, 32.97)} = .36$, $p = .73$) latencies did not slow as sessions progressed. KO choice latencies, however, were significantly slower than WT choice latencies ($F_{(1, 14)} = 10.23$, $p = .006$). The probability by genotype interaction for choice latencies was not significant ($F_{(2.36, 32.97)} = .88$, $p = .44$). KO mice also continued to make significantly slower forced choices than WT mice ($F_{(1, 14)} = 10.02$, $p = .007$). There was also a significant lever by probability interaction ($F_{(2.45, 34.30)} = 5.61$, $p = .005$) and whilst the main effect of probability ($F_{(4, 56)} = 2.37$, $p = .06$) and the probability by genotype interaction approached significance ($F_{(4, 56)} = 2.40$, $p = .06$), there were no other significant effects for forced choice latencies ($F_s = < 1.32$, $P_s = > .27$).

4.3.3.4 Stability analysis

The between genotype difference emerged slowly and only after a significant amount of training had occurred. To further examine this, a stability analysis was performed to establish whether differences arose due to diminishing AUCs in KO mice and also to establish whether discounting performance was comparatively stable in WT mice. To do this, AUC stability was assessed within and between genotypes by comparing AUCs from sessions 30-34 with those from sessions 40-44 by performing a genotype (WT vs KO) by session (30-34 vs 40-44) two-way ANOVA. There was a significant main effect of session ($F_{(1, 14)} = 34.72$, $p = .001$) and also a significant genotype by session interaction ($F_{(1, 14)} = 9.83$, $p = .007$), suggesting that the AUCs of one group were changing more significantly between sessions than the other. Post hoc paired t-tests revealed that mean AUCs produced by KO animals during sessions 40-44 (Fig. 4.4B) were significantly smaller than their mean AUCs during sessions 30-34 ($t_{(7)} = 5.90$, $p = .001$) (Fig. 4.2B). In contrast, there was only a trend toward significantly different AUCs in WT subjects between sessions 30-34 and 40-44 ($t_{(7)} = 2.14$, $p = .07$) which suggests that, in comparison to WT mice, KO mice were developing an increasingly risk sensitive pattern of responses between sessions 30-34 and 40-44. Thus KO mice adjusted their LU choices in an experience-dependent fashion more dramatically than WT mice between these sessions.

4.3.3.5 Sensory specific devaluation

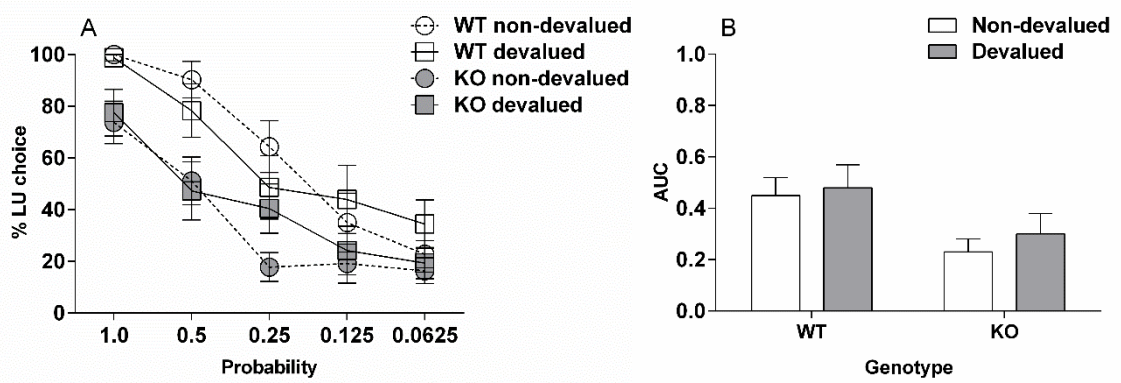


Fig. 4.5 Non-devalued vs devalued % LU choices for WT & KO mice (A) and non-devalued vs devalued AUCs for WT & KO (B) mice.

Sucrose pre-feeding had no clear effects on discounting ($F_{(1, 14)} = .27$, $p = .62$) (Fig. 4.5A). There were, however, significant main effects of probability ($F_{(2.38, 33.38)} = 58.54$, $p = .001$) and genotype ($F_{(1, 14)} = 7.92$, $p = .01$), with KO mice continuing to record a smaller proportion of LU choices than WT mice. None of the interaction terms were significant ($F_s = < 2.46$, $P_s = > .06$). Sucrose pre-feeding similarly did not affect AUCs (main effect of motivational state ($F_{(1, 14)} = .90$, $p = .36$)); genotype by motivational state interaction ($F_{(1, 14)} = .23$, $p = .64$) (Fig 4.5B). The main effect of genotype approached significance with KO mice tending to produce smaller AUCs than WT mice ($F_{(1, 14)} = 4.33$, $p = .060$). By contrast, mice made significantly more magazine omissions during the pre-fed session compared to the food-deprived session ($F_{(1, 14)} = 12.66$, $p = .003$). However, there were no differences between genotypes and the genotype by motivational state interaction was also non-significant ($F_s = < .81$, $P_s = > .38$). In contrast, lever omissions did not differ in any way between motivational states, genotypes or either of these variables in interaction ($F_s = < 2.92$, $P_s = > .11$) (Tabs 4.1 & 4.2). Trial initiation latencies were slower during the devalued session in comparison to the non-devalued session ($F_{(1, 14)} = 15.17$, $p = .002$). Whilst there was no significant main effect of genotype ($F_{(1, 14)} = .07$, $p = .80$), there was a significant motivational state by probability interaction

($F_{(4, 56)} = 4.10, p = .006$) and a significant motivational state by probability by genotype interaction ($F_{(4, 56)} = 3.28, p = .02$). This latter interaction term was followed up with independent samples t-tests to compare performance between genotypes at each probability block separately for each motivational state. None of these tests were significant ($P_s = > .20$). There were no other significant effects for trial initiation latencies ($F_s = < .94, P_s = > .45$). Choice latencies were not significantly affected in any way by pre-feeding and nor were there any significant differences between genotypes in choice latencies either as main effects or interactions ($F_s = < 2.53, P_s = > .13$). There were no significant main effects of motivational state, lever or probability when inspecting forced choice latencies ($F_s = < 2.77, P_s = > .12$). There was, however, a significant main effect of genotype ($F_{(1, 14)} = 4.78, p = .05$) with KO mice continuing to execute slower forced choices than WT mice. The lever by genotype interaction was also significant ($F_{(1, 14)} = 4.46, p = .05$) but the post hoc tests were not significant when controlling for multiple comparisons ($P_s = > .04, \text{sig} = .025$). Whilst there was also a significant lever by probability interaction ($F_{(4, 56)} = 7.78, p = .001$), there were no other significant interaction effects ($F_s = < 2.08, P_s = > .10$).

4.3.3.6 General motivational devaluation

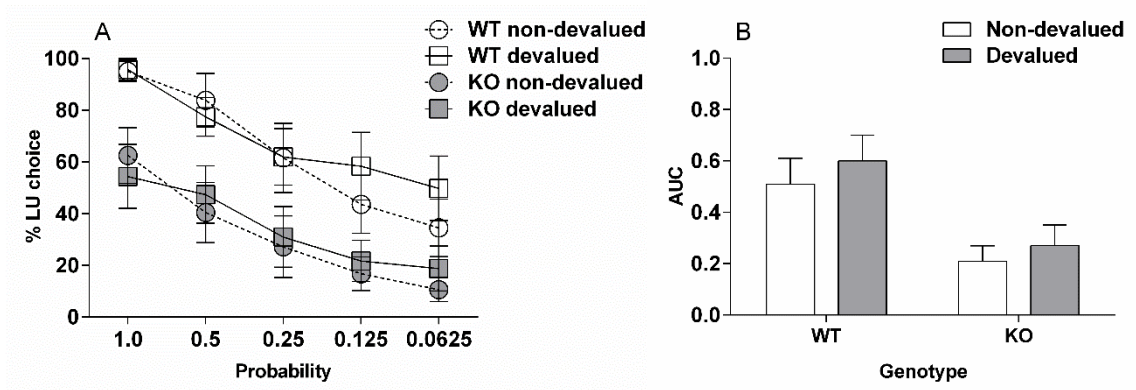


Fig. 4.6 Non-devalued vs devalued % LU choices for WT & KO mice (A) and non-devalued vs devalued AUCs for WT & KO (B) mice.

Chow pre-feeding similarly had no clear effect on % LU choices ($F_{(1, 14)} = 1.35$, $p = .27$) (Fig. 4.6A). There were, however, significant main effects of probability ($F_{(2.20, 30.86)} = 27.35$, $p = .001$) and genotype ($F_{(1, 14)} = 9.72$, $p = .008$), with KO mice continuing to record a smaller percentage of LU choices than WT mice. There were no other significant interaction effects ($F_s = < .94$, $P_s = > .45$). Pre-feeding mice with their maintenance diet similarly did not affect AUCs (main effect of motivational state ($F_{(1, 14)} = 2.88$, $p = .11$); genotype by motivational state interaction ($F_{(1, 14)} = .23$, $p = .64$) (Fig. 4.6B). KO mice did, however, continue to produce smaller AUCs than their WT counterparts ($F_{(1, 14)} = 7.65$, $p = .02$). Pre-feeding mice again significantly increased the number of magazine ($F_{(1, 14)} = 28.47$, $p = .001$), as well as the number of lever omissions ($F_{(1, 14)} = 5.60$, $p = .03$). However, neither of these measures differed between genotypes, either as main effects or interactions ($F_s = < 2.42$, $p = > .14$) (Tabs 4.1 & 4.2). Magazine latencies were significantly slower during the devalued session ($F_{(1, 14)} = 16.73$, $p = .001$). However, there were no other significant main or interaction effects ($F_s = < 2.09$, $P_s = > .09$). Choice latencies were not affected by pre-feeding and there were no significant differences between genotypes either as main or interaction effects ($F_s = < 2.80$, $P_s = > .12$). Aside from a significant lever by probability interaction ($F_{(4, 56)} = 3.21$, $p = .02$), all main effects

and interaction terms relating to forced choice trials during the general motivational devaluation were not significant ($F_s = < 2.72$, $P_s = > .12$).

4.4 Discussion

By the completion of the experiment, mice lacking the DARPP-32 gene appeared to be less tolerant of uncertainty than WT mice. For example, the significant interaction recorded during sessions 40-44 indicated that DARPP-32 KO mice were executing sub-optimal choices during the $p = 0.5$ block, selecting the LU lever on significantly fewer occasions than WT mice, and also selecting the LU lever on significantly fewer occasions than WT mice during the $p = 0.25$ and $p = 0.125$ blocks, when to do so was the more optimal strategy. It is interesting to note that these 3 blocks are the blocks where LU reinforcement is the most uncertain. The likelihood of LU reinforcement is a certainty in the $p = 1$ block, whereas the likelihood of not being reinforced following an LU choice in the $p = 0.0625$ is close to certain. Overall, DARPP-32 KO mice executed a choice strategy that appeared to involve selecting the LU lever significantly less than WT mice did during these sessions.

Nonetheless, significant differences in baseline responding, (see figs. 4.3 & 4.4, and particularly figs. 4.5 & 4.6) when KO mice biased away from the LU lever even when the larger reward was certain, may hint at an issue with reward magnitude discrimination (St Onge & Floresco, 2009). Indeed, such explanations may well account for findings from studies examining the effects of acute drug treatment on probabilistic choices in previously well-discriminating subjects over comparatively few sessions. Thus, the effects of the drug are said to disrupt the subject's ability to discriminate the large reward from the small reward. However, it is more difficult to imagine such an effect in the case of a constitutive genetic manipulation like the one reported here, as subjects in both the

experimental and control group had achieved comparable discrimination of the large reinforcer at baseline early in the experiment but, as the experiment progressed, experimental group subjects gradually began to select the LU lever less at baseline. Nevertheless, it is possible reinforcer magnitude discrimination might have partly contributed to the observations reported in this chapter. Indeed, inactivation of the NAcSh has been shown to minimally but significantly impair reinforcer magnitude discrimination (Stopper & Floresco, 2011).

One possible explanation for the between groups discounting performance is that KO mice might differ from WT mice in their ability to overcome the impact of non-rewarded choices in previous sessions; in a sense, a reward-uncertainty carryover effect. For example, it is possible that KO mice might cumulatively degrade the subjective value of the LU reward following consistent experience with non-reward in low probability blocks which is registered and incorporated into the subjective value of the LU lever and manifests as a continuing adjustment of reward choices. Consistent with this interpretation, the results of the additional stability analysis indicate that there was a greater degree of instability in KO LU choices which diminished more consistently over sessions than WT LU choices.

A similar interpretation of the role of D1 receptor activation was offered by Floresco and colleagues as being responsible for “keeping the eye on the prize” in the face of non-rewarded choices to ensure that subjects maximise long-term gains when faced with reinforcement uncertainty by limiting the impact of negative-feedback (St Onge et al., 2011). For instance, SCH 23390-induced antagonism of either mPFC (St Onge et al., 2011) or accumbal (Stopper et al., 2013) D₁ receptors reduces risky decisions by increasing the likelihood that subjects shift their subsequent choice to the SC lever after a non-rewarded LU choice. Taken together with the present findings then, it is possible

that in addition to maintaining optimal LU choices within sessions by mitigating the impact of non-rewarded choices, accumbal D₁ receptors via DARPP-32 may also mitigate the impact of non-rewarded choices in previous sessions to maintain between-session response stability in low risk blocks in subsequent sessions by 'returning the eye to the prize'.

To some extent, these findings are consistent with our predictions and marry with those by Floresco and colleagues who have consistently reported that dampened DA transmission through D₁ receptors increases probability discounting (Stopper et al., 2013; St Onge et al., 2011; St Onge & Floresco, 2009). They are also consistent with work in humans showing that an allelic variant of DARPP-32 predicted improved performance on a probabilistic choice task (Frank et al., 2007). Targeted nucleus accumbens injections of SCH 23390 have been shown to induce risk aversion (Stopper et al., 2013) and accumbal lesions (Cardinal & Howes, 2005) or temporary inactivation of the NAcSh also induced a risk-averse pattern of choices (Stopper & Floresco, 2011). On the other hand, Cardinal & Howes (2005) reported risk aversion following lesions of the NAcC in a probability discounting task. However, significant portions of the NAcSh were also damaged which makes interpretation of their findings difficult. A recent study showed that temporary inactivation of the NAcSh but not NAcC also impaired performance on a probabilistic reversal task indicating that NAcSh function is also critical for the flexible adjustment of behaviour during instrumental tasks constrained by probabilistic contingencies (Dalton et al., 2014). Taken together, these studies indicate that the absence of DARPP-32 in the NAcSh is the most plausible explanation for the differential performance between genotypes. Despite this proposal, however, DARPP-32 is also distributed in PFC regions associated with the mediation of probabilistic choices, albeit relatively sparsely, so one cannot exclude the possibility that the absence of DARPP-32

in PFC regions shown to influence probabilistic reinforcement choices, such as the OFC and mPFC, did not contribute to our findings.

In conjunction with these reports and those outlined in the introduction to this chapter, it was hypothesised that DA transmission, presumably in the nucleus accumbens shell, stimulates the D₁-PKA-DARPP-32 pathway to flexibly alter behaviour during probabilistic tasks. The present experiment found some support for this hypothesis, though the results may suggest that the mechanism(s) is not straight forward. For example, differential discounting between WT & KO subjects was late onset, but when it did occur it persisted until the completion of the experiment. Cardinal & Howes (2005) initially reported no significant differences between lesioned and sham lesioned subjects in the first 3 discounting sessions post-surgery. However, lesioned subjects became significantly more risk averse than sham lesioned subjects between 10 & 12 sessions post-surgery. Given that one can reasonably expect a greater and possibly more rapid effect on choice behaviour following the introduction of a lesion compared to a subtler manipulation like deletion of an intracellular signalling molecule, it is perhaps not surprising that it took approximately 20 sessions of training following the introduction of more ‘punitive’ probabilities for differences in probabilistic choices to emerge. Indeed, some other studies have subjected rodents to approximately 25 sessions before achieving stable responses (St Onge & Floresco, 2009) suggesting that rodents often require a significant number of sessions to assimilate the probabilistic contingencies and to efficiently organise their behavioural output under conditions of instrumental uncertainty.

In addition to the significant differences in LU choices, KO mice also executed significantly slower choice and forced choice latencies than their WT littermates. These slower forced choice latencies were apparent prior to the emergence of between genotypes discounting. However, the emergence of significantly slower choice latencies

coincided with the significantly different discounting. In general, these data suggest that DARPP-32 deletion increases choice latencies during probability discounting. This is in contrast to intertemporal discounting, where no between genotype differences in either choice or forced choice latencies were apparent. It is unlikely that these effects can be accounted for by discrepancies in motivational state between genotypes because trial initiation latencies, and magazine and lever omissions were similar between genotypes throughout these sessions.

The results from the reinforcer devaluation sessions suggested that pre-feeding with either sucrose or chow had no effect on the overall pattern of discounting and that these manipulations did not produce distinct differences between genotypes in either the number of omissions recorded or choice of the larger but uncertain reward option. However, the results from the general motivational devaluation were complicated by the high level of omissions during the non-devalued session of the chow pre-feeding devaluation. Whilst the mean number of magazine omissions during the food-deprived session from the sucrose devaluation were relatively low for both WT and KO subjects, mean omissions during the food-deprived (non-devalued) session of the chow pre-feeding manipulation were high for both KO and WT mice (Tabs. 4.1 & 4.2). On this basis, and also on the basis that prior research has shown that pre-feeding can increase probability discounting (St Onge & Floresco, 2009), it is difficult to completely exclude the possibility that between-group differences in the sensitivity to motivational shifts were responsible for the low baseline preference for the LU lever in KO mice during the general motivation devaluation. For example, because the mean number of magazine omissions were high during the non-devalued session, it is possible that mice were already in a relatively dampened state of motivation during the non-devalued session. With this in mind, it is difficult to exclude the possibility that motivational floor effects might have

prevented the manipulation from identifying between-group differences in the sensitivity to general motivational devaluation. Thus it is possible that LU choices were actually already devalued in the non-devalued session and, whilst pre-feeding increased magazine omissions, it might have been ineffective at devaluing % LU choices owing to a floor effect.

Despite this interpretational difficulty concerning the chow pre-feeding devaluation, it is unlikely that a shift in motivational state can explain the between-group differences that emerged between sessions 35-39 because magazine and lever omissions were relatively low in both genotypes and nor were there any significant differences between genotypes in omissions (Tabs. 4.1 & 4.2). Moreover, magazine and lever omissions remained low in both KO and WT throughout most of the experiment, including the sucrose devaluation manipulation. Therefore, it is unlikely that any potential differences in the sensitivity to motivational shifts contributed to the between genotype discounting that emerged during sessions 35-39.

