Investigating the molecular substrates of goal-directed and habitual behaviour

by

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Contents

Abstract

According to contemporary learning theory, instrumental actions are controlled, in a large part, by two dissociable systems. Whilst goal-directed actions are driven by an explicit awareness of an anticipated outcome, habitual responses are the product of previously reinforced associations between contextual stimuli and salient outcomes. Although experimental lesion studies and pathological disorders, such as drug addiction and Parkinson's disease, have advanced our knowledge of the brain circuitries important in the regulation of instrumental behaviour, we still do not have a full understanding of the executive mechanisms regulating the expression of these behaviours or the neural processes which underlie the shift from flexible goal-directed actions to stimulus-bound habits that occurs naturally following repeated practice.

The present work focused specifically on the molecular mechanisms involved in this shift. The first experimental section, Chapter 3, established a protocol for modelling goal-directed and habitual instrumental responding in rats. Experiment 1 measured the sensitivity of lever press responding to changes in outcome value, as an index of goal-directed behaviour, in groups of rats sourced from either Charles River or Harlan. After three sessions of instrumental training, only rats sourced from Charles River and devalued in the same context that they were trained in, rather than in a different context, reduced responding for a devalued outcome. Building on these findings, in Experiment 2 a separate group of rats, sourced from Charles River, was trained to level press over 10 sessions. In contrast to the first experiment, lever press responding at test in this group of animals was insensitive to outcome devaluation.

These initial experiments demonstrated evidence consistent with a training-induced shift from goal-directed to habitual instrumental behaviour and were used to inform the design of a second set of experiments, described in Chapter 4, in which region-specific differences in gene expression were compared across groups of rats with different levels of lever press experience. DNA microarray analysis of tissue samples from the dorsolateral (DLS) and posterior dorsomedial striatum (pDMS), using Rat Gene 2.0 ST Affymetrix arrays, revealed both training group- and brain region-specific effects. Evidence of a high proportion of non-linear regulation profiles across the training groups was indicative of experience-dependent shifts in gene expression; furthermore, the high degree of separation between the training-dependent expression profiles of the DLS and pDMS points to the dynamic engagement of distinct, region-specific regulatory networks over the course of instrumental learning.

The experiments presented in Chapter 5 went on to investigate the role of epigenetic mechanisms, specifically histone acetylation, in the regulation of instrumental behaviour by using the histone deacetylase inhibitor sodium butyrate (NaB) to interfere with endogenous chromatin remodelling processes. The effects of systemic injections of NaB on the sensitivity of lever press behaviour to changes in outcome value were assessed in three separate studies, each designed to target a different stage of learning. NaB had no effect on the acquisition or consolidation of goal-directed behaviour. However, after three sessions of training, the instrumental behaviour of

animals receiving an injection of NaB prior to exticntion test was less sensitive to reinforcer devaluation relative to controls, suggesting that histone acetylation may be involved in the retrieval phase of instrumental learning.

In an attempt to address some of the limitations of systemic drug administration, the final experimental chapter, Chapter 6, describes an initial exploratory investigation into the effects of administering NaB directly into the brain using a microinfusion protocol. Western blot analysis showed an increase in histone H4 acetylation in the mPFC following infusions of NaB targeting the infralimbic cortex. Behavioural data suggested that NaB may act to ameliorate some of the damaging effects of the infusion procedure, with NaB-treated animals showing enhanced sensitivity to outcome devaluation relative to controls. These preliminary data act as proof of principle for the development of a microinfusion protocol for studying the role of histone acetylation in instrumental learning and highlight a number of practical issues which will be addressed in ongoing work.

Taken together the experiments presented in this thesis provide evidence for the existence of discrete gene expression changes associated with minimal or extended training regimes, and for highly specific effects of a systemically administered HDACi on components of instrumental behaviour. These data offer new insights into the molecular mechanisms involved in the regulation of instrumental learning in rodents and provide a starting point for further investigations into the role that epigenetic processes may play in the neural plasticity underlying the transition from goal-directed to habitual behaviour.

Chapter 1

General Introduction

Try to remember your drive home today; think about the deceptively simple, but really quite complex, sequence of actions that enabled you to start your car and operate it to get to your destination. Chances are, if you are an experienced driver, you probably weren't even fully conscious of making the necessary actions as you were doing them. Maybe you were planning what to have for dinner, or trying to remember whether it was bin day or not? Now imagine that the manual car you have driven for years needs a service and you are given an automatic to drive until it is ready. It is likely that you will need to think more carefully about your actions when you first drive this car and, initially, may even find your left foot reaching for the clutch. However, eventually, with practice, you will be able to drive the automatic with the same ease as your familiar manual.