Finally, even though task parameters were altered before the commencement of session 15 and, even though DARPP-32 KO mice appear to be impaired at integrating changes in task parameters - as reported in the previous chapter - there is reason to believe that changes in task parameters were not the cause of the findings in this study. In the previous chapter, DARPP-32 KO mice performance was different to WT animals following changes in the task parameters but, with experience, performance was comparable between genotypes. In this experiment, however, DARPP-32 KO mice increasingly diverged in their % LU choices from their WT littermates. This is more indicative of a persistent deficit in probabilistic reinforcement choices as opposed to a transient deficit in integrating changes in the task.

In summary, the findings presented in this chapter provide some evidence which suggests that DARPP-32 is a mediator of probabilistic reinforcement choices. Under ongoing conditions of reward uncertainty, it appears that DARPP-32 is involved in the flexible integration of probabilistic information which allows subjects to direct probabilistic reinforcement choices efficiently. In the absence of DARPP-32, KO subjects were impaired in their ability to sustain optimal reinforcement choices in high probability blocks but significantly better at executing optimal choices in low probability blocks. It is possible that DARPP-32 contributes to overcoming negative feedback in uncertain situations, thus allowing subjects to flexibly alter their choices in the face of changing reinforcement costs.

Chapter 5

Progressive ratio**5.1 Introduction**

In the previous chapters, the relationship between DARPP-32 deletion and intertemporal and probabilistic choices was examined. Although pre-feeding had no clear effects on choice patterns during both of these experiments, and on the basis that mice with targeted interference of the Ser⁹⁷-DARPP-32 phosphorylation residue display impairments in progressive ratio responding (Stipanovich et al., 2008), it was deemed important to establish whether DARPP-32 KO mice similarly display motivational impairments during progressive ratio testing. It was felt that doing so would provide additional insight into whether any behavioural disturbances present in these mice might arise from motivational disturbances or from a learning deficit. Or, indeed, a combination of these factors. Finally, intertemporal and probabilistic choice paradigms assess investment costs along dimensions of time and risk respectively so, by assessing progressive ratio, it provided a platform to identify whether global deletion of DARPP-32 impacted the willingness to invest effort.

DA has a well elucidated role in the provision of instrumental motivation. For example, responding on a food-reinforced FR-5 schedule, but not free-feeding, is associated with increases in extracellular accumbal DA in a pattern that is related to the magnitude of responding (Salamone, Cousins, McCullough, Carriero & Berkowitz, 1994). Interference with accumbal DA transmission by 6-OHDA depletion significantly reduces responding during high but not low-effort instrumental schedules (Aberman & Salamone, 1999; Salamone, Wisniecki, Carlson & Correa, 2001). In addition to this, D₁ & D₂ receptor antagonism reduces the breaking point of responding for food rewards in progressively escalating schedules of reinforcement (Aberman et al., 1998; Barbano, Le

Saux & Cador, 2009) and intra-NAcSh injections of amphetamine significantly enhances the number of active lever responses during a food reinforced PR-2 schedule (Zhang et al., 2003). Furthermore, the DA transporter (DAT) inhibitor MRZ-9547 was recently shown to significantly increase instrumental responding during both a PR task and a PR/chow concurrent choice task in which subjects can opt to exert effort for a highly palatable food reward reinforced under a progressive ratio schedule or to consume freely available but less valued chow (Sommer et al., 2014). Therefore, whilst DA antagonism or depletion reduce the motivation to expend effort during high-effort food-reinforced tasks, increasing DA transmission conversely increases motivation in such tasks.

The postsynaptic effects of DA during progressive ratio responding most likely induce changes in the phosphorylation of DARPP-32. For example, significantly higher levels of Thr³⁴-DARPP-32 labelling in NAcC MSNs were reported in the most vigorously responding subjects during the concurrent choice PR/chow task described briefly above (Randall et al., 2012). DA, however, is not the only effector of DARPP-32 that contributes to the allocation of effort during instrumental tasks. The adenosine and glutamate systems make important contributions to the allocation of instrumental motivation. For example, discrete injections of the adenosine A_{2A} agonist CGS 21680 into the NAc significantly reduces FR-5 responding and increases chow consumption in the concurrent choice procedure described earlier (Font et al., 2008). Systemic administration of MSX-3, an adenosine A_{2A} receptor antagonist, blocks the effort-dampening effects of haloperidol in a T-maze effort-based choice task (Mott et al., 2009) and A_{2A} receptor null mice are less sensitive to the effects of haloperidol in the same procedure (Pardo et al., 2012). The mGluR₅ receptor antagonist MPEP reduces the breaking point of responding in instrumental procedures reinforced with either cocaine, nicotine or food (Paterson & Markou, 2005). As discussed previously, both adenosine A_{2A} and mGluR₅ receptors

contribute to the phosphorylation of DARPP-32 in co-dependent fashion. Whilst additional work is required to directly establish whether mGluR₅ & A_{2A} receptors contribute to the exertion of effort by influencing the phosphorylation of DARPP-32, these findings do suggest that DA, adenosine and glutamate, all of which influence DARPP-32 phosphorylation, significantly impact the motivation to expend effort.

Because Thr³⁴-DARPP-32 phosphorylation increases in response to the magnitude of effort subjects produce and, as mentioned briefly above, because mice with targeted interference of the Ser⁹⁷-DARPP-32 phosphorylation residue were impaired during a single session test of progressive ratio responding (Stipanovich et al., 2008), this experiment had 2 main purposes. Firstly, the previously reported disturbance in progressive ratio responding in Ser⁹⁷-DARPP-32 mutant mice suggests that DARPP-32 KO mice might also possess motivational disturbances. Therefore, the first aim of this study was to establish whether DARPP-32 KO mice similarly display impairments in instrumental motivation. However, whilst PR schedules of reinforcement are routinely referred to as measures of instrumental motivation, it is important to establish the sensitivity of the procedure to measure motivation by directly manipulating motivational state (e.g. pre-feeding devaluation). As well as providing a mechanism to establish the sensitivity of the procedure to measure instrumental motivation, it was hoped that reinforcer devaluation sessions might also provide insight into whether DARPP-32 KO mice are more sensitive than WT mice to direct manipulations of motivational state. In accordance with this aim, mice were exposed to 3 reinforcer devaluation manipulations at various times throughout testing. The second aim of this study relates to the Stipanovich et al (2008) study which adopted a single session of progressive ratio testing but, because of this, it is not possible to interpret the precise nature of the deficits they reported (i.e. whether interference with DARPP-32 induces a transient, acquisitive deficit

or a persistent impairment in instrumental performance during tasks that directly measure incentive motivation). For example, mice with a non-phosphorylatable knockin Thr³⁴-DARPP-32 alanine residue were impaired in the acquisition of instrumental responding for cocaine, however, once these mice acquired stable performance, they eventually responded significantly more vigorously than controls (Zhang et al., 2006).

Mice were subjected to an extended testing protocol which examined a number of progressive ratio schedules of reinforcement to establish whether any potential impairments or enhancements in food-reinforced instrumental responding varied as a function of the schedule (e.g. high effort vs low effort requirements). Mice were also provided with training on different instrumental devices associated with unique response components (levers and nose pokes) in separate phases of the experiment. This was done to establish whether responses associated with different effort requirements might uncover between genotype differences in PR responding. Previous research has revealed different breaking points of responding as a function of the effort required to produce a response (e.g. lever height) (Skjoldager, Pierre & Mittleman, 1993). Additionally, Clemens, Caillé & Cador (2010) found that nose pokes supported higher levels of FR-5 instrumental responding than levers. Session length was also adjusted at various times throughout the experiment to establish whether short vs long sessions would uncover differences in instrumental motivation between genotypes.

5.2 Methods

5.2.1 Subjects

Eight DARPP-32 KO mice aged 15 to 35 weeks (mean age = 24 weeks; mean weight = 28.95 g) and 8 WT counterparts aged 13 to 38 weeks (mean age = 25 weeks; mean weight = 27.19 g) were used for this study. Each group was composed of 5 males and 3

females. Genotyping was performed prior to study commencement using the procedure outlined in chapter 2. Subjects were at least the 6th generation of backcrossed mice bred at the University of Sussex from a C57BL/6J background in the way previously described. Mice were food restricted to 85% of their free-feeding weight for the duration of the experiment (except when explicitly noted). Mice were granted *ad libitum* access to tap water in their home cages and housed under the same conditions as those listed previously. Each mouse was handled for 5 min during 3 consecutive once a day sessions to reduce handling stress at the time of training and testing.

5.2.2 Apparatus

All testing was conducted in the conditioning chambers described in chapter 3. For the first phase of the experiment, ultra-sensitive levers were used as the instrumental manipulanda and, for the second phase of the experiment, these were replaced with nosepokes.

5.2.3 Procedure

5.2.3.1 Magazine training

Mice were first provided with 3 sessions of magazine training which were identical to those listed previously except mice were given boluses of 17.7 µl of 10% sucrose.

5.2.3.2 Phase one: PR responding for sucrose

5.2.3.2.1 Continuous reinforcement

Mice were trained for 4 sessions under a continuous reinforcement (FR-1) schedule to encourage reliable and similar responding in both genotype groups. Sessions ended after 60 min had passed or when mice achieved 50 reinforcements. One KO mouse failed to

show appreciable lever responding (i.e. it failed to achieve in excess of 10 responses) and was omitted from the experiment. Genotypes were counterbalanced with respect to the position of the ‘reinforced’ active lever or nosepoke port (left vs right) for all portions of the experiment.

5.2.3.2.2 Fixed ratio training

Mice were next provided with 7 sessions of fixed ratio-5 (FR-5) training. Sessions were terminated after 60 min or before if mice acquired 50 reinforcements. FR-5 training continued until all subjects in each group had acquired at least 20 reinforcements for 3 consecutive sessions.

5.2.3.2.3 Progressive ratio training

The number of responses required to achieve reinforcement was progressively increased after each preceding reinforcement event throughout these sessions. Mice were first tested under a schedule calculated by Richardson and Roberts (1996) using the following formula ($5e^{(\text{reinforcement number} * j)} - 5$) where $j = 0.2$. Response requirements for this schedule were as follows: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328. Mice received a total of 16 sessions of testing under this schedule. The first 12 sessions were terminated after 240 min or before that if mice failed to complete a schedule within 30 min. The final 4 sessions of testing conducted at this schedule were 60 min in length and had no timeout.

In order to examine the sensitivity of the PR schedule to changes in motivation, and potential interactions with genotype, mice undertook a ‘general motivational’ reinforcer devaluation manipulation during the final 6 sessions of the $j = 0.2$ PR sessions. These reinforcer devaluation manipulations were near identical to those previously described with the exception that mice were granted *ad libitum* chow access for an extended period

(minimum of 16 hr). That is, for the first 3 sessions, half of the mice, counterbalanced with respect to genotype and active lever position, underwent PR training whilst being granted *ad libitum* access to their maintenance chow diet in their home cages, beginning the evening before their first devaluation session. The other half of the mice remained food-deprived whilst conducting PR training during these 3 sessions. After these 3 sessions, the mice that were food-deprived were granted *ad libitum* home cage access to chow and were then exposed to 3 sessions of PR training whilst pre-fed. The other half of the mice that were previously fed *ad libitum* conducted the final 3 of these 6 sessions in a state of food deprivation. A period of 5 days was allocated to return those mice granted *ad libitum* access during the first 3 sessions to a state of food deprivation before the final 3 sessions of the devaluation manipulation were conducted.

After again returning mice to a state of food deprivation, a single 60 min session of testing was conducted with all mice food restricted under this same PR schedule and that included a stringent 10 min timeout (TO) period to examine whether including a relatively short timeout period would differentially affect responding between genotypes. A final 4 sessions of testing under this same schedule were also 60 min in duration but contained no timeout period. Mice were finally given 4 sessions of testing during which the response requirements were doubled (PR x 2) after each reinforcement (e.g. 1, 2, 4, 8, 16, etc.). These sessions were terminated after 240 min or when mice failed to complete a schedule within 30 min. The breaking point was designated as the last completed schedule for all schedules used in both phases of the experiment.

5.2.3.3 Phase two: PR responding for milk

To establish whether using manipulanda associated with unique effort requirements would uncover differences between genotypes in progressive ratio performance mice were trained to make nosepoke responses to receive a 10% condensed milk solution.

5.2.3.3.1 Continuous reinforcement

Mice were again first trained under continuous reinforcement (FR-1) for 3 sessions using novel instrumental manipulanda (nosepokes) and a novel reinforcer (10% condensed milk). These sessions were identical to FR-1 lever training sessions except levers were replaced with nosepokes.

5.2.3.3.2 Fixed ratio training

Mice were then provided with FR-5 training using the same parameters as those listed in the FR-5 lever training protocol.

5.2.3.3.3 Progressive ratio training

Mice were first tested for 6 sessions using the Richardson and Roberts (1996) schedule described before (i.e. $j = 0.2$). These sessions were 60 min long and contained no timeout period. Mice were then tested for 6 sessions on a variant of the abovementioned schedule; this schedule was also first calculated by Richardson and Roberts (1996) and used the following equation ($5e^{(\text{reinforcement number} * j)} - 5$), where $j = 0.12$. This calculation generated the following schedule of reinforcement (1, 1, 2, 3, 4, 5, 7, 8, 10, 12, 14, 16, 19, 22, 25, 29, 33, 38, 44, 50, 57, 65, 74, 84, 95, 108, 123, 139, 157, 178, 201, 228). Four of these sessions were reinforcer devaluation sessions (2 *ad libitum*, 2 food-deprived) which were implemented in the same way described previously. Sessions were 60 min long and

contained no timeout. Two additional sessions were conducted at this schedule which were 120 min long and contained a 30 min timeout. Finally, mice were tested for 8 sessions on a shallow PR-1 schedule of reinforcement which increased the response requirements by 1 response after each reinforcement. PR-1 sessions were 90 min long and contained a 30 min TO period. The final 4 sessions were reinforcer devaluation manipulation sessions (i.e. 2 *ad libitum*, 2 food-deprived) which were implemented in the way previously listed.

5.2.4 Data analysis

In order to confirm that lever performance was equivalent between genotypes during the final FR-5 training session, independent t-tests were conducted to compare performance for each lever/nosepoke separately.

Progressive ratio performance was compared by conducting genotype (WT vs KO) by session repeated measures ANOVA separately for breaking point, active responses, and inactive responses. Because in all instances, analyses of active lever/nosepoke responses and breaking point provided identical information, and because inactive lever/nosepoke responses did not yield any significant effects, the results of the statistical analyses of active and inactive lever/nosepoke responses are not presented and are graphically presented only.

The effects of pre-feeding were analysed by conducting genotype (WT vs KO) by session (e.g. test day) by motivational state (food-deprived vs pre-fed) mixed model ANOVAs separately for each variable of interest.

5.3 Results

5.3.1 Phase one

5.3.1.1 Instrumental training

Neither responses on the active or inactive lever were significantly different between genotypes during the final session of FR-5 lever training (active lever ($t_{(8.42)} = .85$, $p = .42$); inactive lever ($t_{(13)} = .93$, $p = .37$)).

5.3.1.2 $j = 0.2$ schedule (240 min sessions 30 min TO)

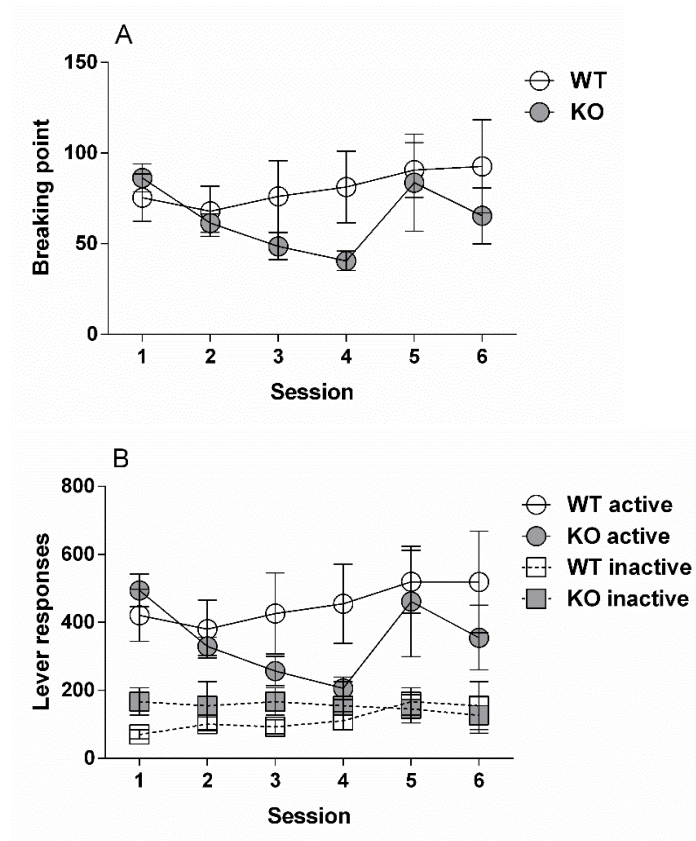


Fig. 5.1 Mean WT vs KO breaking points (A) and mean WT vs KO active & inactive lever responses (B).

Breaking points were not significantly different across sessions or between genotypes, either as main effects or interactions, when mice were provided with 6 sessions of 240 min ($j = 0.2$, 30 min TO) progressive ratio testing ($F_s = < 1.92$, $P_s = > .15$) (Fig. 5.1A).

5.3.1.3 $j = 0.2$ schedule (240 min sessions 30 min TO) pre-feeding manipulation

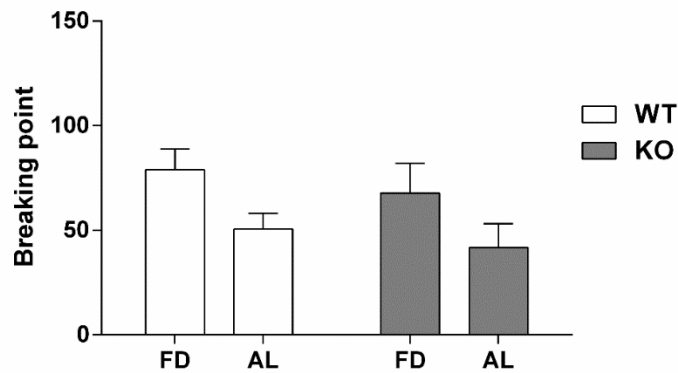


Fig. 5.2 Collapsed mean WT vs KO breaking points during the 3 food-deprived (FD) & 3 *ad libitum* (AL) feeding sessions.

In comparison to 3 sessions of 240 min ($j = 0.2$, 30 min TO) progressive ratio testing conducted when food-deprived, 3 days *ad libitum* access to maintenance chow significantly reduced the breaking point of responding during the corresponding sessions ($F_{(1, 12)} = 25.17$, $p = .001$) (Fig. 5.2). However, breaking points did not significantly differ between genotypes or sessions and nor were there any significant interaction effects ($F_s = < 2.01$, $P_s = > .16$).