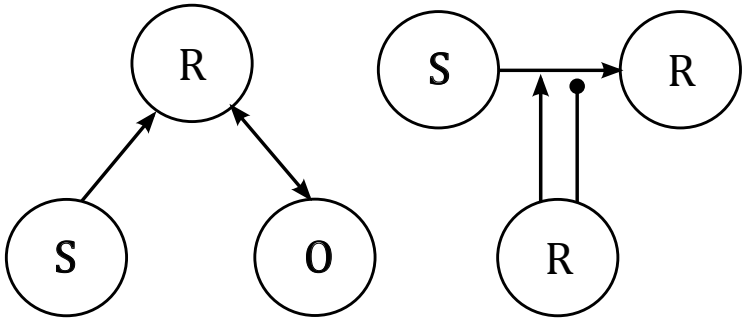
This everyday example illustrates the fact that the more a given action is performed the more automatic, or 'habitual', it becomes. Whilst this process allows us to carry out routine tasks rapidly, using minimal cognitive resources, the common aphorism *"Practice makes perfect"* belies the fact that such habitual behaviours are not always appropriate, as the above example demonstrates. Therefore, a system is required in which well practiced responses to familiar situations can be adapted to accommodate

new environments and changing motivational needs. Loss of such behavioural flexibility has been implicated in disorders such as obsessive compulsive disorder (OCD) and drug addiction (Everitt and Robbins, 2005; Everitt et al., 2008; Fontenelle et al., 2011), highlighting the importance of attempting to understand the processes involved in the transition from flexible, purposive actions to reflexive, stimulus driven responses and the executive mechanisms that monitor and allow switching between the two forms of behaviour.

1.1 Dissociable systems for instrumental behaviour: actions and habits

Historically, instrumental behaviour was thought to reflect the probabilistic association of environmental stimuli with specific outcomes (Thorndike, 1911; Skinner, 1938). However, there is now clear evidence that instrumental responses can be moderated by their consequences (Adams and Dickinson, 1981; Adams, 1982). Thus, according to contemporary learning theory, instrumental actions are controlled by at least two dissociable systems, which interact to provide the required behavioural flexibility described above. Whilst goal-directed actions are driven by an explicit awareness of an anticipated outcome, habitual responses are the product of previously reinforced associations between contextual stimuli and salient outcomes (Dickinson, 1985). Crucially, because the outcome is not encoded in the stimulus-response (S-R) associations underlying habitual behaviour, responding is controlled solely by antecedent stimuli, independent of any explicit motivation to obtain a specific 'goal' (see Figure 1.1.1 b). Conversely, as shown in Figure 1.1.1 a, the associative structures underlying goal-directed actions contain a representation of the expected outcome and its causal association with the action (Balleine and Dickinson, 1998). This means that, whilst goal-directed responses are sensitive to changes in both the

action-outcome (A-O) contingency (i.e. contingency learning) and outcome value (i.e. incentive learning), habits are not (Balleine and Dickinson, 1998). Thus, manipulations of these factors, action-outcome contingency and outcome value, have been used to experimentally dissociate the two types of instrumental responding in animal models.



(a) Structure of action-outcome associations

(b) Structure of stimulus-response associations

Figure 1.1.1: Graphical representation of the associative structures underlying goal-directed (a) and habitual (b) responses. O: Outcome; R: Response; S: Stimulus.

Manipulations of instrumental outcome value typically involve devaluation of the outcome through specific satiety, achieved by pre-feeding, or through lithium chloride-induced conditioned taste aversion. An animal performing in a goal-directed manner will reduce instrumental responding for a devalued outcome, whilst animals performing habitually will continue responding at rates comparable to those of animals for which the outcome is still valued. Importantly, tests of the effect of outcome devaluation on responding are conducted in extinction (i.e. in the absence of reward delivery). Sensitivity of responding to changes in outcome value in the absence of any consummatory feedback indicates that the outcome is explicitly encoded in the associative structure driving behaviour and that the representation of the outcome is updated to reflect the change in value (Balleine and O'Doherty, 2009). Similarly, manipulations of the causal association between an action and its outcome can be achieved by introducing non-contingent, "free" rewards. Degradation of the causal

contingency between a response and its outcome in this way is predicted to result in a reduction in goal-directed responding, but will not influence habitual responding (Balleine and Dickinson, 1998).

A number of studies have used these manipulations to investigate the learning conditions favouring the expression of goal-directed and habitual responding respectively. Early work suggested that it was simply the number of times an action had been performed that determined whether it was goal-directed or habitual. For example, Adams (1982) showed that animals that made 100 rewarded lever presses during training were sensitive to outcome devaluation, whilst animals that performed 500 rewarded presses were not. However, Dickinson (1985) later argued that it is not the number of rewarded responses an animal has made that determines the nature of instrumental behaviours, but rather the experienced correlation between response rate and reward rate. On a continuous reinforcement schedule, where each lever press is rewarded, there is a perfect correlation between response rate and reward rate. However, because the variability in response rate across training sessions declines with increased training, the range of response rates across which the animals experience the instrumental contingency also decreases. Because awareness of the instrumental contingency is critically dependent on the experience of a variation in behaviour giving rise to a variation in reward rates, the reduced variability in responding with increased training means that the perceived correlation between action and outcome decreases. Thus, Dickinson (1985) claims, behaviour becomes less dependent on this relationship with the result that changes in action-outcome contingency and outcome value do not impact on responding.

Support for this theory comes from evidence that animals display habitual performance much earlier on in training when trained on an interval schedule of reinforcement, where outcome delivery is time-bound, than when trained on a ratio schedule, where outcome delivery is response-bound. Whilst ratio schedules maintain a lin-

ear response-outcome relationship, under interval schedules this correlation varies with response rate. Thus, at low levels of responding the correlation between responses and rewards is relatively high. However, at higher levels of responding, variability in response rate has less effect on outcome rate, weakening the experienced response-outcome relationship. Therefore, Dickinson (1985) argues, whilst over-training can lead to habitual responding, it is this weakening of the experienced response-outcome relationship which is the crucial factor underlying this transition rather than increased experience of this relationship per se. Despite this, more recent work has shown that rats do display goal-directed instrumental behaviour after training on interval schedules of reinforcement, but only if training is minimal (Dickinson et al., 1995; Nelson and Killcross, 2006). For example Nelson and Killcross (2006) demonstrated that, after only 120 exposures to the action-outcome contingency across three sessions of training on a random interval reinforcement schedule, lever press responses remained sensitive to outcome devaluation by lithium chloride (LiCl).

In the context of human behaviour, habits are often viewed as a form of procedural memory. A study by Tricomi et al. (2009) demonstrated that humans exhibit the same insensitivity to outcome devaluation following extensive training as rodents. In this study subjects were trained to respond to different symbols by pressing specific buttons in order to 'earn' small amounts of one of two palatable snack foods. At the end of training subjects were allowed to eat one of the snacks food to satiety before completing a final test stage of button pressing. Like the rats in the studies presented in the preceding section, human subjects that had received only a few sessions of training reduced responding on the button associated with the snack they had just consumed. Conversely, after extensive training, levels of button pressing were comparable for the two foods. Similarly, human subjects also exhibit sensitivity to contingency degradation, although the effects of over-training on this sensitivity

remain to be investigated (Balleine and O'Doherty, 2009).