5.3.1.4 PR x 2 (240 min sessions 30 min TO)

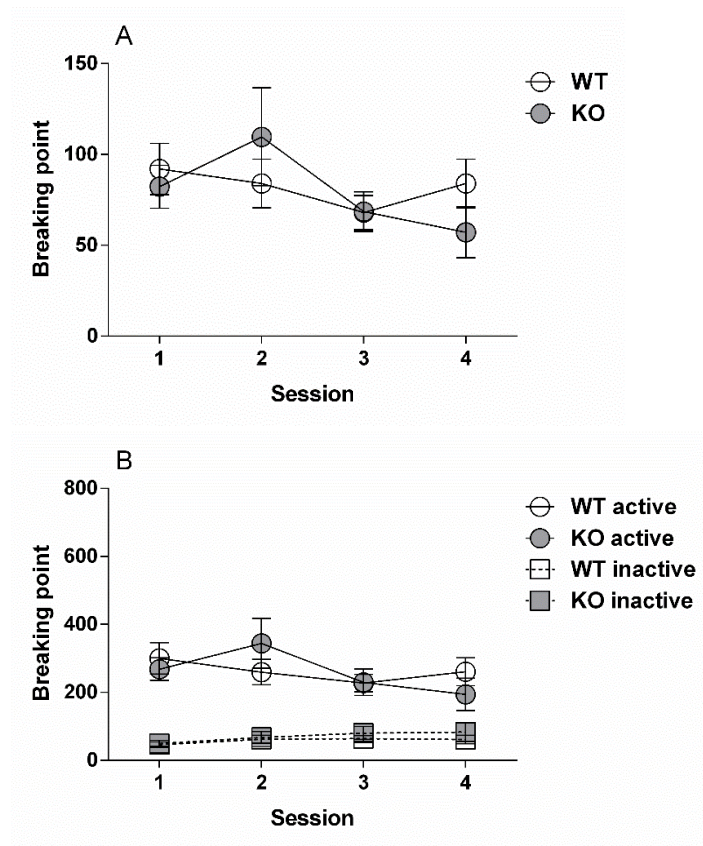


Fig. 5.3 Mean WT vs KO breaking points (A) and mean WT vs KO active & inactive lever responses (B).

Mean breaking points recorded during 4 sessions of 240 min (PR x 2, 30 min TO) progressive ratio testing were not significantly different between genotypes when collapsing across session ($F_{(1, 13)} = .02$, $p = .89$) (Fig. 5.3A). There was, however, a significant genotype by session interaction ($F_{(3, 39)} = 2.86$, $p = .05$). Despite this significant interaction, post hoc independent t-tests comparing breaking points between genotypes for each session separately revealed no significant differences during any of these sessions ($P_s = > .19$). There was also a significant main effect of session ($F_{(3, 39)} = 4.4$, $p = .01$). However, none of the Bonferroni post hoc tests were significant ($P_s = > .11$).

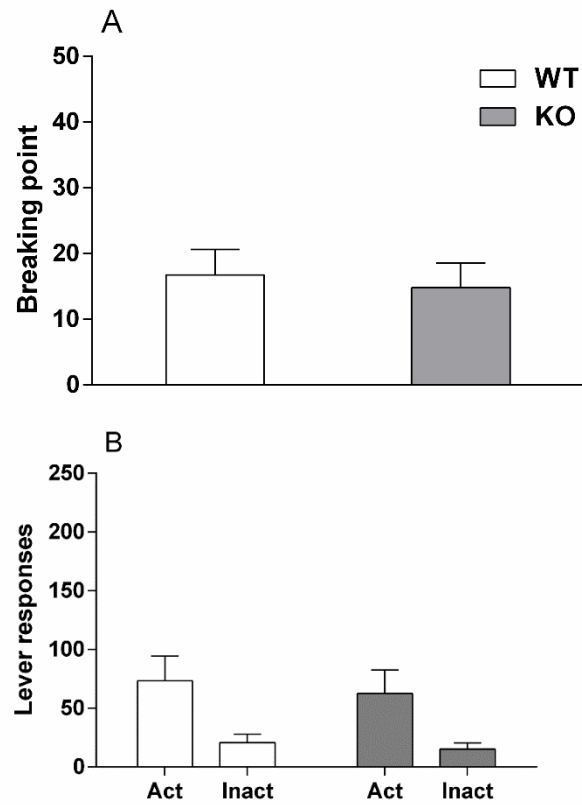
5.3.1.5 $j = 0.2$ schedule (60 min sessions 10 min TO)

Fig. 5.4 Mean WT vs KO breaking points (A) and mean WT vs KO active & inactive lever responses (B).

Exposing mice to a comparatively brief 10 min timeout did not differentially alter the breaking point of responding between genotypes during a single 60 min ($j = 0.2$) session of progressive ratio testing ($t_{(12)} = -.35$, $p = .74$) (Fig. 5.4A).

5.3.1.6 $j = 0.2$ schedule (60 min sessions no TO)

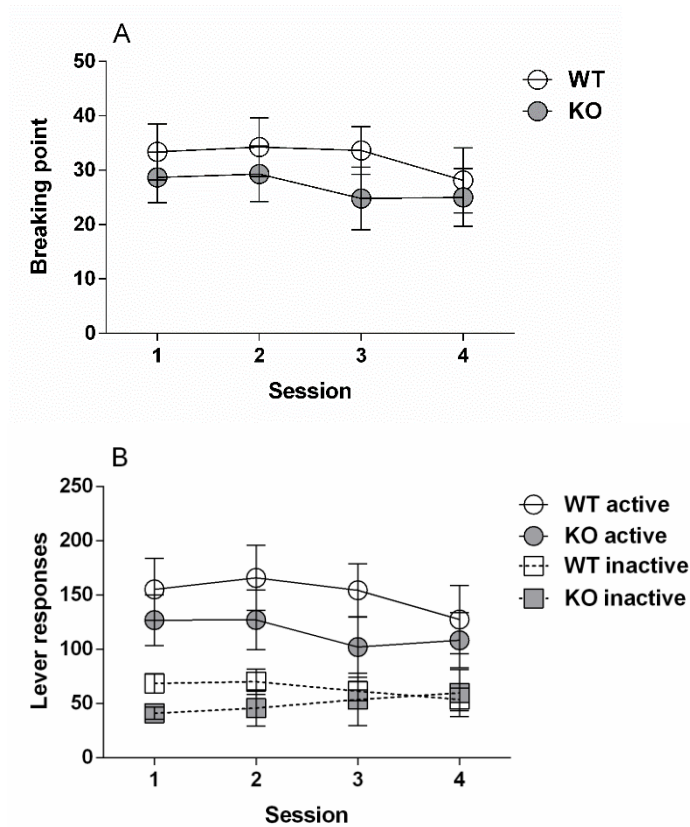


Fig. 5.5 Mean WT vs KO breaking points (A) and mean WT vs KO active & inactive lever responses (B).

Breaking points were not significantly different in any way across sessions or between genotypes when mice were provided with 4 sessions of 60 min ($j = 0.2$, no TO) progressive ratio testing ($F_s = < 1.12$, $P_s = > .36$) (Fig. 5.5A).

5.3.2 Phase 2: Nosepokes

5.3.2.1 Instrumental training

The mean number of active or inactive nosepoke responses were not significantly different between genotypes during the final stage of FR-5 training (active nosepokes ($t_{(9.30)} = .25$, $p = .81$); inactive nosepokes ($t_{(10.76)} = 1.56$, $p = .15$).

5.3.2.2 $j = 0.2$ schedule (60 min sessions no TO)

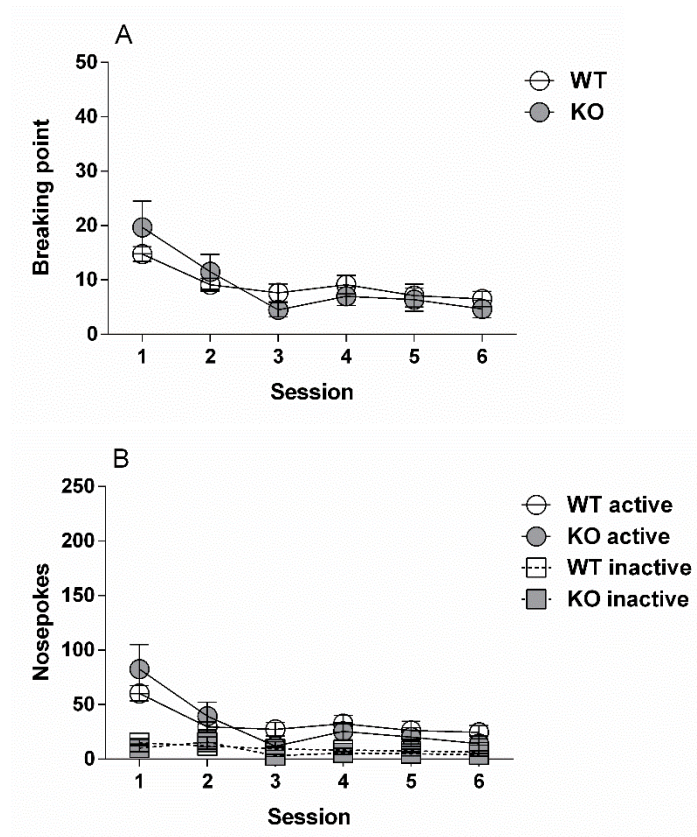


Fig. 5.6 Mean WT vs KO breaking points (A) and mean WT vs KO active & inactive nosepoke responses (B).

Breaking points were not significantly different between genotypes either as a main effect of genotype or as an interaction between session and genotype when mice were provided with 6 sessions of 60 min ($j = 0.2$, 60 min TO) progressive ratio testing ($F_s = < 1.28$, $P_s = > .3$) (Fig. 5.6A). There was, however, a significant main effect of session ($F_{(2.97, 35.67)} = 9.96$, $p = .001$) with mice recording significantly higher breaking points

during session 1 than during all other sessions at these parameters (Bonferroni post hoc tests, $P_s = < .05$).

5.3.2.3 $j = 0.12$ schedule (60 min sessions no TO) pre-feeding manipulation

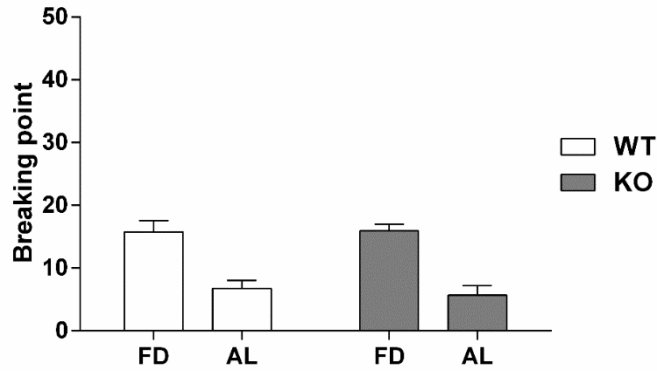


Fig. 5.7 Mean WT vs KO breaking points during the 2 food-deprived (FD) & 2 *ad libitum* (AL) feeding sessions.

The breaking points recorded when mice were provided with 2 sessions of progressive ratio ($j = 0.12$, 60 min TO) testing in a pre-fed (*ad libitum* chow access) state were significantly lower than breaking points recorded during the corresponding 2 sessions conducted in a food-deprived state ($F_{(1, 12)} = 49.68$, $p = .001$) (Fig. 5.7). There were, however, no significant main effects of session or genotype nor any significant interaction terms ($F_s = < 1.71$, $P_s = > .22$).

5.3.2.4 $j = 0.12$ (120 min sessions 30 min TO)

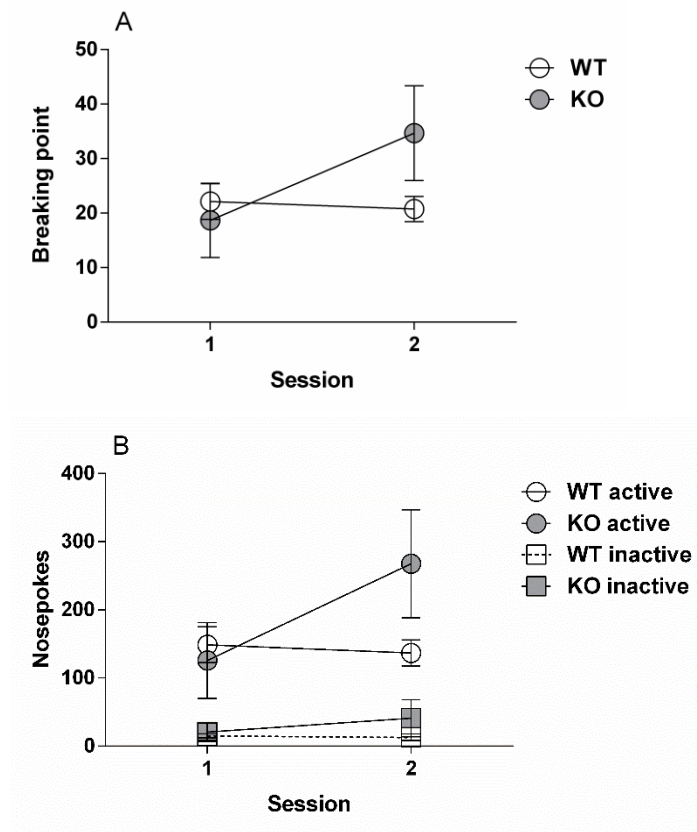


Fig. 5.8 Mean WT vs KO breaking points (A) and mean WT vs KO active & inactive nosepoke responses (B).

Breaking points were not significantly different between genotypes during 2 sessions of 120 min ($j = 0.12$, 30 min TO) progressive ratio testing when collapsing across session ($F_{(1, 12)} = .57$, $p = .46$) (Fig 5.8A). There was, however, a significant session by genotype interaction ($F_{(1, 12)} = 9.99$, $p = .01$) but post hoc independent t-tests comparing breaking points between genotypes for each session revealed no significant differences ($P_s = > .10$). There was also a main effect of session ($F_{(1, 12)} = 7.08$, $p = .02$) with Bonferroni post hoc tests revealing that breaking points were significantly higher during session 2 ($p = .02$).

5.3.2.5 PR-1 (90 min sessions no TO)

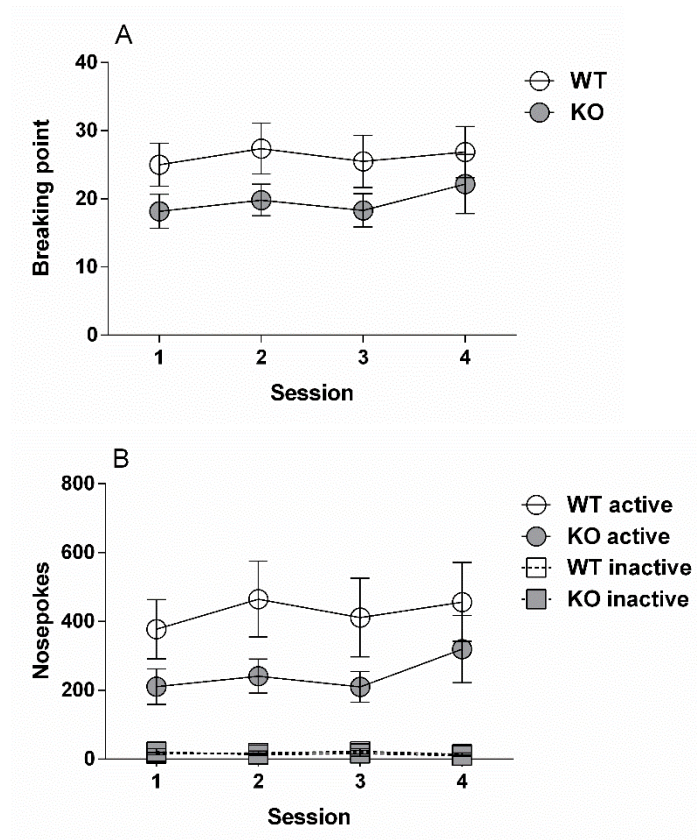


Fig. 5.9 Mean WT vs KO breaking points (A) and mean WT vs KO active & inactive nosepoke responses (B).

Breaking points recorded during 4 sessions of 90 min (PR-1, no TO) progressive ratio testing constrained by a shallow schedule were not significantly different between genotypes either as a main effect or as an interaction between session and genotype ($F_s = < 1.90$, $P_s > .19$) (Fig. 5.9A). The main effect of session was significant but the Bonferroni post hoc tests did not approach significance ($F_{(3, 36)} = 3.19$, $p = .04$) (Bonferroni post hoc $p = > .31$).

5.3.2.6 PR-1 (90 min sessions no TO) pre-feeding manipulation

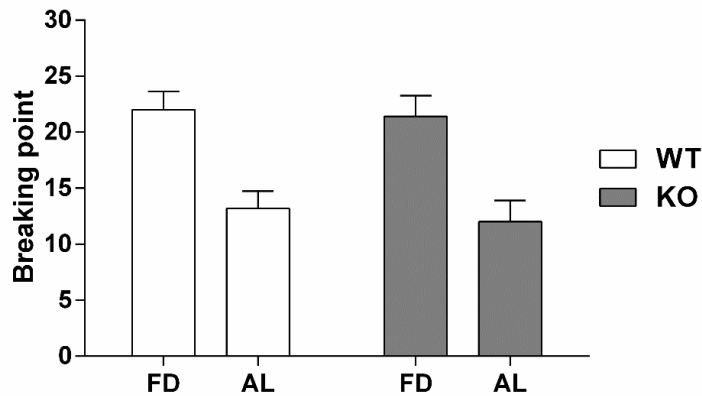


Fig. 5.10 Collapsed mean WT vs KO breaking points for the 2 food-deprived (FD) & 2 *ad libitum* (AL) feeding sessions.

Breaking points recorded when mice were in a pre-fed state (*ad libitum chow access*) during 2 sessions of 90 min (PR-1, no TO) testing were significantly lower than breaking points recorded during the corresponding food-deprived sessions ($F_{(1, 12)} = 129.37$, $p = .001$) (Fig. 5.10). There were, however, no significant main effects of genotype and session and there were no significant interaction terms ($F_s = < 1.20$, $P_s = > .29$).

5.4 Discussion

The performance of mice without the DARPP-32 gene during a food reinforced progressive ratio task was indistinguishable from DARPP-32 WT mice. Both the breaking point of responding and active response measures were indistinguishable between WT and KO mice during all of the schedules that were employed. Nor were there any performance differences as a function of the response format (i.e. levers or nose pokes). These findings contrast with those reported by Stipanovich et al (2008) who identified significantly reduced nose poke responding during a single session of food-reinforced progressive ratio testing in mice with a targeted mutation to the Ser⁹⁷-DARPP-32 phosphorylation residue. At the outset, we can conclude then that global deletion of the DARPP-32 gene can produce differential effects to interference with selected phosphorylation residues of this protein.