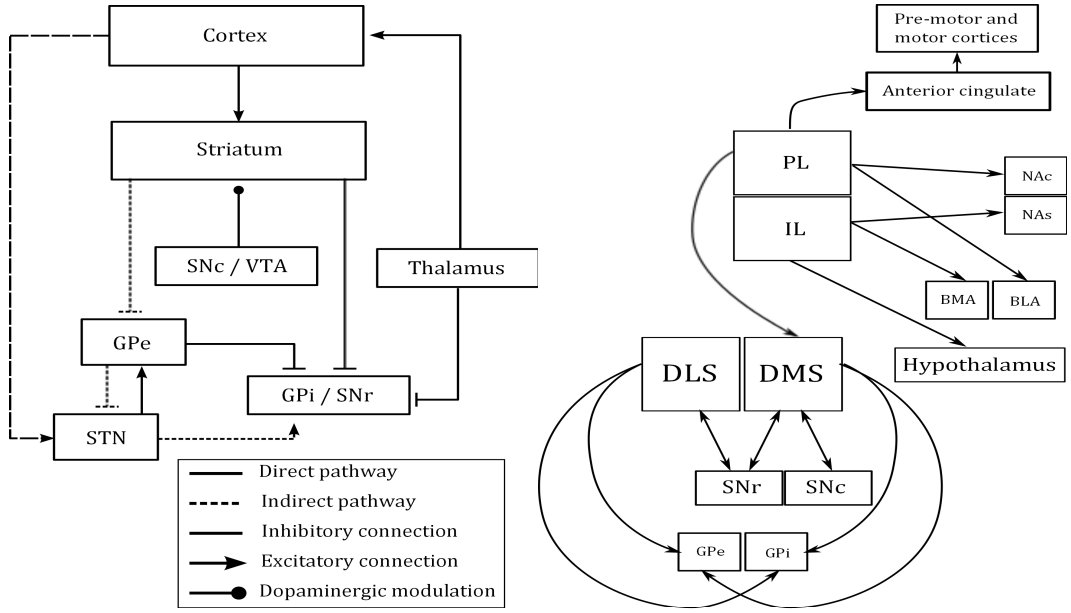
1.2 Functional neurobiology of actions and habits

The establishment of a robust set of behavioural tasks for studying goal-directed and habitual responding has enabled the investigation of the respective neurobiological mechanisms mediating these behaviours. Using a mixture of experimental studies in animal models and clinical observations, it has been possible to start to explore the functional, neuroanatomical and neurophysiological substrates underlying these psychologically distinct components of instrumental behaviour.

1.2.1 Functional Neuroanatomy

Several key brain areas and circuitries have been highlighted by a series of lesion studies investigating the anatomical substrates of instrumental learning, in particular structures of the basal ganglia, the medial pre-frontal cortex (mPFC) and sensorimotor regions (Killcross and Coutureau, 2003; Yin and Knowlton, 2006). The following section provides an overview of the structural and functional architecture of these key regions and outlines the main findings to date regarding the proposed role they may play in instrumental behaviour.

Figure 1.2.1a shows a simplified circuit of the major connections between structures of the basal ganglia and cortical regions. The basal ganglia are a collection of brain nuclei which includes the striatum, the pallidum (globus pallidus and ventral pallidum), the substantia nigra and the subthalamic nucleus. These nuclei are found at the base of the forebrain and are highly conserved across vertebrate species (Medina and Reiner, 1995; Wicht and Northcutt, 1998). A series of topographical loops connect the structures of the basal ganglia with the neocortex, forming distinct functional units that have been implicated in a number of cognitive and



(a) Schematic diagram of basal ganglia connectivity (adapted from Yin and Knowlton, 2006)

(b) Simplified illustration of major mPFC and DS connections

Figure 1.2.1: Illustration of the major anatomical connections involved in instrumental motor learning. BMA/BLA: basomedial and basolateral amygdala respectively; DS: Dorsal striatum; DLS/DMS: dorsolateral and dorsomedial striatum respectively; GPe/GPi: globus pallidus external and internal respectively; mPFC: medial prefrontal cortex; NAc/NAs: nucleus accumbens core and shell respectively; IL: infralimbic cortex; PL: prelimbic cortex; SNr/SNc: substantia nigra pars reticulata and pars compacta respectively; STN: subthalamic nucleus; VTA: ventral tegmental area.

behavioural processes (Middleton and Strick, 2000). These parallel, semi-closed circuits are thought to communicate with each other, in part, via striatal interneurones and Nucleus Accumbens core (NAc) projections to the dorsal striatum via Substantia Nigra pars compacta (SNc)/Ventral tegmental area (VTA) dopaminergic fibers (Pennartz et al., 2009).

At least two distinct corticostriatal pathways have been delineated. The direct pathway comprises of inhibitory striatal projections to the Globus pallidus internal (GPi) which act to reduce pallidal GABAergic inhibition in associated cortical regions. Conversely, the indirect pathway, which connects with the GPi via inhibitory connections from the Globus pallidus external (GPe) and subthalamic nuclei (STN) increases pallidal inhibitory output to the cortex (Alexander and Crutcher, 1990).

The activity of these circuits is modulated by dopamine (DA) signals from the SNc and the VTA (Joel and Weiner, 2000). More recently a hyperdirect pathway connecting cortical regions directly to the STN has been described (Nambu et al., 2002).

Of all the basal ganglia structures, the striatum has received the most attention to date in relation to instrumental behaviour. In both rodents and primates the striatum is grossly divided into ventral and dorsal regions. The ventral striatum modulates the activity of dorsal regions via a series of spiralling connections (Alexander et al., 1986, 1990; Miyachi, 2009). The majority of the ventral striatum is comprised of the nucleus accumbens and receives primarily limbic inputs (Kunishio and Haber, 1994; Groenewegen et al., 1996). In primates, the dorsal striatum has traditionally been subdivided further, along the boarder of the internal capsule, into the caudate and putamen . However, this purely structural division does not map directly on to the functional dissociations described in the literature (Cragg et al., 2002). Instead, the more rostral portion of the putamen, which receives projections from the prefrontal association cortex, is commonly referred to as the associative striatum, whilst the more caudal part of the putamen, which receives cortical projections from sensorimotor areas, is referred to as the sensorimotor striatum (Joel and Weiner, 2000). In rodents these subdivisions appear to correspond, at least in terms of functional connectivity, to the dorsomedial (DMS) and dorsolateral striatum (DLS) respectively (Alexander et al., 1990; Groenewegen et al., 1990). In addition to their cortical inputs, these two regions differ in the dopaminergic inputs they receive (Gerfen et al., 1987), their receptor expression patterns and the mechanisms of plasticity that they exhibit (Joel and Weiner, 2000; Partridge et al., 2000).

Medium spiny neurones (MSNs), the predominant cell type in the striatum, can be grouped into two classes based on the ratio of D1 and D2 dopamine receptor subtypes they express. These two neuronal subgroups appear to play opposing roles in DA-dependent glutamatergic plasticity in the striatum. Whilst D1 MSNs are associated

with DA-dependent long-term potentiation (LTP), D2 MSNs are associated with DA-dependent long-term depression (LTD; Reynolds et al., 2001; Shen et al., 2008). Moreover, Burkhardt et al. (2009) used receptor sub-type specific antagonists to dissociate the effects of D1 and D2 receptor blockade on basal ganglia neural activity. Blocking D1 activity significantly reduced neuronal synchrony in the striatum, whilst blocking D2 activity reduced both the firing rate of MSNs and the strength of local field potential oscillations, highlighting the different effects of direct and indirect pathway activation.

Structures of the basal ganglia have long been implicated in motor control (Mink, 1996). Even as early as 1664 the striatum was referred to as the *sensorium commune*[1] by anatomist Thomas Willis (Parent, 1986). More recently, the advancement of ideas regarding the nature of the associative processes driving instrumental behaviour, combined with ever growing anatomical knowledge, have significantly increased our understanding of the role that the striatum and other structures in the basal ganglia play in the acquisition, expression and control of motor and cognitive behaviour (Balleine et al., 2007).

Receiving diverse projections from the association and premotor cortices and mid-brain dopamine neurons and sending projections to the sensorimotor cortex, the striatum is well placed for incentive learning and motor control (Nakano et al., 2000; Nakano, 2000). Early work demonstrated that striatal lesions prevented the acquisition and expression of instrumental actions. Specifically, these early studies suggested that damage to the striatum blocked the ability to link instrumental actions to their consequences (McDonald and White, 1993).

More recently, however, this view has been refined following evidence that sub-regions of the dorsal striatum play dissociable roles in behavioural control. For example, an-

[1] A term first used by Aristotle to describe the locus of communication between sensory and motor processes

imals with pre-training lesions of the posterior region of the dorsomedial striatum (pDMS) never exhibit sensitivity to either outcome devaluation or degradation of an instrumental contingency (Yin et al., 2002). Furthermore, lesions and temporary inactivation of this region *after* training also blocks sensitivity to devaluation and contingency degradation, indicating that the pDMS is required for both the acquisition and expression of goal-directed behaviour (Yin et al., 2005; Corbit et al., 2012). Conversely, the lateral part of the dorsal striatum (DLS) appears to be involved in S-R learning. Animals with lesions to this region remain goal-directed after being trained on a reinforcement schedule that produced habitual responding in control animals (Yin et al., 2002) and temporary inactivation of this region post-training has been shown to reinstate goal-directed responding for drug and alcohol rewards (Corbit et al., 2012; Zapata et al., 2010). Similar results have been obtained by inactivating homologous regions in the non-human primates (Miyachi et al., 1997).