As noted, DARPP-32 regulates the integration of at least 3 neurotransmitter systems implicated in the motivation to press for food reward reinforced under demanding schedules. Activation of the DA system enhances motivation to press for food (Zhang et al., 2003), whereas activation of the adenosine system dampens the motivation to exert effort to receive food reward (Font et al., 2008). Moreover, inhibition of the glutamate system similarly reduces the motivation to press for reward, suggesting that the glutamate system promotes incentive motivation (Paterson & Markou, 2005). Because DARPP-32 moderates the integration of these transmitter systems within brain regions that facilitate incentive motivation, it is possible that selective interference with one phosphorylation residue upsets the equilibrium that exists between these neurotransmitter systems and promotes behavioural disturbances to a greater extent than globally deleting the entire DARPP-32 protein does.

Two major aims of this experiment were to establish the sensitivity of the procedure to measure instrumental motivation by implementing reinforcer devaluation manipulations, and in doing so, to identify whether there were any between-group differences in the sensitivity to direct manipulations of motivational state. However, whilst all reinforcer devaluation manipulations were effective at significantly reducing the breaking point of responding, there were no significant main effects of genotype or any significant genotype by motivational state interactions. It is therefore unlikely that the lack of genotype effects were due to a relative insensitivity of the PR procedures to measure changes in motivation since both genotypes showed significant and equivalent reductions in breaking points and active responses following *ad libitum* chow access during all of the reinforcer devaluation manipulations.

Furthermore, and perhaps more importantly, the findings from the reinforcer devaluation manipulations presented in this chapter, in conjunction with those presented

in earlier chapters, indicate that DARPP-32 KO mice do not possess significant impairments in instrumental motivation. These manipulations ranged from comparatively brief (e.g. 2 hours pre-feeding) in previous chapters, up to 3 days *ab libitum access* to maintenance chow in the current experiment. Said simply, these manipulations were effective at reducing motivation but not at delineating a role of DARPP-32 in motivated performance.

It is also unlikely that the different observations reported here and those reported by Stipanovich and colleagues (2008) can be explained by the difference in the schedules recruited by the respective studies. Subjects were exposed to a variety of schedules in this study, 1 of which (PR x 2) was more demanding than the schedule employed by Stipanovich et al (2008) whilst others were similar in their requirements, and other schedules less arduous (PR-1).

Instrumental performance was also indistinguishable between genotypes across 2 distinct manipulanda each associated with different responses. Although it was not always possible to directly compare nosepoke performance of mice with lever performance, it was possible to compare performance across devices for some sessions at least. The final 4 sessions of PR lever training and the first 6 sessions of PR nosepoke training were conducted under identical task parameters (i.e. $j = 0.2$, 60 min duration, no timeout). Visual inspection of the figures from these sessions (Figs. 5.5B & 5.6B) shows that mice made markedly fewer active and inactive nosepokes than the corresponding lever responses which suggests that mice found nosepoking more arduous than lever pressing. These results contrast with a previous report which identified increased responding in nosepoke versus levers (Clemens et al., 2010).

One simple explanation for the different levels of responding between levers and nosepokes concerns the different reinforcers that were associated with either response

device. For example, sucrose might preferentially sustain higher levels of instrumental responding than condensed milk. What makes this less likely is testing conducted in our laboratory has found that 10% condensed milk typically sustains higher levels of lever responding in C57BL/6J mice than 10% sucrose when these animals have been exposed to both the sucrose and condensed milk reinforcers during instrumental training. This does not account for other differences, of course, including that the custom developed levers in our lab have a significantly greater surface area than levers typically used in commercial designs.

In summary, and in contrast to selective interference with the Ser⁹⁷-DARPP-32 gene, deletion of the DARPP-32 gene has no significant effects on the willingness of mice to work for food reward. These data also suggest that the DARPP-32 KO mouse does not possess impaired motivation for food reward. Given that evidence in this thesis has identified that DARPP-32 KO mice perform differently to WT mice during food reinforced intertemporal and probabilistic choice tasks, these data additionally suggest that dampened motivation for food reward does not alternatively explain such differences.

Pavlovian-to-instrumental transfer

6.1 Introduction

Data presented in the previous chapters identified differential performance between DARPP-32 KO mice and their WT littermates in 2 distinguishable forms of instrumental choice but no such role in the motivation to work for food rewards. These 3 assays measured the extent to which incentive value decays as a function of time, risk and effort independently. The purpose of the current chapter, however, was to establish whether the previously identified absence of incentive salience in DARPP-32 KO mice (Crombag et al., 2008), as determined by general PIT, could be rescued.

Neurobiologically, PIT is underpinned by a circuit comprising of the OFC (Ostlund & Balleine, 2007), the nucleus accumbens (Corbit & Balleine, 2011; Hall, Parkinson, Connor, Dickinson & Everitt, 2001), the amygdala (Corbit & Balleine, 2005; Hall et al., 2001) and the VTA (Corbit et al., 2007). The anatomical subdivisions where PIT effects are mediated in the nucleus accumbens and the amygdala depends entirely on the precise experimental arrangement of conditioned cues, instrumental devices and USs/reinforcers. For instance, different amygdala and accumbal subregions are recruited when a solitary reward predictive stimulus (CS1) is superimposed over an instrumental device that delivered a single reward (R1) than compared to when multiple reward associated cues (e.g. CS1, CS2) that predict unique USs (US1, US2) are superimposed over multiple instrumental devices (e.g. R1, R2) that delivered these reward outcomes independently (O1, O2). The former of these 2 arrangements is referred to as general PIT; CS1 enhances R1 but this cue-potentiated effect does not require the subject to discriminate the reinforcer on the basis of its sensory properties. It is a purely motivation driven

phenomenon. The latter of these 2 arrangements is referred to as selective outcome PIT because CS1 selectively enhances responding on R1 whereas CS2 enhances responding on R2. Unlike the general form of PIT, this selective arrangement requires subjects to discriminate CSs and responses on the basis of the sensory features of the rewards that are encoded within the memorial representations of the stimuli and devices that predicted these rewards. Lesions to either the NAcC (Corbit & Balleine, 2011; Hall et al., 2001) or CeA (Corbit & Balleine, 2005; Hall et al., 2001) abolish the general form of PIT but leave the selective form intact, whereas lesions to either the NAcSh (Corbit & Balleine, 2011) or BLA (Corbit & Balleine, 2005) abolish the selective form of PIT but leave the general form of PIT intact. In contrast to the nucleus accumbens and the amygdala, reversible VTA inactivation does not distinguish between the selective and general forms of PIT. Both kinds of PIT are abolished following discrete reversible VTA inactivation with a baclofen/muscimol cocktail, which is consistent with a major DAergic influence over the attribution of this form of incentive salience (Corbit et al., 2007).

DA and glutamate transmission converge in DARPP-32 expressing regions known to mediate PIT effects. Microinjections of amphetamine into the nucleus accumbens core or shell enhance cue-potentiated instrumental responding (Peciña, & Berridge, 2013; Wyvell & Berridge, 2000) whilst systemic administration of DA antagonists attenuate PIT (Dickinson, Smith & Mirenowicz, 2000). Direct NAcC or NAcSh injections of either the D₁ receptor antagonist SCH 23390 or the D₂ receptor antagonist raclopride abolish the PIT effect, although this effect is most pronounced in response to SCH 23390 (Lex & Hauber, 2008).

Much of what is known about the glutamatergic influence on PIT has been derived from molecular studies targeting post-synaptic glutamate substrates. For instance, whilst global deletion of the AMPA GluR₁ subunit did not disrupt general PIT (Mead &

Stephens, 2003), Crombag and colleagues identified a necessary role for this AMPA subunit in the general PIT assay by isolating the precise phosphorylation residues of the AMPA GluR₁ receptor subunit that are required for the performance of general PIT (Crombag, Sutton, Takamiya, Holland, et al., 2008). Simultaneous genetic interference of the Ser⁸⁴⁵ and Ser⁸³¹ phosphorylation residues of the AMPA GluR₁ subunit, but not single mutations of either of these residues, abolishes the PIT effect. In the absence of the entire AMPA GluR₁ subunit, compensatory mechanisms appear to rescue the instrumental enhancing effects of Pavlovian stimuli whereas interference with selected components of this receptor subunit critically disturbs the ability of conditioned associations to potentiate instrumental responding.

Whilst simultaneous interference with the Ser⁸³¹ and Ser⁸⁴⁵ GluR₁ phosphorylation residues was required to abolish PIT, interference with components associated with only 1 of those pathways similarly suppresses the PIT effect. For example, Ser⁸³¹ GluR₁ phosphorylation is stimulated by calcium and calmodulin dependent protein kinase (CaMKII) and striatal interference with this protein abolishes PIT (Wiltgen, Law, Ostlund, Mayford & Balleine, 2007). Disturbing selected components in the Ser⁸⁴⁵ GluR₁ pathway also abolishes PIT. Crombag & collaborators (2008) have previously identified an absence of PIT in DARPP-32 KO mice. In light of this disturbance in DARPP-32 KO mice, the purpose of the present study was to establish whether acute treatment with methylphenidate prior to the PIT test could rescue this behaviour. Interestingly, certain behaviours which are disturbed in the DARPP-32 KO mouse, such as acute locomotor activity to cocaine and novel object recognition, have been rescued by increasing DA transmission with cocaine (Fienberg et al., 1998) and methylphenidate respectively (Heyser et al., 2013). On the basis of previous work by Crombag and colleagues, it is predicted that DARPP-32 KO mice will display indistinguishable instrumental

performance during variable interval instrumental training for a food reward, normal conditioned approach behaviour but crucially, will exhibit selective impairments in the ability of conditioned stimuli to potentiate instrumental responding when administered saline 20 min prior to test. It is not yet known whether the absence of PIT in DARPP-32 KO mice reflects a disturbance in the acquisition or expression of incentive salience. Should the acute administration of methylphenidate prior to the PIT test rescue PIT in DARPP-32 KO mice, one can reasonably speculate that the impairment reflects a deficit in the expression of incentive salience rather than a disturbance in the acquisition of incentive salience. Should methylphenidate have no effect, however, it would not be reasonable to suggest that this impairment reflects a disturbance in the acquisition of incentive salience since this effect could represent a general failure of the drug to influence responding.

6.2 Methods

6.2.1 Subjects

Twelve DARPP-32 KO mice aged 7 to 18 weeks old, consisting of 9 males and 3 females, (mean weight 22.3 g) and 12 WT littermates aged 6 to 20 weeks (mean weight 27.3 g), consisting of 7 females and 5 males, were used for this study. Genotyping was conducted prior to study commencement using the method described earlier. Mice were at least the 8th generation of backcrossed mice bred from a C57BL/6J background at the University of Sussex in the manner previously described. Mice were granted *ad libitum* access to tap water in their home cages but were food restricted to 90% of their free-feeding weight for the duration of the experiment. Subjects were housed in the same conditions as those previously reported. To reduce handling stress, each mouse was handled for 5 min per day on 3 consecutive occasions prior to study commencement.

6.2.2 Drug

Methylphenidate hydrochloride (Sigma Aldrich, Dorset, UK) (MPH), at 2.5mg/kg or 5 mg/kg doses was dissolved in 0.9% saline (SAL) and administered prior to PIT testing. SAL was administered as a control. See below for procedural details.

6.2.3 Apparatus

All testing was conducted in the conditioning chambers described previously. A sonalert tone (4500 Hz, Med Associates, Georgia, VT, USA) approximately 8 dB above background and a solenoid clicker approximately 6 dB above background were both located on the ceiling of the sound-attenuating cabinet and these functioned as conditioned stimuli. Two ultra-sensitive levers were the instrumental devices for the instrumental training and PIT test sessions. A 10% liquid sucrose solution served as the reinforcer in all phases of the experiment.

6.2.4 Procedure

6.2.4.1 Magazine training

Mice were provided with a single session of magazine training using the exact procedure described in the progressive ratio chapter.

6.2.4.2 Pavlovian training

Mice were provided with a total of 14 sessions of Pavlovian conditioning. For half of the mice, the CS+ was the sonalert tone and the CS- was the clicker and, for the remaining half of the mice, the CS+ was the clicker and the CS- was the tone. For the first 3 sessions, the CS+ and CS- were each presented on 5 occasions, with each stimulus presentation lasting 120 s. For all remaining sessions, both stimuli were presented on 4 occasions per

session. CS+ presentations were reinforced with 13.3 μ l of sucrose dispensed on an RI-30 schedule. ITI length was 180 s on average but never less than 120 s or more than 240 s. Conditioned stimuli were counterbalanced with regards to genotype and lever position. Two consecutive sessions of Pavlovian training, 1 session per day, were followed by 2 consecutive sessions of instrumental training, 1 session of training per day. This pattern continued for the duration of the experiment.

6.2.4.3 Instrumental training

For all instrumental sessions, 1 lever, counterbalanced with respect to position, functioned as the active lever so that presses delivered 13.3 μ l of sucrose on a schedule dependent basis. The other lever was designated as the inactive lever and its activation had no programmed consequences. Mice were first given 2 60 min sessions, 1 session per day, of instrumental training reinforced on a continuous (FR-1) reinforcement schedule. All subsequent sessions were 30 min long and the schedule of reinforcement was a progressively increasing variable interval (VI) schedule such that the interval between reinforcements was extended across sessions. Mice received 2 sessions of VI-15 training, 1 session of VI-20 training, 2 sessions of VI-30 training, 1 session of VI-45 training and 4 sessions of VI-60 training prior to the first PIT test.

6.2.4.4 Pavlovian-to-instrumental transfer test

Each mouse was provided with 3 PIT tests under extinction conditions on separate days. Mice were given 1 session of instrumental training (VI-60) and 1 Pavlovian training session between each PIT test to mitigate the impact of repeated testing under extinction conditions. Mice were administered intraperitoneally either SAL, 2.5 mg/kg MPH or 5 mg/kg MPH dissolved in SAL 20 min before each test. This time was selected on the basis of previous findings describing the time course of MPH (Gerasimov et al., 2000),

as suggested by Heyser et al (2013), and on the basis that MPH rescued novel object recognition in DARPP-32 KO mice (Heyser et al., 2013) when administered at this time point. Drug was administered according to a Latin square design. PIT test sessions began with the illumination of the houselight and the presentation of both levers. The first 2 min of each test were designated as an extinction period during which responses were recorded but were not included in the statistical analysis. After this extinction period came a 120 s ITI period. This period was followed by the presentation of the first conditioned stimulus which was presented for 120 s. The selection of the first stimulus (tone vs click) occurred pseudorandomly but all subsequent stimulus presentations occurred on an alternating stimulus basis (e.g. tone ITI click or click ITI tone). The number of lever responses that occurred during the presentation of the CS+, the CS- and the ITI periods were recorded to establish the effects of conditioned reward associations on instrumental responding. Magazine entries and time spent in the magazine during each period (ITI, CS+ & CS-) were also recorded.

6.2.5 Data analysis

Instrumental performance during training was compared between genotypes by conducting a genotype (WT vs KO) by session (14 levels) repeated measures ANOVA of lever response rates for each lever separately.

Pavlovian training performance was compared between genotypes by conducting a genotype (WT vs KO) by session (14 levels) repeated measures ANOVA of a magazine entry discrimination index (i.e. % of the total magazine entries that were attributable to the CS+ period). The magazine entry discrimination index was calculated by dividing the number of entries which occurred during the CS+ period by the total number of entries. This was then multiplied by 100 to provide the percentage of the total number of

magazine entries which occurred during the CS+ period (i.e. $\text{CS+ entries} / (\text{CS+ entries} + \text{CS- entries} + \text{ITI entries}) * 100$).

Pavlovian training performance was similarly compared between genotypes by conducting a genotype (WT vs KO) by session (14 levels) repeated measures ANOVA of a magazine time discrimination index (i.e. % of the total magazine time that was attributable to the CS+ period). This discrimination index was calculated in the same way as described above, except magazine time was used in all components of the equation rather than magazine entries.

Performance during each PIT test was compared by calculating change scores for each lever in response to each stimulus presentation (i.e. responses during stimulus period - responses during ITI period) and then by comparing these change scores between genotypes by conducting genotype (WT vs KO) by stimulus (CS+ vs CS-) by dose (SAL vs 2.5 mg/kg MPH vs 5 mg/kg MPH) mixed model ANOVAs for each lever separately.

Magazine entries during the PIT tests were compared between genotypes by conducting a genotype (WT vs KO) by stimulus (CS+ vs CS-) by dose (SAL vs 2.5 mg/kg vs 5 mg/kg) mixed model ANOVA.

6.3 Results

6.3.1 Instrumental training

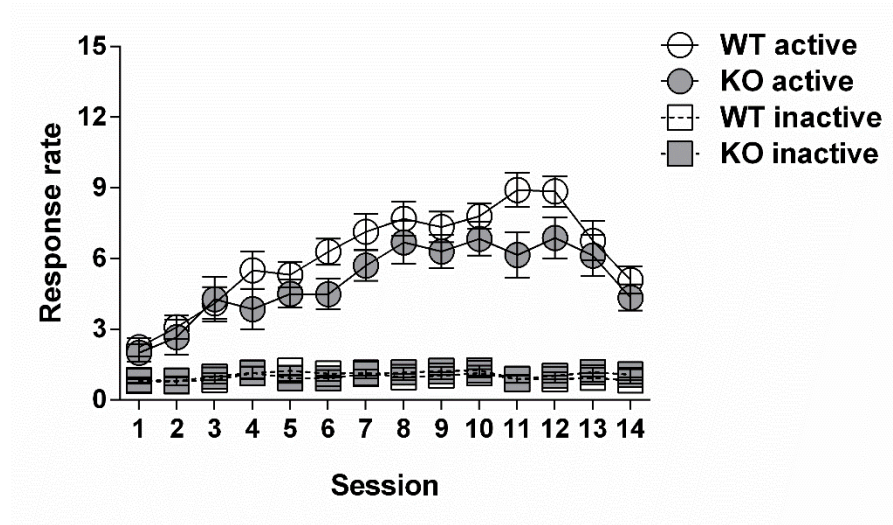


Fig. 6.1 Active vs inactive lever response rates for WT vs KO mice during training.

Active lever responses were significantly different across sessions ($F_{(4.69, 103.12)} = 29.28$, $p = .001$) but there were no significant differences between genotypes and no significant interaction between genotype and session ($F_s = < 1.98$, $P_s = > .17$) (Fig. 6.1). Inactive lever responses did not differ significantly across sessions or between genotypes ($F_s = < 1.94$, $P_s = > .08$) (Fig 6.1). Thus, deletion of DARPP-32 does not impact the ability of mice to acquire robust instrumental responding under increasingly sparse variable interval schedules of reinforcement.