The data from rodent and non-human primate studies is supported by functional imaging studies in humans. The anterior portion of the caudate appears to play a homologous role to the DMS in rodents in the control of goal-directed behaviour (Balleine and O'Doherty, 2009). In particular, this portion of the associative striatum appears to be involved in encoding action-outcome contingencies (Tricomi et al., 2004). Conversely, Tricomi et al. (2009) found an increase in task related activity in the posterior dorsolateral region of the putamen with extended training. Importantly, this region appeared to be specifically involved in processing behaviourally relevant stimuli, indicating a role in S-R learning. Interestingly, both associative and sensorimotor striatal regions are active early on in training, indicating that the processes underlying the acquisition of goal-directed and habitual responding occur concurrently at the start of training (Brovelli et al., 2011). Further support for a functional dissociation between striatal subregions in instrumental learning comes from other neuroimaging studies, not explicitly looking at habit formation, which have shown

an increasing involvement of the sensorimotor striatum (DLS homologue) with the development of automatic motor skills (Lehéricy et al., 2005; Poldrack et al., 2005).

Pre-frontal regions, most notably the medial PFC (mPFC), have been shown to be involved in the more executive aspects of instrumental control (Killcross and Coutureau, 2003). The two regions that have received the most attention in relation to instrumental learning are the prelimbic cortex (PL) and the more ventral infralimbic cortex (IL). Using retrograde tracers, studies in rats have shown that these two sub-regions have different patterns of connectivity (see Figure 1.2.1b), consistent with their apparent oppositional role in instrumental learning. The PL projects primarily to the basolateral amygdala (BLA; McDonald et al., 1999), lateral and posterior hypothalamus (Jones et al., 2011) ventral striatum (NAc; Gorelova and Yang, 1997), the premotor cortex, via the anterior cingulate (Lu et al., 1994), and the DMS (Berendse et al., 1992), whilst the IL sends afferents mainly to the Nucleus Accumbens shell (NAs), dorsomedial hypothalamus and the basomedial amygdala (Sesack et al., 1989; Hurley et al., 1991; Vertes, 2004). In addition to these external projections, the IL and PL share extensive interconnected internal connections (Fisk and Wyss, 1999).

Pre-training lesions of the entire mPFC block the expression of goal-directed responding (Ostlund and Balleine, 2005). This finding is recapitulated in animals with lesions restricted to the PL (Killcross and Coutureau, 2003; Balleine and Dickinson, 1998). However, lesions of the more ventral IL have the opposite effect, preventing the transition to habits (Killcross and Coutureau, 2003). Interestingly, whilst post-training lesions of the PL cortex have no effect on behaviour (Ostlund and Balleine, 2005), post-training inactivation of the IL cortex reinstates goal-directed responding in extensively trained animals (Coutureau and Killcross, 2003). Together these findings suggest that, whilst the PL is necessary for the acquisition of A-O associations, the IL is involved in the transition to habitual responding that occurs with over-

training. Importantly, these studies indicate that this transition is not an absolute process; S-R associations do not come to replace A-O associations over the course of training, rather, both associative structures may be acquired in parallel and it is their relative influence over behavioural output that changes across the course of training. Indeed Tricomi et al. (2009) reported that, in human subjects, ventromedial PFC (vmPFC) activity did not diminish with over-training and interpret this as evidence that the associative processes underlying goal-directed and habitual behaviour may be simultaneously activated but the relative balance of activation determines behavioural output.

Building on the findings of the lesion studies in rodents discussed above, researchers have started to investigate the neural pathways mediating communication between cortical and subcortical regions in the co-ordination of instrumental behaviour. For example, Bradfield et al. (2013) have shown that altering the activity of the thalamic cholinergic input to the pDMS disrupts goal-directed behaviour and impairs the ability to update previously learnt action-outcome contingencies. Conversely, connections between the amygdala central nucleus (CeN) and the DLS are required for habit formation (Lingawi and Balleine, 2012). In humans, a fascinating study by de Wit et al., 2012 showed that differences in the structure of specific corticostriatal white matter pathways are associated with individual differences in instrumental behaviour. Using diffusion tensor imaging (DTI), they found that the integrity of white matter tracts connecting premotor cortical regions to the posterior putamen was positivity correlated with a bias towards habitual responding, whilst the integrity of connections from the vmPFC to the caudate was related to goal-directed behaviour. These, and similar studies (e.g. Faure et al., 2005; Lex and Hauber, 2010), highlight the importance of complex corticostriatal connections and basal ganglia pathways in the control of instrumental behaviours.