6.3.2 Pavlovian training

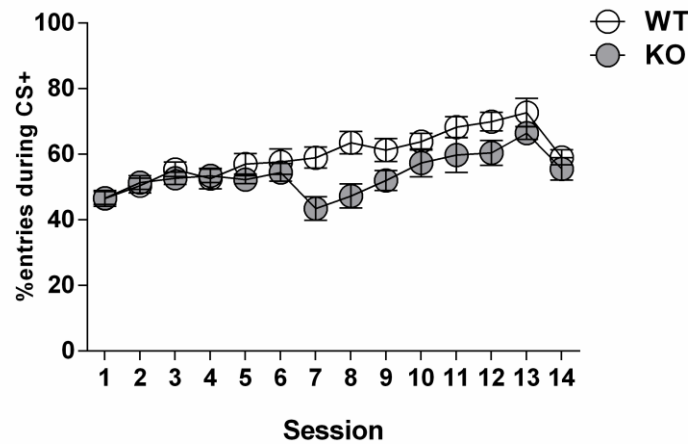


Fig. 6.2 % total magazine entries occurring during the CS+ for each Pavlovian training session.

% entries during CS+: there was a significant main effect of session with mice making a greater proportion of entries during the CS+ as training progressed ($F_{(13, 286)} = 12.62$, $p = .001$) (Fig. 6.2). There was unexpectedly a significant main effect of genotype with WT mice making a significantly greater proportion of total entries during the CS+ than KO mice ($F_{(1, 22)} = 4.81$, $p = .04$). There was also a significant session by genotype interaction ($F_{(13, 286)} = 2.50$, $p = .003$) which was followed up with independent t-tests to compare Pavlovian performance between genotypes for each session. These tests revealed that WT mice made a significantly greater proportion of entries during the CS+ than KO mice during sessions 7 ($t_{(22)} = 3.24$, $p = .004$) and 8 ($t_{(22)} = 3.25$, $p = .004$) but not during session 9 ($p = .03$) and session 12 ($p = .06$) when adjusting the significance value for multiple comparisons. The significance value was constrained to $p = .013$ by dividing the ordinary $p = .05$ significance value by only those sessions reasonably expected to uncover a difference (i.e. 4 sessions (7, 8, 9 & 12)), rather than all 14 training sessions, to control for type 2 error. In addition to this, Pavlovian performance was compared across the final 3 sessions of training by conducting a genotype by session repeated measures ANOVA. Whilst this test revealed a significant main effect of session ($F_{(2, 44)} = 14.06$, $p = .001$), there was no significant main effect of genotype ($F_{(1, 22)} = 3.12$, $p = .09$) and no significant

genotype by session interaction ($F_{(2, 44)} = .81, p = .45$). Therefore, whilst there were 2 sessions during which Pavlovian performance was different between genotypes, this difference was a transient occurrence and, crucially, there were no significant differences between genotypes in the sessions immediately prior to the PIT tests.

% magazine time during CS+: The percentage of total time spent in the magazine that occurred during CS+ presentations increased as training progressed ($F_{(13, 286)} = 26.16, p = .001$). However, whilst there was no significant main effect of genotype ($F_{(1, 22)} = .73, p = .40$), there was a significant session by genotype interaction ($F_{(13, 286)} = 2.04, p = .02$). Despite this significant interaction, none of the post hoc independent t-tests comparing this measure of Pavlovian performance between genotypes separately for each session were significant ($P_s = > .07$) suggesting that deletion of DARPP-32 had no impact on this measure of associative learning.

6.3.3 Pavlovian-to-instrumental transfer test

6.3.3.1 Active lever change scores

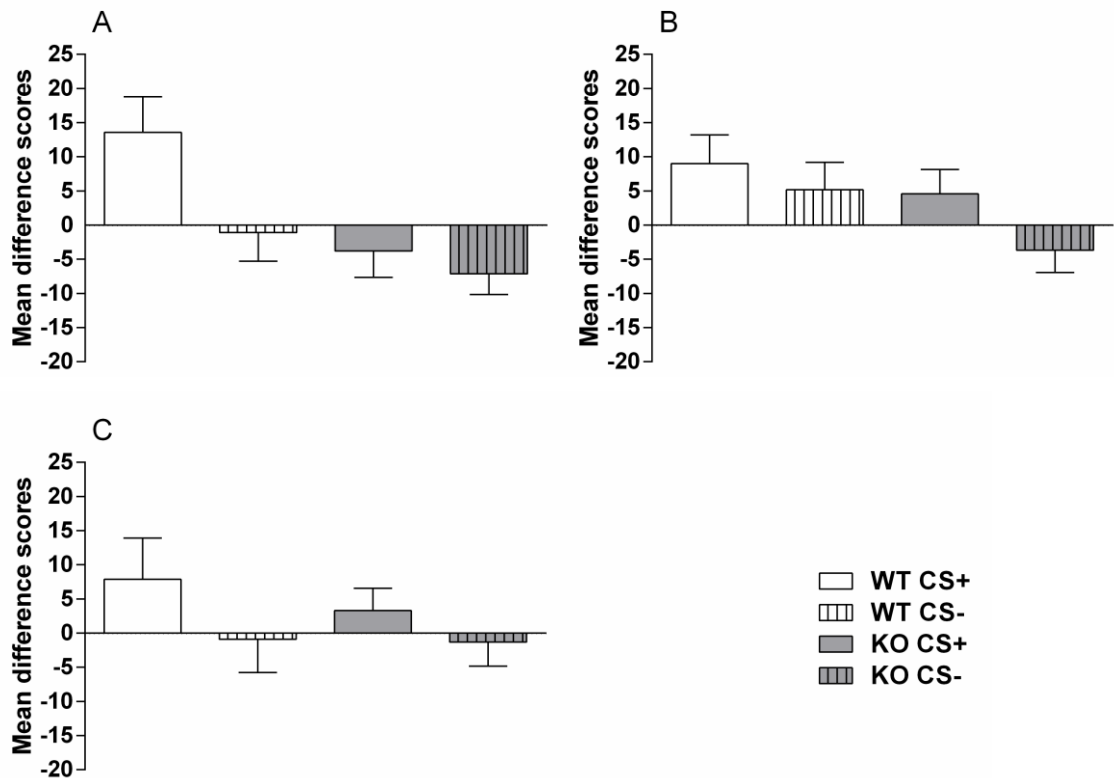


Fig. 6.3 Active lever CS+ vs CS- change scores during SAL (A), 2.5 mg/kg MPH (B) & 5 mg/kg MPH (C) PIT tests.

CS+ presentations increased active lever responding significantly more than CS- presentations did ($F_{(1, 22)} = 7.47$, $p = .01$) (Figs. 6.3A, 6.3B & 6.3C). There was also a significant main effect of genotype with WT mice producing significantly higher change scores than KO mice ($F_{(1, 22)} = 5.46$, $p = .03$). However, the stimulus by genotype interaction was not significant (stimulus by genotype interaction ($F_{(1, 22)} = .49$, $p = .49$). MPH produced no significant main effects nor any significant interactions ($F_s = < 1.37$, $P_s = > .27$). This was an unexpected result because it implies that both genotypes are able to perform PIT. However, inspection of figure 6.3A clearly shows that KO mice showed no PIT effect during the SAL condition and there was no real evidence of a PIT effect in the 2.5 mg/kg (Fig. 6.3B) or 5 mg/kg (Fig. 6.3C) conditions whereas WT mice showed a

clear PIT effect during the SAL condition (Fig. 6.3A), a less robust effect during the 5 mg/kg condition (Fig. 6.3C) but no real effect during the 2.5 mg/kg condition (Fig. 6.3B).

This was explored further by conducting a stimulus by dose repeated measures ANOVA separately for each genotype. These results revealed a significant main effect of stimulus for WT mice ($F_{(1, 11)} = 4.92$, $p = .05$) with CS+ presentations elevating active lever responses significantly higher than CS- presentations. There was no such main effect of stimulus in KO mice ($F_{(1, 11)} = 2.58$, $p = .14$) which suggests that WT but not KO mice were sensitive to the incentive salience properties of conditioned stimuli. No effects of MPH were uncovered in either WT or KO mice ($F_s = < 2.41$, $P_s = > .11$).

6.3.3.2 Inactive lever change scores

There was a significant main effect of stimulus with CS- presentations increasing inactive lever responses more than CS+ presentations ($F_{(1, 22)} = 13.77$, $p = .001$). There were, however, no other significant effects of conditioned stimulus presentation on inactive lever responding ($F_s = < 1.33$, $P_s = > .27$).

6.3.3.3 Magazine entries

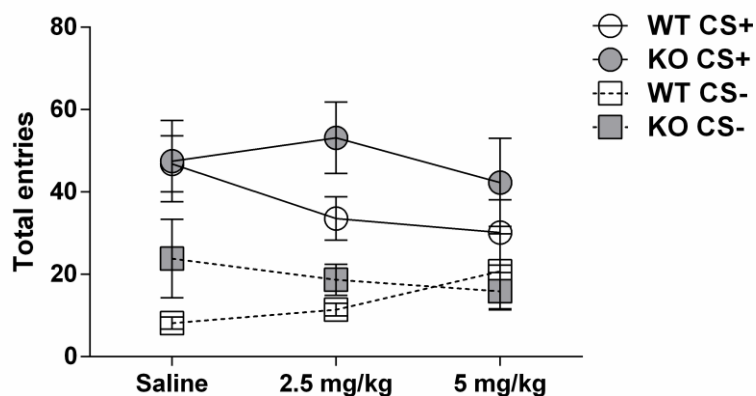


Fig. 6.4 WT vs KO magazine entries during CS+ & CS- periods for all PIT tests.

Mice made significantly more magazine entries during CS+ presentations than CS- presentations ($F_{(1, 22)} = 46.34$, $p = .001$) (Fig. 6.4) but there were no significant main

effects of genotype or dose ($F_s = < 1.59$, $P_s = > .22$). There was a significant stimulus by dose by genotype interaction ($F_{(2, 44)} = 4.79$, $p = .01$) and a significant stimulus by dose interaction ($F_{(2, 44)} = 3.12$, $p = .05$). The stimulus by dose by genotype interaction was followed up with separate genotype by dose two-way ANOVAs to inspect the effects of dose on CS entries for each stimulus separately but neither of these tests produced significant main effects or interactions (CS+ genotype by dose ANOVA ($F_s = < 1.49$, $P_s = > .24$); CS- genotype by dose ANOVA ($F_s = < 1.78$, $P_s = > .18$)). Because the main effect of dose was not significant for either of these post hoc tests, the stimulus by dose interaction was not explored any further. No other significant interaction effects were found for magazine entries ($F_s = < .43$, $P_s = > .53$).

6.4 Discussion

There was a modest PIT effect and a significant main effect of genotype which was unfortunately smaller than anticipated. Whilst the results from the main statistical analysis suggested there was no difference between genotypes in the magnitude of change in instrumental responding between the CS+ and CS-, further statistical exploration by looking at test performance separately in each genotype established PIT effects in WT but not KO mice. Thus, in conjunction with the significant main effect of genotype, it appears that KO mice were impaired in incentive salience attribution which rendered them insensitive to the instrumental enhancing effects of reward-paired Pavlovian stimuli. These results are broadly in keeping with both one's predictions and previous observations recorded by Crombag and coworkers who identified an absence of PIT in DARPP-32 KO mice (2008). These results are perhaps also commensurate with results obtained using mice with a double mutation to the Ser⁸³¹ and Ser⁸⁴⁵ residues of the AMPA GluR₁ subunit which display a complete absence of PIT (Crombag, Sutton, Takamiya,

Holland, et al., 2008). However, although DARPP-32 KO mice were reported to have attenuated phosphorylation of both of these residues, the profile by which this occurred suggested that the influence of DARPP-32 to affect the Ser⁸³¹ AMPA GluR₁ residue occurs in a regionally selective way. For example, Ser⁸⁴⁵ GluR₁ phosphorylation is affected by loss of DARPP-32 in a fairly universal way throughout DARPP-32 expressing regions. However, attenuated Ser⁸³¹ GluR₁ phosphorylation has been observed in the hippocampus but not striatum or PFC of DARPP-32 KO mice (Svenningsson, Tzavara, Witkin, et al., 2002). It is therefore unknown as to whether the loss of function in DARPP-32 KO mice results from a combined disturbance in the Ser⁸³¹ and Ser⁸⁴⁵ AMPA GluR₁ phosphorylation residues or from a loss of signalling selectively through the Ser⁸⁴⁵ GluR₁ pathway. Furthermore, it is possible that diminished function in other DARPP-32 targets (e.g. NMDA receptors, Ca²⁺ channels etc) may have contributed to the failure of Pavlovian stimuli to potentiate instrumental responses. Nevertheless, given the widely established relationship between the AMPA GluR₁ subunit and the attribution of incentive salience, as well as its tightly bound relationship with DARPP-32, it is most likely that deletion of DARPP-32 promoted disturbances in AMPA GluR₁ signalling resulting in the failure of Pavlovian cues to enhance instrumental responding.

Given that methylphenidate was ineffective at all doses, it remains unclear as to whether the deficit represents a failure in DARPP-32 KO mice to acquire the entire spectrum of conditioned incentive motivational features that occur during Pavlovian training or whether it is a failure of the expression of incentive salience. Previous work had suggested that increased DA transmission can rescue behavioural deficits in DARPP-32 KO mice (Fienberg et al., 1998; Heyser et al., 2013). However, there was no compelling statistical evidence that the drug restored PIT in DARPP-32 KO mice or that it had any effect in potentiating responding in WT mice. There are few published studies

involving the use of methylphenidate to enhance incentive motivation in the mouse. Whilst Methylphenidate was ineffective at enhancing CRf in C57BL/6J mice along a dose-range (2.5 mg/kg, 3.5 mg/kg & 5 mg/kg) similar to the range used in this study, the 3.5 mg/kg dose, but not the 2.5 mg/kg or 5 mg/kg doses, enhanced CRf in CD-1 mice. This raises the possibility that psychostimulant drugs exert differential effects on the incentive motivational properties of conditioned stimuli as a function of the background strain of the mouse but it also suggests that the response enhancing effects of methylphenidate might exist within a fairly narrow dose range. In addition to the possibility that strain differences or dosing parameters might have influenced the ability of methylphenidate to elevate incentive salience in the PIT assay, it is important to consider the nature of the injection protocol, as well as the possibility that methylphenidate might have induced other behaviours such as stereotypy or hyperlocomotion, which might have disrupted methylphenidate's effects on incentive salience. With regards to the timing of the injections, Heyser et al (2013) injected DARPP-32 KO mice 20 min prior to a test of novel object recognition on the basis that methylphenidate's effects have been shown to be highest in rodents 20 min post IP injection, with drug effects persisting for a total of 80 min (Gerasimov et al., 2000). Heyser et al (2013) successfully rescued novel object recognition in the DARPP-32 KO mouse using this protocol. Therefore, it seems unlikely that the drug was ineffective because it was injected 20 min pre-test. Nor does it seem likely that repeated testing might have diminished the efficacy of methylphenidate. Browne and colleagues (2014) repeatedly tested their subjects using multiple doses of methylphenidate and found dose-dependent CRf responding in CD-1 mice. However, given that methylphenidate has been shown to induce its maximal response 20 min post-injection, it raises the possibility that subjects were in their maximal state of hyperlocomotion or stereotypy at the

commencement of the test. This might have had the unintended consequence of disrupting the attribution of incentive salience. Unfortunately, no measures of locomotor activity or stereotypy were recorded in the chambers during test. On this basis, the presence of competing behaviours cannot be wholly discounted as an alternative explanation for the failure of methylphenidate to potentiate responding during PIT. Nonetheless, Browne and coworkers (2014) reported higher levels of locomotor activity in the CD-1 group than in the C57BL/6J group to doses of methylphenidate during their CRf study that were similar to the doses administered in this study. If competing behaviours such as methylphenidate-induced locomotor activity are a potential cause of disrupted incentive salience attribution one might expect, then, that a group of mice undergoing higher levels of locomotor activity would experience diminished not elevated CRf, yet this was not the case in the Browne study. Lastly, there are no existing studies – to the author’s knowledge – which have shown that methylphenidate is effective at enhancing PIT in rodents. Further research is therefore required in order to establish whether this psychostimulant is effective at enhancing PIT in rodents.

One other important consideration that warrants discussion concerns the nature of the PIT arrangement employed in this study. General PIT is neurobiologically underpinned by the NAcC and the CeA and, as previously suggested, there is a dissociation in the expression of DARPP-32 within subcompartments of the amygdala (see chapter 2). That is, DARPP-32 is present in the CeA but not the BLA of the mouse amygdala. Thus, one might reasonably expect that the loss of DARPP-32 within the mouse amygdala is a contributing factor to the failure of Pavlovian cues to potentiate instrumental responding in DARPP-32 KO mice. It has been suggested that the CeA might interact indirectly with the NAc, most likely via the medial thalamus, to augment instrumental responding during the presentation of reward associated stimuli (El-Amamy & Holland, 2007; Holland &

Gallagher, 2003). Further work is needed, however, to establish whether the expression of DARPP-32 in the CeA mediates the Pavlovian enhancement of instrumental responding. To test this proposal, it would be beneficial to interfere with DARPP-32 expression in a regionally selective way either with antisense oligonucleotide probes or by viral mediated knockdown. Given the reported anatomical dissociation of amygdala subcompartments in the production of the general and reinforcer selective varieties of PIT, it would be additionally beneficial to identify whether DARPP-32 KO mice are able to produce reinforcer selective but not general PIT. One notes, however, the difficulties in isolating selective PIT in the mouse. To the best of the author's knowledge, there is 1 published report of selective PIT in the mouse (Wiltgen et al., 2007).

Finally, although there was a significant main effect of genotype during Pavlovian training when using % CS+ entries as a measure of performance, this difference predominantly reflected brief genotype differences in specific sessions of training. Importantly, there were no significant differences during Pavlovian training sessions prior to the PIT tests. Thus, Pavlovian performance was comparable between genotypes prior to testing and there were no differences in Pavlovian performance between genotypes using any of the other measures. Furthermore, previous studies examining conditioned behaviours (e.g. CRf and PIT) in DARPP-32 KO mice have not reported any disturbances in conditioned approach (Crombag et al., 2008). Therefore, it is unlikely that the failure of DARPP-32 KO mice to display a PIT effect arose from a deficit in the acquisition of conditioned approach. Nor do DARPP-32 KO mice possess deficits in instrumental responding as assessed by progressive ratio or interval schedules of reinforcement. Clearly, data regarding the acquisition of conditioned associations are not altogether instructive about the attribution of incentive salience.

In summary, DARPP-32 KO mice are impaired in the ability of reward-paired Pavlovian stimuli to enhance instrumental responding in the general PIT arrangement. These findings add to previous studies identifying a link between DARPP-32 and the attribution of incentive salience.

Chapter 7

Amphetamine psychomotor sensitisation**7.1 Introduction**

So far, we have examined the effects of DARPP-32 deletion on intertemporal and probabilistic choice patterns, as well as its effects on PIT, and the motivation to respond for food during a progressive ratio task. In this chapter, we will directly examine the role of DARPP-32 deletion in the acquisition and expression of d-amphetamine psychomotor sensitisation. Prior work has established a role for DARPP-32 in the acquisition of cocaine psychomotor sensitisation (Hiroi et al., 1999; Valjent et al., 2005; Zachariou et al., 2006) and reports citing unpublished data have also suggested that DARPP-32 KO mice display locomotor impairments to an acute dose of amphetamine (as cited in Fienberg et al., 1998). In addition to this behavioural evidence, DARPP-32 mutant mice display abnormalities in amphetamine induced gene expression (Fienberg et al., 1998). However, there are no published reports assessing the role of DARPP-32 deletion in the acquisition and/or expression of amphetamine psychomotor sensitisation.

Psychomotor sensitisation is underpinned by the coincident activity of midbrain DA and cortical glutamate projections converging on striatal MSNs. For instance, intra-VTA, intra-striatal injections or systemic blockade of either the DA or glutamate systems is sufficient to attenuate or abolish cocaine- or amphetamine-induced psychomotor sensitisation (Vanderschuren & Kalivas, 2000). DAergic projections from the VTA to the NAc undergo a series of changes in response to repeated psychostimulant administration. For instance, repeated psychostimulant treatment enhances/sensitises psychostimulant induced extracellular DA in the ventral striatum (Kalivas & Duffy, 1993). Glutamatergic projections to the VTA and NAc undergo similar drug induced adaptations following repeated psychostimulant treatment. In similar fashion to drug-induced changes in

extracellular DA, repeated cocaine treatment sensitises NAc glutamate release (Reid & Berger, 1996). Furthermore, systemic or intra-VTA microinjections of NMDA receptor antagonists block the acquisition of cocaine and amphetamine induced psychomotor sensitisation (Kalivas & Alesdatter, 1993; Vezina & Queen, 2000).

To recapitulate the importance of co-incident DAergic and glutamatergic signalling as effectors of DARPP-32, striatal DA and glutamate transmission is directed towards the post-synaptic regulation of gene expression and plasticity in MSNs. Post-synaptic DA and glutamate activity influence distinct molecular tracts that eventually undergo considerable convergence at DARPP-32. As previously mentioned, DARPP-32 mediates some key electrophysiological characteristics of MSNs such as LTD and LTP (Calabresi et al., 2000), AMPA (Yan et al., 1999) and NMDA channel currents (Flores-Hernandez et al., 2002) and phosphorylation of AMPA GluR₁ (Snyder et al., 2000) and NMDA NR₁ (Snyder et al., 1998) glutamate receptor subunits. These 2 glutamate receptor subunits are important aspects of psychostimulant sensitisation (Beutler et al., 2011; Boudreau & Wolf, 2005). Acute and chronic drug treatment stimulate dynamic changes in the phosphorylation of DARPP-32 and its downstream targets. The phosphorylation of DARPP-32 is bi-directionally affected by dose chronicity; acute psychostimulant treatment induces a concomitant increase in Thr³⁴ phosphorylation and a decrease in Thr⁷⁵ phosphorylation whereas this pattern is reversed following repeated treatment (Bibb et al., 2001; Nishi et al., 2000). Repeated cocaine-stimulated increases in Thr⁷⁵-DARPP-32 phosphorylation are mediated by activity at the mGluR₅ receptor (Scheggi et al., 2007). Co-administration of the mGluR₅ antagonist MPEP with cocaine reduces stereotypy in cocaine sensitised animals and restores the DARPP-32 profile (e.g. PP-1 inhibitory form) to that observed in previously cocaine naïve animals to a challenge dose of cocaine. Furthermore, psychostimulant-induced phosphorylation of the AMPA GluR₁ and NMDA

NR₁ subunits is absent in rats repeatedly treated with cocaine and this effect is similarly restored following MPEP pretreatment (Scheggi et al., 2007). In addition to these molecular effects, DARPP-32 KO mice (Hiroi et al., 1999) or Thr³⁴-alanine knockin mutant mice (Zachariou et al., 2006) display exaggerated cocaine sensitisation to an extended injection protocol, whilst cocaine sensitisation is blocked in Thr⁷⁵-alanine knockin mutant mice (Zachariou et al., 2006), and two-injection protocol cocaine sensitisation is blocked in Thr³⁴-DARPP-32 mutant mice (Valjent et al., 2005).

In comparison to the breadth of literature describing the relationship between cocaine administration and DARPP-32, little is known about the corresponding relationship between amphetamine administration and DARPP-32, particularly the role of DARPP-32 in facilitating amphetamine sensitisation. Amphetamine-evoked GABA efflux is attenuated in synaptosomes and striatal slices derived from DARPP-32 KO mice (Fienberg et al., 1998). Acute amphetamine-induced striatal c-fos expression is also severely affected by DARPP-32 deletion (Fienberg et al., 1998) and it has also been reported that DARPP-32 KO mice are impaired to the acute locomotor activating effects of amphetamine (as cited in Fienberg et al., 1998). Like cocaine, acute amphetamine administration increases Thr³⁴-DARPP-32 and decreases Thr⁷⁵-DARPP-32 phosphorylation, and also promotes increases in Ser⁸⁴⁵-GluR₁ phosphorylation (Svenningsson et al, 2003; Valjent et al., 2005). It was recently shown that selective blockade of CK1 prevents the acute locomotor activating effects of acute intra-accumbal amphetamine and associated increases in Thr³⁴-DARPP-32 and Ser⁸⁴⁵-GluR₁ phosphorylation (Li et al., 2011). The reader is reminded that CK1 phosphorylates Ser¹³⁰-DARPP-32 which opposes the dephosphorylation of Thr³⁴-DARPP-32 by PP-2B. Selective interference with Ser⁸⁴⁵-GluR₁ subunit phosphorylation similarly blocks the locomotor activating effects of accumbal amphetamine (Li et al., 2011). However, as

suggested, there are no known data to have established the role of DARPP-32 deletion in the acquisition of amphetamine sensitisation. The purpose of the present study was to address this lacuna.

7.2 Methods

7.2.1 Subjects

Eighteen DARPP-32 KO mice (15 males), aged between 8 and 47 weeks old, (9 mice were aged > 30 weeks) and 16 WT littermates (14 males), aged between 8 and 47 weeks old, (8 mice aged > 30 weeks) were used as subjects for this study. As before, animals were genotyped using the protocol described in chapter 2. All animals used in this study were at least the 4th generation of backcrossed mice bred from a C57BL/6J background in the manner previously reported. Mice were given *ad libitum* access to food and tap water in their home cages and were singly housed in a temperature ($21^{\circ}\text{C} \pm 1.5^{\circ}\text{C}$) and humidity ($50\% \pm 10\%$) controlled environment, with lights maintained on a 12 hr dark/light cycle (lights on 7am).

7.2.2 Apparatus

Locomotor testing was conducted in 9 identical circular runways constructed from polypropylene (H = 25.5cm, D = 24.5cm). Locomotor cylinders were placed atop a semi-transparent plexiglas table and behavioural data were recorded via a camera located directly underneath the plexiglas table (Mead, Vasilaki, Spyraiki, Duka & Stephens, 1999). Locomotor data were quantified from video recordings using Matlab (Version 15, The MathWorks Inc., Natick, Mass, USA) and computed as the total distance travelled (m) during each session.

7.2.3 Drug

All substances were administered IP at a volume of 5ml/kg. d-amphetamine hydrochloride (AMPH) (Sigma Aldrich, Dorset, United Kingdom) was dissolved in 0.9% SAL.

7.2.4 Procedure

At the start of the experiment, equal proportions of each genotype were pseudorandomly designated to either SAL (control) or AMPH pretreatment group. Animals first underwent 2 60 min sessions on separate days, during which all received a SAL injection in the locomotor cylinders, to habituate them to the treatment protocol. All subsequent sessions commenced with 30 min habituation during which subjects were placed into the locomotor cylinders and no drug was administered. Animals received their designated treatment immediately afterwards and were then returned to their locomotor cylinder for a further 60 min, during which their locomotor activity was recorded. Mice were returned to their colony room immediately after each daily session. For session 1, AMPH pretreatment subjects received 1.0mg/kg. However, owing to a relatively poor locomotor response to this dose of AMPH, the dose for all subsequent training sessions was increased to 2.0 mg/kg. Mice next received a total of 10 daily injections of SAL or 2.0 mg/kg AMPH before undergoing a within-session dose-response test following a 7 day washout period. During this test, all subjects first received 30 min habituation as before. Immediately after the 30 min habituation period, all animals received SAL injections and were returned to the locomotor apparatus for 60 min. Animals were then treated with escalating doses of AMPH (0.5mg/kg, 1.0 mg/kg and 2.0 mg/kg) administered at approximately 65 min intervals.

7.2.5 Data analysis

Habituation data were subjected to a mixed genotype (WT vs KO) by session (first habituation session vs final habituation session) by pretreatment (SAL vs AMPH) ANOVA. Habituation data from the dose-effect test were analysed separately with a genotype (WT vs KO) by pretreatment (SAL vs AMPH) repeated measures ANOVA.

Only data from training sessions using the 2mg/kg dose (i.e. sessions 2 to 11) were analysed. Any reference to sensitisation refers explicitly to the sensitisation of locomotor responding to the adjusted 2.0 mg/kg dose.

The acquisition of psychomotor sensitisation was analysed by conducting a genotype (WT vs KO) by session (second vs last) by pretreatment (SAL vs AMPH) mixed factorial ANOVA on data representing the total distance covered during these training sessions.

The acquisition of AMPH sensitisation was also compared between groups by calculating regression slope values derived by fitting a line to values representing the total distance covered during each session of training for each mouse. Higher slope values indicate a more rapid and pronounced acquisition of sensitisation. These values were then subjected to a two-way genotype (WT vs KO) by pretreatment (SAL vs AMPH) two-way ANOVA.

The expression of AMPH sensitisation in WT and DARPP-32 KO mice was assessed by comparing the distance travelled during each treatment administered in the dose-effect test. To this end, a mixed genotype (WT vs KO) by pretreatment (SAL vs AMPH) by dose (SAL vs 0.5 mg/kg vs 1 mg/kg vs 2mg/kg) ANOVA was conducted.

Because of the broad age range of subjects used in this study, mice were allocated to 1 of 2 groups based on their age and additional analyses were conducted (data not included) to inspect the impact of using a population of mixed age mice on the acquisition and expression of AMPH sensitisation as a function of genotype and pretreatment.

Animals aged < 30 weeks were designated as normal aged adults whereas animals aged > 30 weeks were designated as elderly. No significant age effects were uncovered.

7.3 Results

7.3.1 Habituation

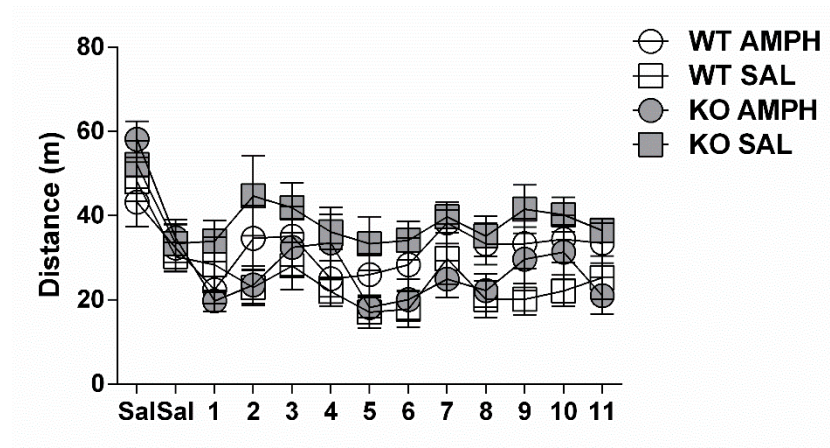


Fig. 7.1 Distance travelled (m) during habituation for each treatment group.

Mice increasingly habituated to the test apparatus as training progressed ($F_{(1, 30)} = 48.40$, $p = .001$). Although both AMPH and SAL pretreated mice habituated to the test apparatus, AMPH pretreated animals travelled significantly further than SAL pretreated animals during the final habituation session suggesting the development of conditioned locomotion in AMPH pretreated mice (session by pretreatment interaction ($F_{(1, 30)} = 7.90$, $p = .01$); post hoc independent t-test comparing distance travelled during the final habituation session between AMPH and SAL pretreatment groups ($t_{(32)} = -2.85$, $p = .01$). However, there were no significant main effects of genotype or pretreatment and no significant interactions involving genotype, pretreatment and/or session ($F_s = < 2.72$, $P_s = > .11$) (Fig. 7.1).

7.3.2 Acquisition of amphetamine sensitisation

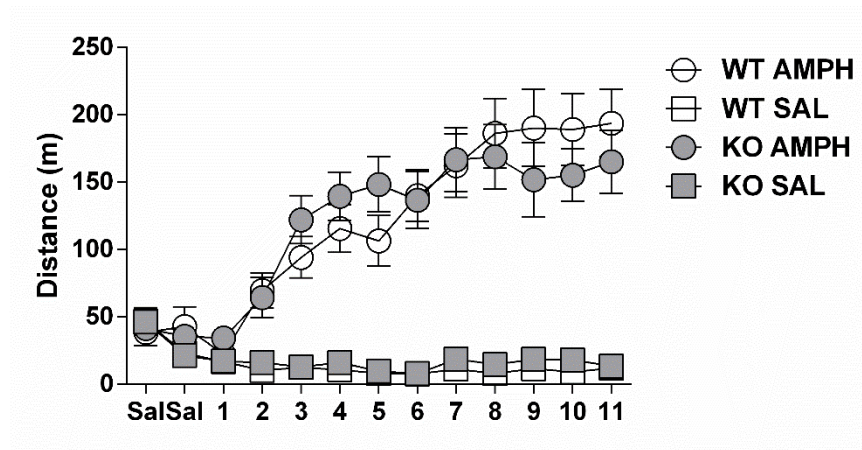


Fig. 7.2 Distance travelled (m) during each training session.

As expected, AMPH but not SAL pretreatment robustly induced psychomotor sensitisation (main effect of pretreatment ($F_{(1,30)} = 85.61$, $p = .001$); session by treatment interaction ($F_{(1,30)} = 53.79$, $p = .001$); post hoc paired t -tests comparing total distance covered in session 2 with session 11 in AMPH pretreated ($t_{(16)} = -7.45$, $p = .001$) and SAL ($t_{(16)} = .28$, $p = .78$) pretreated mice (Fig. 7.2). There were, however, no other significant main or interaction effects ($F_s = < .82$, $P_s = > .37$). These results therefore suggest that deletion of DARPP-32 has no significant impact on the acquisition of amphetamine sensitisation at the doses used in this study.

7.3.3 Amphetamine sensitisation acquisition slope values

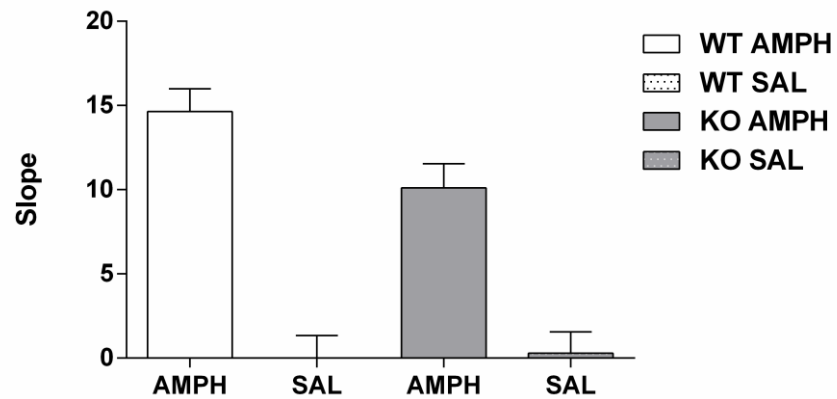


Fig. 7.3 Slope values of AMPH and saline pretreated WT and KO mice.

In light of the non-significant difference between AMPH pretreated WT and KO animals in the total distance travelled during session 11, potential group differences concerning the rate at which animals acquired psychomotor sensitisation were assessed by analysing the regression slope scores of each animal during the acquisition phase of this experiment. Slopes were subjected to logarithmic transformation and statistical analysis was conducted on these transformed values. All reported descriptive statistics represent the non-transformed values. The results from the slope analysis were consistent with those from the analysis of raw distance travelled. AMPH pretreated mice developed robust sensitisation ($F_{(1, 30)} = 160.10$, $p = .001$). There was no significant difference between genotypes in slope values, and no significant difference between AMPH pretreated KO mice and AMPH pretreated WT mice ($F_s = < 3.32$, $p = > .08$) (Fig. 7.3).

7.3.4 Dose-effect test habituation

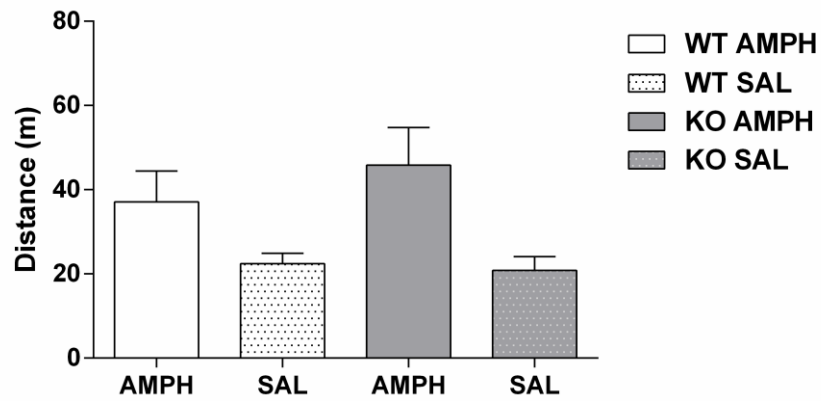


Fig. 7.4 Distance travelled during dose-effect test habituation.

AMPH pretreatment group mice travelled significantly further than SAL pretreatment group mice during the habituation phase prior to the dose-effect test indicating the presence of conditioned locomotion in these mice ($F_{(1, 30)} = 10.11$, $p = .003$) (Fig. 7.4). However, DARPP-32 deletion had no significant effects in either SAL or AMPH pretreated mice on distance travelled during this phase of the experiment ($F_s = < .69$, $P_s = > .41$).