1.2.2 Neural activity and cellular plasticity

Electrophysiological studies have provided further insights into the neural mechanisms underlying the regulation of goal-directed and habitual responding. Although much of the research to date has focused on the learning of simple motor responses, rather than on more complex instrumental behaviours, evidence from studies comparing patterns of neural activity in the dorsal striatum during the early and later stages of training are clearly of relevance to the study of instrumental habit formation.

During motor skill learning there is a rapid increase in task-related corticostriatal activity early on in training which is followed by more gradual changes during the later stages of training (Costa et al., 2004). Importantly, although the absolute proportion of task-related neuronal firing in the striatum remains relatively stable after this initial rise, there is evidence that the number of neurons correlated with particular aspects of task performance changes over the course of training, indicating striatal activity patterns continue to exhibit a degree of plasticity with the repeated performance of an action (Costa, 2007). For example, the proportion of neurones exhibiting activity related to movement speed increases substantially with training, which, Costa et al. (2004) suggests, may reflect a gradual refinement of neural responses in this region to task demands. Together, these findings point to an immediate, large-scale recruitment of striatal neuronal populations following the initial acquisition of a motor response, followed by gradual organisation and refinement of task-related neural processing (Costa, 2007).

Importantly, the functional dissociation between the DLS and DMS, highlighted by the lesion work discussed previously, is further reinforced by evidence of region-specific changes in task-related neural activity in these striatal subregions. For example, there is some evidence that task related activity in the DLS decreases with training (Carelli et al., 1997; Tang et al., 2007, 2009), perhaps reflecting an increasing

efficiency in circuits subserving habit learning (Costa, 2007). Similarly, recordings taken from the brains of non-human primates have shown that the proportion of task specific neurons firing early on in procedural learning was greater in the DMS than in the DLS, whilst the reverse was true later on in training (Miyachi et al., 2002). Extending this finding, Thorn et al. (2010) showed that there is also a marked difference in learning-related changes in activity between the two regions; whilst task relevant activity in the DLS gradually increased over the course of training, firing patterns in the DMS followed an inverted-u shaped function, increasing during the early stages of training but decreasing as training progressed.

Others claim that the co-ordination of goal-directed and habitual actions in the dorsal striatum is the result of qualitative changes in the *nature* of task-relevant activity over the course of training, rather than of gross changes in neural activity levels. For example, Jog et al. (1999) reported a decrease in 'response' related firing and an increase in firing to cues associated with trial start and end points (what Schultz (2003) refers to as "movement initiating" stimuli [pp. 325]) in a spatial learning task and argues that this may reflect a shift away from response-focused learning towards the context-driven learning that characterises motor habits. This interpretation is supported by evidence from Kimchi et al. (2009) showing that DLS neuron firing rate can be modulated by an auditory cue signalling reward delivery. Thus, consistent with a role for this region in stimulus-driven habitual responding, neural activity in the DLS appears to be modulated by the presentation of a conditioned stimulus. Conversely, behavioural adaptation to a new reward contingency is preceded by increases in contingency-related activity in the DMS (Kimchi and Laubach, 2009), again consistent with the the lesion data positing a role for this region in action evaluation and flexible responding. This evidence of dissociable, experience-dependent changes in ensemble activity in dorsal striatal sub-regions points to a shift in the relative weight afforded to different aspects of task-relevant information. Furthermore,

taken together with the data from lesion studies, these findings strongly suggest that distinct neural circuits are involved in the control of goal-directed and habitual responses respectively, and that the relative influence of these circuits over a given action can vary as a function of experience.

1.2.3 Molecular signalling pathways

The data reviewed above strongly implicate corticostriatal plasticity in the regulation of instrumental learning. By targeting the molecular mechanisms underlying this plasticity, it has been possible to directly investigate whether these processes play a necessary causal role the co-ordination of goal-directed and habitual actions.