7.3.5 Dose-effect test following 7 day wash-out period

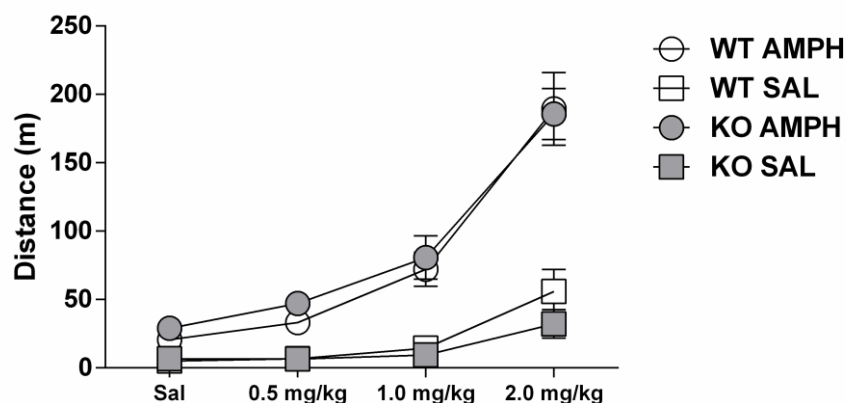


Fig. 7.5 Distance travelled (m) to each treatment during dose-effect test

Results from the dose-effect test were consistent with results from the acquisition phase. A significant main effect of pretreatment ($F_{(1, 30)} = 60.15, p = .001$) and a significant dose by treatment interaction ($F_{(1.18, 35.40)} = 44.05, p = .001$) revealed that AMPH pretreated mice travelled significantly further than SAL pretreated mice (Fig. 7.5). Post hoc independent t-tests indicated that the magnitude of locomotor responding was significantly greater in repeated AMPH pretreated animals at all levels of dose, including SAL, than animals that were SAL pretreated during the training sessions (SAL ($t_{(17.43)} = -4.99, p = .001$); 0.5 mg/kg ($t_{(18.65)} = -7.24, p = .001$); 1 mg/kg ($t_{(19.15)} = -6.23, p = .001$); 2.0 mg/kg ($t_{(26.72)} = -7.94, p = .001$). The significantly greater locomotor responding in AMPH pretreated mice compared to SAL pretreated mice following SAL administration additionally identifies the expression of conditioned locomotor responses in AMPH pretreated mice. A significant main effect of dose ($F_{(1.18, 35.40)} = 118.88, p = .001$) indicated that animals, when collapsing across pretreatment and genotype, displayed dose dependent increases in locomotor responding to AMPH administered during the test compared with SAL treated distances (Bonferroni post hoc tests ($P_s = < .001$)). There were no other significant main or interaction effects, indicating that regardless of pretreatment and/or the test dose administered, DARPP-32 deletion had no significant

effect in mediating the expression of AMPH psychomotor sensitisation ($F_s = < 1.25$, $P_s = > .28$). These data establish the robust expression of psychomotor sensitisation in AMPH pretreated animals but also show that DARPP-32 deletion does not mediate the expression of AMPH sensitisation at the dose(s) and/or the treatment protocol recruited in this study.

7.4 Discussion

Deletion of the DARPP-32 gene did not alter the acquisition or expression of psychomotor sensitisation to amphetamine in the current study. These results are the first known data – to the author – to establish that global deletion of the DARPP-32 gene has few consequences for amphetamine psychomotor sensitisation. This was a surprising finding given the augmenting effects of DARPP-32 deletion on cocaine psychomotor sensitisation (Hiroi et al., 1999). This absence of between groups differences in amphetamine psychomotor sensitisation, along with the data reported elsewhere in this thesis, indicates that global DARPP-32 deletion does not drastically impair reward-based learning and performance. Nevertheless, interpreting the results of psychomotor sensitisation studies recruiting DARPP-32 mutant mice is a complicated task. For example, Valjent and colleagues (2005) reported that cocaine sensitisation is abolished in Thr³⁴-DARPP-32 alanine knockin mice using the two-injection protocol sensitisation procedure, whilst Zachariou et al (2006) reported that Thr³⁴-DARPP-32 mutant mice show enhanced cocaine sensitisation using an extended injection protocol, similar in nature to the one recruited in this study. Valjent and collaborators (2005) similarly showed that cocaine sensitisation in a two-injection protocol is blocked in the DARPP-32 KO mouse whilst Hiroi et al (1999) reported potentiated cocaine sensitisation using an extended treatment protocol. One possible explanation for this effect is that it is a time-dependent process. However, closer inspection of these studies reveals that Thr³⁴-

DARPP-32 knockin mice and DARPP-32 KO mice displayed cocaine sensitisation to the second dose of cocaine in the studies by Zachariou et al (2006) and Hiroi et al (1999). Furthermore, Valjent et al (2005) administered their second dose of cocaine at 2 different time points. One group received their second dose the day after their first treatment and another group received their second treatment 7 days after their first treatment; neither acquired psychomotor sensitisation. Thus it is difficult to reconcile these discrepancies with a simple time-dependency explanation. One notes, therefore, that the interpretation of the results in this chapter, as well as those of some previous studies, are difficult to reconcile on the basis of a common, unifying explanation. The expectation was to observe enhanced amphetamine sensitisation in KO mice but, rather than observe a potentiation or even a loss of amphetamine sensitisation, DARPP-32 KO mice displayed normal amphetamine sensitisation. A potential explanation for this result concerns the dose-dependent locomotor activating effects of psychostimulants in DARPP-32 mutant mice. It has been reported that DARPP-32 mice display dose dependent impairments to acutely administered psychostimulants (Fienberg et al., 1998), therefore, there is presumably a dose range whereby these mutants might acquire sensitisation normally. It would, thus be beneficial, if future studies examined the relationship between dose and the magnitude of psychomotor sensitisation in DARPP-32 mutant mice.

In addition to this, impairments in DARPP-32 KO mice to the acute locomotor response of drugs of abuse do not always translate to impairments in sensitisation. For example, DARPP-32 KO & Thr³⁴-DARPP-32 alanine knockin mice are impaired to the acute locomotor activating properties of morphine but display morphine sensitisation that is indistinguishable from WT mice when exposed to an extended administration protocol (Borgkvist et al., 2007). In contrast to these reports, however, Valjent et al (2010) reported that morphine sensitisation was blocked in Thr³⁴-DARPP-32 mutant mice. Previous

unpublished results (as cited in Fienberg et al., 1998) suggest that the locomotor activating effects of acutely administered amphetamine are attenuated in DARPP-32 KOs. Unfortunately, the absence of more detailed methods (especially dose) and results makes comparison with the current study difficult. In this chapter, there was no increase in locomotor activity, relative to SAL, in either wildtype or knockout mice following the first (1.0 mg/kg) dose of amphetamine and the dose was accordingly increased to 2.0 mg/kg. Both acute locomotor activity and robust psychomotor sensitisation occurred in response to this dose but there were no differences between genotypes in the distance travelled following acute 2.0 mg/kg treatment.

Whilst broad similarities exist between cocaine and amphetamine in the neural circuitry these drugs influence to establish psychomotor sensitisation, subtle differences in the processes these drugs stimulate to produce psychomotor sensitisation do exist (see Vanderschuren & Kalivas, 2000 and Wolf & Ferrario, 2010). Whilst speculative, differences between the effects that repeated cocaine and repeated amphetamine treatment have in stimulating modifications in the AMPA GluR₁ subunit might account for the observations in this study. Cocaine sensitisation is associated with a variety of post-synaptic modifications to AMPA GluR₁ receptor properties, including AMPA GluR₁ receptor surface expression and GluR₁ subunit phosphorylation in the nucleus accumbens (Wolf & Ferrario, 2010). The latter of these modifications is partially regulated by DARPP-32. Whilst recurrent cocaine treatment has been found to up-regulate AMPA GluR₁ surface expression in accumbal neurons 21 days post withdrawal, this finding was not replicated in animals repeatedly treated with amphetamine suggesting that the role of AMPA receptors in promoting psychomotor sensitisation might diverge as a function of drug type (e.g. cocaine vs amphetamine) (Boudreau & Wolf, 2005; Nelson, Milovanovic, Wetter, Ford & Wolf, 2009). In light of these findings, it was proposed in a review by

Marina Wolf, in which she and her colleague outlined the relationship between AMPA receptor plasticity and behavioural assays commonly used to investigate drug related behaviours, that cocaine and amphetamine might differ in the mechanisms they stimulate to influence experience-dependent changes in AMPA receptor function (Wolf & Ferrario, 2010).

Despite the divergent mechanisms that cocaine and amphetamine stimulate to induce experience-dependent changes in the AMPA receptor, the extent to which these drug induced changes in the AMPA GluR₁ subunit contribute to differences in the acquisition and expression of psychomotor sensitisation is not yet fully understood. Much of the work describing the different capabilities of these 2 kinds of psychostimulant to modify the AMPA receptor has been conducted following a significant withdrawal period and it is not yet known whether post-withdrawal GluR₁ modifications have a causal role in the acquisition of psychomotor sensitisation, whether they are a component of the long-term expression of psychomotor sensitisation, or if they are solely a consequence of drug withdrawal distinct from the sensitising effects of psychostimulants.

In the current study, mice were subjected to a 7 day withdrawal period prior to administering a dose-effect test. However, in the study conducted by Hiroi in which they examined the relationship between DARPP-32 deletion and cocaine sensitisation, mice were not exposed to a challenge dose of cocaine following a withdrawal period. As a result, it was not known whether DARPP-32 influenced the expression of already acquired psychomotor sensitisation following a withdrawal period. There were no differences between KO and WT amphetamine pretreated mice to escalating doses of amphetamine during the dose-effect test in this experiment. It should be noted that animals were exposed to a 7 day withdrawal period in the current study but experience-dependent modifications to repeated psychostimulant administration may continue to

occur beyond this point (Boudreau & Wolf, 2005). Therefore, it would be instructive to directly examine whether DARPP-32 deletion mediates the AMPA GluR₁ modifications that occur in response to repeated psychostimulant administration by including a number of withdrawal periods (e.g. 0 days vs 7 days vs 14 days vs 21 days) prior to implementing a psychostimulant challenge.

In addition to the dosing parameters, the apparatus used in this study warrants discussion. A low-moderate dose of amphetamine was selected on the basis that the locomotor apparatus used for this experiment do not allow one to capture psychostimulant induced stereotypy. Higher doses of amphetamine are associated with the induction of significant stereotyped motor responses and even repeatedly administered moderate (2.5 mg/kg) doses can produce stereotypic responding in rodents (Rebec & Segal, 1980). Furthermore, metabotropic glutamate receptor antagonist mediated reductions in stereotypic responding in cocaine sensitised rats correlate with changes in DARPP-32 phosphorylation (Scheggi et al., 2007). On the basis that psychomotor sensitisation can involve the induction and expression of stereotyped responses as well as locomotor enhancements, it is impossible to exclude a possible role for DARPP-32 in mediating sensitisation to the stereotypy inducing effects of amphetamine.

Although somewhat speculative, age-dependent effects may also account for some of the observed differences between the current study and those reported by Hiroi and co-workers. Mice were aged between 2-5 months in the experiment conducted by Hiroi, whereas mice were aged between 2-10 months in the current study. Many correlates of neuroplasticity such as LTP undergo developmental transformation over the lifespan (see McCutcheon & Marinelli (2009) for a short review on age-dependent effects in common behavioural neuroscience assays). Age-dependent effects have been reported in many behavioural assays aimed at investigating addictive behaviours in the rodent. Indeed,

younger mice are more susceptible to the sensitising effects of repeated amphetamine administration but these sensitivities were reported in mice considerably younger than those recruited in either this study or the study conducted by Hiroi and colleagues (Kameda et al., 2011). However, to the best of the author's knowledge, there have been no reported age-dependent differences in the capability of psychostimulants to influence the phosphorylation of DARPP-32 or any age-dependent changes in the efficacy of DARPP-32 to mediate psychostimulant induced gene expression and receptor modifications. Furthermore, the average age of mice used in the respective groups in this study was equal and statistical analysis (data not shown) uncovered no age-dependent effects.

In summation, DARPP-32 has previously been shown to mediate the locomotor augmenting effects of recurrent cocaine treatment yet, in the current study, no such role for DARPP-32 in the psychomotor sensitising effects of amphetamine was observed. This discrepancy might reflect subtle differences in the post-synaptic modifications that occur between repeated cocaine and repeated amphetamine treatment. It might, however, also reflect variations in the procedures used (e.g. dose, age of mice etc.). Additional work is required to fully understand whether DARPP-32 has distinct roles in facilitating cocaine but not amphetamine sensitisation. Such work could recruit mice with targeted disruptions of specific phosphorylation residues to attempt to overcome the limitations inherent in using mouse models with global protein deletion. In addition to this, Cre-recombinase technology can be used to selectively delete DARPP-32 in specific populations of striatal MSNs (in D₁ vs D₂ expressing MSNs). A more specific disruption of DARPP-32 phosphorylation residues might be possible following the development of a virus to knockdown DARPP-32 in discrete nuclei known to underpin psychomotor sensitisation. For instance, selective disruption of the NMDA NR₁ gene *Grin 1* in D₁

receptor expressing MSNs attenuated amphetamine psychomotor sensitisation whereas widespread virally-mediated striatal disruption of Grin 1 rescued amphetamine sensitisation (Beutler et al., 2011). Therefore, region specific genetic deletion or regionally selective viral-mediated knockdown of DARPP-32 may advance one's understanding of any role this protein has in amphetamine psychomotor sensitisation.

Summary of findings and general discussion

8.1 Introduction

Despite the widespread enrichment of DARPP-32 throughout brain regions underpinning incentive learning and motivation, in many ways, the DARPP-32 KO mouse is a remarkably normal animal capable of learning/performing interval and ratio schedules of reinforcement and of learning Pavlovian associations. In an ecological setting, however, the relations between reinforcement contingencies and the outcomes they produce are rarely predictable but change on an ongoing basis. For example, one might wait in the queue in a hamburger outlet for 30 seconds before being tended to on one occasion but, on the subsequent occasion, one might experience a wait of 5 min, and on another occasion one might wait 10 min before deciding that the hamburger is not worth waiting any longer for. Similarly, the prudent investor has to be sensitive to the inherently uncertain and fluctuating nature of the stock market lest they face financial ruin. Likewise, the effort costs associated with reinforcement often fluctuate rather than remain fixed, predictable costs. With this in mind, optimising reinforcement requires animals to rapidly integrate a multitude of information concerning investment costs (e.g. time, risk and effort) and to also integrate information conveyed by reward-predictive cues. The integration of this information allows animals to make flexible cost-benefits decisions about competing choices and to adapt behaviour when presented with information (e.g. reward paired stimuli) which predicts the presence of impending reward. Although the nature of the behavioural disturbances reported in this thesis were not always straightforward, there is some indication that DARPP-32 is involved in the efficient allocation of behaviour under environmentally changing conditions. Two studies reported in this thesis suggest that DARPP-32 is needed to rapidly adapt to unpredictable

changes in delay parameters in the intertemporal discounting task and to adapt flexibly to uncertain reinforcement in the probability discounting assay. Furthermore, prior research (Crombag et al., 2008), as well as work contained in this thesis, suggests that DARPP-32 is intimately involved in the multiplicative enhancement of instrumental reward value that occurs when Pavlovian cues are superimposed over the presence of instrumental response devices during the general PIT test. Prior work has also shown that mice with a targeted interference of the Ser⁹⁷-DARPP-32 phosphorylation residue are less capable of adapting their behavioural output following increases in the effort costs needed to achieve reinforcement (Stipanovich et al., 2008) and that DARPP-32 KO mice are less responsive to external change (Heyser et al., 2000; Heyser et al., 2013). Overall, these data suggest that DARPP-32 is involved in behavioural adaptation following reward related change, either in response to changes in the task, or to the Pavlovian inflation of reward value that occurs during PIT.

8.2 DARPP-32 and intertemporal discounting

Although the results pertaining to this chapter identified a significantly different intertemporal choice profile in DARPP-32 KO mice at long delays, there is reason to believe that this difference might represent a failure to rapidly integrate changes in the task rather than a true genotype difference in the sensitivity to delayed reinforcement. As discussed earlier, DARPP-32 KO mice were, for the most part, capable of executing similar intertemporal choice profiles to WT mice. However, KO mice were relatively inflexible following changes in task parameters (e.g. when the delay order was reversed and when delays were increased significantly). Moreover, DARPP-32 KO mice achieved comparable performance to WT mice following extended testing at long delays. These data are in accordance with the suggestion that the accumbens mediates the sensitivity to

unpredictable changes in delays (Acheson et al., 2006), as well as learning about time-based action outcome contingencies (Cardinal & Cheung, 2005) and in mediating certain kinds of behavioural flexibility (Annett et al., 1989; Haluk & Floresco, 2009).

Cardinal et al (2003) suggested that, in non-human subjects, it is important to dissociate the learning of task parameters from the performance of the task once the contingencies have been fully learned. The data in this study exemplify their point by showing that changes in the task parameters can induce transient disturbances in intertemporal choices which eventually disappear with extended experience. To the best of one's knowledge, these data are the first to identify a role for DARPP-32 in the intertemporal discounting task.

8.3 Probability discounting

In contrast to the previous chapter, the data captured during this experiment identified a persistent performance difference between DARPP-32 WT & KO mice during probability discounting. DARPP-32 WT and KO mice were initially indistinguishable in their probabilistic choices but, later in the experiment, KO mice became significantly less tolerant of uncertainty than WT mice. An interesting finding from this experiment is that by the end of the study, DARPP-32 KO mice were less capable of optimising reinforcement in high probability blocks but better at executing choices in low probability blocks than WT mice. These data indicate that, in comparison to WT mice, DARPP-32 KOs were executing an inflexible strategy which consisted of avoiding uncertainty, rather than an optimal pattern of choices that would entail maximising the acquisition of the large reinforcer in high probability blocks but avoiding it in low probability blocks. These data make a novel contribution to the literature by identifying a role for DARPP-32 in the rodent probability discounting task and they also provide a certain degree of translational

relevance by producing parallel findings to those observed in humans which have also identified a role for DARPP-32 in a probabilistic learning task (Frank et al., 2007).

8.4 Progressive ratio

In this chapter, deletion of DARPP-32 had no effect on the willingness of mice to invest effort to acquire a food reinforcer. DARPP-32 KO mice achieved comparable performance with WT mice throughout all periods of the experiment and between genotypes instrumental performance was unaffected by changes in the reinforcement schedule (e.g. making the schedule shallower or steeper). This finding suggests, that in comparison to mice with a targeted interference of the DARPP-32 gene (Stipanovich et al., 2008), global deletion does not induce deficits in instrumental motivation and that DARPP-32 KO mice are able to adapt rapidly to changes in effort costs. However, because DARPP-32 is a complicated intracellular signalling protein, and on the basis of previous work comparing the effects of targeted interference of specific phosphorylation residues on specific behaviours (e.g. psychomotor sensitisation) (Valjent et al., 2005; Zachariou et al., 2006), one can reasonably predict that different effects might occur depending on the method taken to disrupt DARPP-32 (e.g. global deletion vs phosphorylation residue specific disruption). It is clear, therefore, that DARPP-32 is involved in the provision of instrumental motivation. However, global deletion of the DARPP-32 protein does not induce any significant deficits in this measure of instrumental motivation.

8.5 Pavlovian-to-instrumental transfer

Although the effects were much smaller than anticipated, the data presented in this chapter lend support to previous findings indicating that DARPP-32 KO mice do not perform the general form of PIT (Crombag et al., 2008). Whilst WT mice increased their

response rates during the presentation of the CS+ significantly above baseline, KO mice displayed no evidence of such an effect. The precise nature of this disturbance, however, is not yet known. For example, it is not clear whether the failure to perform PIT represents a failure of the acquisition of incentive salience or its expression. These data could indicate a disturbance in Pavlovian incentive motivation which would be entirely commensurate with the role of DA in this process. However, an alternative interpretation of these results is that DARPP-32 KO mice could lack the behavioural flexibility necessary to modify their behaviour when cues are superimposed over the presence of the instrumental manipulanda.

Mice were also administered MPH during this experiment in an attempt to rescue this deficit in DARPP-32 KO mice. However, MPH had clear no effects on either WT or KO mice which makes it impossible to determine whether acute administration of DAergic compounds can or cannot rescue this deficit in KO mice. Nonetheless, these data are the first to show that MPH does not rescue general PIT in DARPP-32 KO mice at the doses administered.

8.6 Amphetamine psychomotor sensitisation

Unlike previous findings which have established that repeated administration of cocaine induces a more profound psychomotor sensitisation in DARPP-32 KO mice (Hiroi et al., 1999), repeated amphetamine treatment did not induce different levels of sensitisation between genotypes. DARPP-32 KO mice were indistinguishable from their WT counterparts in both the acquisition and expression of psychomotor sensitisation. This was unexpected. However, and as noted earlier, the findings relating to psychomotor sensitisation in mice with disruptions to the DARPP-32 gene have been inconsistent and also vary as a function of the administration protocol. It might also be that repeated cocaine treatment stimulates subtly different molecular adaptations compared to repeated

amphetamine treatment (e.g. AMPA GluR₁ subunit changes). However, without additional work it is impossible to determine the precise mechanisms responsible for the different abilities of cocaine and amphetamine to promote psychomotor sensitisation in DARPP-32 KO mice.

8.7 DARPP-32 and the effect of motivational shifts

Because previous reports had suggested that Ser⁻⁹⁷-DARPP-32 mutant mice displayed impairments in the motivation to exert effort (Stipanovich et al, 2008), and because DARPP-32 is expressed in brain regions associated with habit formation and the sensitivity to reinforcer devaluation, mice were exposed to a variety of devaluation manipulations to directly assess the impact of inducing motivational shifts on reinforcement choices (e.g. intertemporal or probabilistic choice) and the motivation to exert effort. Whilst these manipulations significantly reduced the motivation to initiate trials during the intertemporal and probabilistic choice tasks, and also the breaking point of responding during the progressive ratio experiment, they did not differentially affect the motivation to initiate trials between genotypes and nor did they differentially affect intertemporal or probabilistic choices. Nor did reinforcer devaluation differentially affect breaking points of responding during the progressive ratio experiment. Overall, these results indicate that DARPP-32 KO mice do not possess significant deficits in instrumental incentive motivation. Therefore, one can reasonably infer that the abovementioned findings are broadly indicative of learning impairments rather than motivational disturbances. However, as noted above, this does not imply that DARPP-32 has no role in provisioning instrumental incentive motivation but, rather, that the method taken in this thesis (e.g. global deletion), did not induce any discernible differences in motivation between genotypes in the behavioural tasks recruited in this thesis.

8.8 Behavioural conclusion

In many ways, DARPP-32 KO mice are remarkably normal animals apparently capable, for the most part, of learning and performing numerous motivated behaviours. The deficits that are apparent in these mice, most especially those deficits which do not involve tasks supported by drug-reinforcement, appear to be subtle and might involve failures to rapidly reorganise behaviour in environmentally dynamic conditions (e.g. when task parameters are altered or when Pavlovian cues are superimposed over instrumental devices). In the broadest of senses, the data in this thesis indicate that unlike their wildtype littermates, DARPP-32 KO mice lack the behavioural plasticity necessary to rapidly reorganise or modify their behaviour under changing circumstances. This conclusion is based on the findings that 1) DARPP-32 KO mice were slow to update their intertemporal choice profiles in response to changes in the task parameters. 2) DARPP-32 KO mice developed a less flexible strategy during the probability discounting task which consisted of avoiding the LU lever significantly more than WT mice in the blocks where reinforcement following the selection of the LU was most uncertain. 3) Mice lacking DARPP-32 failed to flexibly modify instrumental responding during the presentation of Pavlovian cues in the PIT tests. 4) Findings from previous research indicates that DARPP-32 KO mice lack novel object recognition and are impaired during simple instrumental reversal learning. These latter 2 tasks directly measure the response to external change. Overall then, DARPP-32 KO mice are less capable of integrating reward-related information to flexibly modify their behaviour in environmentally fluctuating conditions.

Finally, the failure to observe differences between WT & KO subjects in amphetamine psychomotor sensitisation raises additional questions about the role DARPP-32 might have in incentive motivational processes. Repeated amphetamine treatment has been

shown to potentiate incentive motivation (Mead et al., 2004; Wyvell & Berridge, 2001). This is entirely consistent with the DAergic influence of amphetamine and its subsequent effects on incentive motivation. However, the absence of between-group differences in locomotor responding to repeated amphetamine, as well as the indistinguishable performance of WT & KO subjects in progressive ratio of reinforcement testing, and the subtle group differences reported in this thesis, suggests that global deletion of DARPP-32 has a relatively modest impact on reward-based learning and performance.

8.9 Biological considerations and limitations

Drawing firm conclusions, especially conclusions which precisely elucidate the underlying molecular mechanisms and the biological loci responsible for the behavioural perturbations that were reported earlier is a difficult, if not impossible task, given the nature of the experimental approach taken to interfere with DARPP-32 (e.g. global deletion). Phosphorylation residue specific mutants can produce different effects to global mutants and studies performing targeted deletion of DARPP-32 in the direct vs indirect pathway suggest that DARPP-32 may have differential consequences for behaviour depending on the nature of the DA receptor which is present on MSNs. Selective deletion of DARPP-32 in the direct striatonigral pathway diminishes baseline locomotor activity and cocaine's hyperlocomotive effects, whilst deletion of DARPP-32 in striatopallidal neurons increases baseline locomotor activity and attenuates haloperidol's cataleptic effects (Bateup et al., 2010). Global deletion has no effect on baseline locomotor activity but dose-dependently affects cocaine's hyperlocomotive effects (Fienberg et al., 1998). The distinction between D₁ and D₂-expressing MSNs has important psychological consequences. For instance, D₁-expressing MSNs are believed to be important for reward related incentive learning (Beninger & Miller, 1998) and limited forms of behavioural flexibility such as set-shifting (Haluk & Floresco, 2009), whilst D₂-expressing MSNs are

important for aversive learning, reward omission and behavioural flexibility in the domain of learning to overcome previously rewarded contingencies which are no longer so (e.g. reversal learning) (Nakanishi, Hikida & Yawata, 2014).

Although the most plausible explanation for the differential probability discounting is due to the absence of DARPP-32 in D₁-expressing accumbal MSNs, this task is also mediated by both D₁ and D₂ receptors in the mPFC (St Onge et al., 2011), rendering precise mechanistic conclusions impossible. The complex nature of disturbances in the intertemporal discounting task and the approach taken to interfere with DARPP-32 also makes it difficult to understand precisely whether some of the disturbances in that task arose from disruptions to DARPP-32 in D₁-expressing MSNs, D₂-expressing MSNs or a combination of both. Little is known about the anatomical locus of receptor populations responsible for intertemporal discounting. However, systemic administration of either D₁ (Koffarnus et al., 2011) or D₂ (Wade et al., 2000) receptor antagonists alters delayed reinforcement choices. General PIT is also affected by either D₁ or D₂ receptor antagonists (Lex & Hauber, 2008). The regional pattern of DARPP-32 expression also precludes precise neurobiological conclusions because, in addition to being expressed in both striatal D₁ and D₂-expressing MSNs, DARPP-32 is expressed in PFC (mPFC, OFC & ACC) regions and the CeA, and the behavioural tasks in this thesis are underpinned by the striatum and one or more of these regions. Therefore, on the basis of the data presented in this thesis, it is impossible to determine which DARPP-32 null region(s) or which phosphorylation residues were responsible for the deficits in any given task.

8.10 Disturbances in the electrophysiological properties of DARPP-32 expressing neurons

As discussed previously, DARPP-32 deletion has been shown to disrupt the electrophysiological properties of MSNs in both D₁ and D₂ expressing populations

(Bateup et al., 2010; Calabresi et al., 2000; Fienberg et al., 1998). For example, NMDA (Fienberg & Greengard, 2000) and AMPA subunit phosphorylation (Snyder et al., 2000), as well as NMDA (Flores-Hernandez et al., 2002) and AMPA (Yan et al., 1999) channel currents, Ca^{2+} and Na^{+} channel currents and D_1 receptor mediated changes in Na^{+} , K^{+} ATPase activity (Fienberg et al., 1998) are disturbed in MSNs from DARPP-32 KO mice. Striatal MSNs from DARPP-32 KO mice also lack both LTD and LTP (Calabresi et al., 2000) and regionally selective deletion of DARPP-32 disrupts LTP in either the direct or indirect pathway (Bateup et al., 2010). It has been suggested that co-incident DA and glutamate activity converging on MSNs stimulates synaptic alterations (e.g. LTP & LTD) which are crucial to reinforcement learning, action selection and perhaps underlie the transition from controlled to compulsive drug use (Hyman, Malenka & Nestler, 2006; Redgrave, Vautrelle & Reynolds, 2011; Wickens, 2009). Computational models of DARPP-32 function have suggested that brief glutamate stimulated Ca^{2+} influx strengthens DA stimulated cAMP-PKA signalling leading to PP-1 inhibition and the insertion of AMPA receptors into the surface membrane and eventually LTP (Lindskog, Kim, Wikström, Blackwell, & Kotaleski, 2006). It is most likely that most if not all of the disturbances in DARPP-32 KO mice arise from failures in neuroplasticity and associated disturbances in the electrophysiological properties of DARPP-32 expressing neurons. As noted elsewhere, the balance of neural activity within distinct populations of striatal MSNs may differentially affect behavioural output. However, it is not yet clear which processes and which regions mediate the distinct disturbances reported in this thesis.

In summary, DARPP-32 KO mice display reward-related learning and memory impairments, as well as disturbances in biological correlates of memory and associated electrophysiological processes. The balance of neural activity through direct and indirect basal ganglia networks are important determinants of goal-directed behaviour. DARPP-

32 is a molecular pivot that can influence the balance of neural activity throughout both the direct and indirect pathways and, in light of the data presented in this thesis, it seems that this function, in some instances, facilitates adaptation to change, most probably by integrating coincident DA and glutamate signals that are crucial to reward related learning and memory. These experience-dependent electrophysiological changes in striatal MSNs allow for the coherent and coordinated control of basal ganglia output signals and, accordingly, the generation of efficient and rewarding goal-directed behaviour.

8.11 Limitations

The most obvious limitations of the work conducted in this thesis concern the use of a mouse model that involved global deletion of a protein that is 1) widely distributed throughout incentive motivational regions involved in the behaviours that were examined, thus making precise neurobiological conclusions difficult 2) that is expressed in different populations of DA receptor expressing cells which, to a certain degree, have different physiological and behavioural responses to DA 3) that has such a complicated phosphorylation profile. These limitations were discussed in greater detail above.

Some limitations relating to the behaviour also merit discussion. In particular, the results from the intertemporal discounting task were difficult to interpret. This was mainly due to the fact that the experiment was not designed to uncover differences in the ability of mice to flexibly adjust behaviour following unpredictable (to the subjects) changes in the parameters, but to establish whether there were consistent differences in within-session discounting. Some of the behavioural differences were either smaller than anticipated (e.g. general PIT) or transient (e.g. intertemporal discounting) which prevented the generation of strong conclusions.

8.12 Human relevance

Studies conducted in humans have established a role for DARPP-32 in the updating of outcome-expectancies (Hämmerer et al., 2013) and performance in complex probabilistic reinforcement tasks (Frank et al., 2007/2009). The work in this thesis is broadly commensurate with those studies in that DARPP-32 KO mice showed disturbances in the probability discounting task and disturbances following the changes in task parameters in the intertemporal discounting task. A preference for risk is associated with pathological gambling (Kräplin et al., 2014), ADHD (Groen et al., 2013) and addiction (Bornovalova et al., 2005; Brand et al., 2008). Likewise, a preference for instant gratification is a hallmark of addiction (Petry, 2001), ADHD (Scheres, Lee & Sumiya, 2008), bipolar and schizophrenia (Ahn et al., 2011). Thus understanding instrumental decisions along dimensions of risk and time has relevance for clinical populations. In addition to this, the relatively inflexible profile of DARPP-32 KO mice suggests this protein might have clinical relevance for compulsive disorders such as OCD and addiction. On the basis that prior research has identified a role for conditioned associations in the stimulation of drug craving (Carter & Tiffany, 1999) and, on the basis that it has been suggested that reward-paired cues might contribute to relapse (Jentsch & Taylor, 1999; Robinson & Berridge, 1993), the findings from the PIT experiment indicate relevance for DARPP-32 in addictive behaviour.

8.13 Recommendations for future research

8.13.1 Regional specific targeting of DARPP-32

Given that the work in this thesis has identified a number of disturbances which add to a growing literature suggesting that DARPP-32 assists in the rapid and flexible modification of reward-related behaviour, it would be beneficial to establish the

neurobiological regions where DARPP-32 expression is essential for the normal performance of the behaviours examined in this thesis. This could be achieved by selectively interfering with DARPP-32 in distinct regions by introducing antisense oligonucleotides to disrupt the translation of DARPP-32 or by disrupting DARPP-32 by viral mediated knockdown. This approach could allow one to compare the behavioural effects of regional interference of DARPP-32. In addition to this, recruitment of mice with targeted interference of specific phosphorylation residues or mice with receptor population specific (e.g. D₁ vs D₂ expressing populations) deletion of DARPP-32 would help identify the molecular and regional bases of these behaviours.

8.13.2 DARPP-32 and intertemporal choices

Owing to the nature of the task and the behavioural differences that were uncovered between DARPP-32 KO and WT mice, it would be beneficial to implement a between-session discounting procedure similar to the one adopted by Acheson and coworkers (2006) in which the delays were manipulated between sessions pseudorandomly. If DARPP-32 KO mice are impaired in the rapid integration of changes in delay parameters, then they should be less sensitive to unpredictable between session changes in delays than WT mice. In ecological settings, intertemporal reinforcement choices are not fixed and can fluctuate during any particular instance. Therefore, DARPP-32 might contribute to the extent that animals can make rapid and efficient intertemporal choices in a fluctuating environment.

8.13.3 DARPP-32 and probabilistic reinforcement choices

Prior research has suggested that certain behavioural disturbances in DARPP-32 KO mice can be rescued by increasing DA transmission (Fienberg et al., 1998; Heyser et al., 2013). It would be interesting then, to train DARPP-32 KO mice on a probability

discounting task until differential performance emerges between genotypes. After establishing differential performance, the introduction of DAergic compounds might then return choice patterns in DARPP-32 KO mice to patterns comparable to WT mice when tested drug free.

8.13.4 DARPP-32 and the exertion of effort

Prior work has established a role for Ser⁹⁷-DARPP-32 alanine knockin mice in the provision of instrumental effort (Stipanovich et al., 2008) and changes in DARPP-32 phosphorylation have been reported in the NAc in relation to the magnitude of effort in an effort-based task (Randall et al., 2012). It might therefore prove fruitful to establish whether genetic interference with DARPP-32 disrupts complex effort-based choice procedures, such as the T-Maze effort-based choice task or the concurrent choice task. DARPP-32 is also expressed in the ACC and this structure has been implicated in effort-based decisions rather than the exertion of effort (Schweimer & Hauber, 2005). In addition to this, it might be beneficial to examine whether DARPP-32 has a role in mediating the effort-choice altering effects of DAergic compounds such as haloperidol and in mediating the exertion of effort in response to DAergic compounds in progressive ratio testing.

8.13.5 DARPP-32, incentive salience and incentive sensitisation

Whilst some of the behaviour in this thesis and, many of those previously reported as disturbed in DARPP-32 mutant mice, involve the adaptation of behaviour in response to change, the absence of general PIT in DARPP-32 KO mice indicates that DARPP-32 is involved in the attribution of incentive salience. It would be beneficial to establish whether DARPP-32 is involved in the performance of outcome-selective PIT. Given the

absence of DARPP-32 in the BLA and its presence in the CeA, these mice might exhibit a dissociation in the ability to perform the outcome-selective and general forms of PIT.

It would also be interesting to examine whether DARPP-32 is involved in other forms of Pavlovian incentive learning such as autoshaping. Autoshaping is sensitive to both DAergic and glutamatergic manipulations (Dalley et al., 2005) and DARPP-32 is expressed in 2 nuclei (e.g. NAcC and CeA) which are necessary for the normal acquisition of autoshaping.

Finally, whilst DARPP-32 KO mice display normal CRf, they develop an exaggerated sensitisation to repeated cocaine administration. Because of these 2 features, it could be beneficial to establish whether the exaggerated psychomotor sensitisation reported in DARPP-32 KO mice translates to a significantly greater sensitisation of responding during the CRf test than WT mice.

Conducting these studies would help us understand whether the disturbances in DARPP-32 KO mice broadly represent a failure to rapidly adjust to changing conditions or whether these mice also display broad-ranging disturbances in incentive salience. For example, autoshaping does not involve a change in a previously learned arrangement in the way that PIT does. Prior to the PIT test, the animals have never experienced the cue in the presence of the instrumental devices and, in order to perform PIT, mice must adapt to the change in the environmental circumstance. However, if DARPP-32 KO mice possess disturbances in autoshaping and/or the potentiation of CRf by drug, then it is most likely that these animals are both impaired in the rapid-updating of behaviour and in acquiring/expressing incentive salience.

8.13.6 DARPP-32 and behavioural flexibility

In light of previous findings (Heyser et al., 2000), as well as those reported in this thesis, it might be beneficial to examine a broader range of behaviours using the DARPP-

32 KO mouse in the domain of behavioural flexibility. These experiments could include set-shifting, as well as probabilistic and/or serial reversal learning.

8.14 Conclusion

In an ecological setting, where the relations between costs and benefits frequently change, maximising reinforcement requires the flexible and efficient allocation of resources by executing profitable cost-benefits computations, by integrating information conveyed by conditioned associations and by modifying reinforcement choices in an environmentally fluctuating setting. Both prior work and the work presented in this thesis suggests that DARPP-32 might be involved in some of these processes. For example, DARPP-32 KO mice struggled to flexibly adapt their intertemporal choices in response to task changes and these mice were also less willing to invest risk during instrumental choice tasks. In addition to this, DARPP-32 KO mice lack the incentive salience driven potentiation of instrumental responding which entails the presentation of Pavlovian stimuli in the general PIT test. Prior work has also established that DARPP-32 mutant mice are less flexible to external change and less capable of summoning the motivation needed to achieve reinforcement in progressive ratio testing. Overall, these data suggest that DARPP-32 contributes to reinforcement optimisation by allowing animals to make flexible reward-based decisions and investments and to integrate Pavlovian associations, and to use the information conveyed by such stimuli to modify instrumental responding. Disruptions to the electrophysiological properties of MSNs most plausibly account for these deficits. Future work should focus on clarifying the nature of these behavioural disturbances and on disentangling the molecular pathways and anatomical locations where DARPP-32 mediates reward-based decision making and incentive salience

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