The interaction of dopamine and glutamate signalling plays a central role in striatal plasticity and has been shown to mediate both long term potentiation (LTP) and long term depression (LTD) within striatal circuits via an N-Methyl-D-aspartate receptor (NMDAR) dependent mechanism (Calabresi et al., 1992b; Lovinger et al., 2003; Lovinger, 2010). Consistently, manipulations targeting NMDARs have been shown to have both brain region-specific and experience-dependent effects on instrumental responding. For example, NMDAR antagonists impair goal-directed Pavlovian learning when infused into the NAc (Dalley et al., 2005). Similarly, when infused into the pDMS, NMDAR antagonists block striatal LTP and prevent goal-directed instrumental responding (Yin et al., 2005). Conversely, DA neuronal-specific knock-down of NMDAR1 expression in mice impairs habit formation (Wang et al., 2011a), highlighting the brain-region specific roles that NMDA signalling plays in co-ordinating instrumental behaviour.

Interestingly, it has been shown that ethanol exposure impairs NMDAR-dependent LTP in the DMS in a dose dependent manner and, at high doses, ethanol not only blocks striatal LTP but leads to an extended period of depressed activity in the DMS (Yin et al., 2007). It remains to be seen whether or not these changes in

plasticity effect behaviour, but there is evidence that alcohol consumption facilitates habit formation (Corbit et al., 2012; Ostlund et al., 2010). Similarly, exposure to psychostimulant drugs, such as cocaine and amphetamine, has been shown to both effect structural plasticity in the striatum (Gerdeman et al., 2003; Canales, 2005; Jedynak et al., 2007) and to alter the nature of instrumental behaviour (Schoenbaum and Setlow, 2005; Nelson and Killcross, 2006).

Both psychostimulants and alcohol[2] alter dopamine signalling in the brain (Grace, 2000; Letchworth et al., 2001; Dalley and Everitt, 2009) and these changes may underlie the parallel effects of these drugs on instrumental behaviour (Everitt et al., 2001; Everitt and Robbins, 2005). Indeed, dopaminergic signals from the VTA and SNc are a crucial moderator of plasticity in the basal ganglia (Calabresi et al., 1992a,b) and have been shown to play a central role in both Pavlovian and instrumental learning (Costa, 2007; Dalley and Everitt, 2009).

Choi et al. (2005) and others (see Costa, 2007 for a comprehensive review) have shown that, whilst blocking D1 activity early on in training impairs Pavlovian responding, responses become more resistant to D1 blockade following extensive training. Consistently, DA neuron responses to appetitive stimuli decrease with repeated presentation (Ljungberg et al., 1992). There is a lack of agreement regarding the mechanisms underlying this shift towards DA-independence; whilst some argue that it reflects a strengthening of task-relevant connections within corticostriatal networks (Wickens et al., 2007), others have suggested that the associative processes driving habitual behaviour may come to be mediated solely by cortical pathways, without the involvement of striatal dopaminergic pathways (Ashby et al., 2007).

Despite evidence that established habitual behaviours are DA-independent, dopamine signalling does appear to be required for the *acquisition* of S-R responses both in

[2]The effect of alcohol on dopamine transmission are indirect: ethanol effects VTA DA-neuron activity via it's affect on GABAergic afferents (Xiao and Ye, 2008).

animal models and in humans (Costa, 2007). For example, the death of DA neurones in the SNc that occurs in Parkinson's disease (PD) has been associated with impairments in S-R learning (Knowlton et al., 1996). Conversely, as noted above, chronic exposure to psychostimulant drugs accelerates habit formation, an effect which may reflect the sensitizing effects these drugs have on the dopaminergic system (Nelson and Killcross, 2006; Schoenbaum and Setlow, 2005). Indeed, similar results have been observed following intrastriatal infusion of DA-agonists (Packard and White, 1991). Therefore, whilst over-training appears to lead to a gradual independence of instrumental actions from DA transmission, DA is required for the initial learning of S-R associations.

Importantly, the role of dopamine in the regulation of instrumental behaviour is region-specific. 6-hydroxy dopamine[3] (6-OHDA) lesions of the pDMS and the prelimbic cortex impair goal-directed responding (Naneix et al., 2009; Lex and Hauber, 2010), whilst 6-OHDA lesions to the lateral portion of the nigrostriatal pathway block the expression of habitual responses (Faure et al., 2005). Furthermore, the effects of blocking DA transmission in different brain regions on instrumental behaviour depends on the extent to which the behaviour has been trained. Blocking DA activity in the pDMS, but not the DLS, early on in training renders responding habitual. Conversely, blocking DA transmission in the DLS late on in training, when behaviour would normally be habitual, results in goal-directed responding for cocaine, whilst pDMS DA-blockade has no effect (Murray et al., 2012).

These complex findings have provided a starting point for investigations into the downstream signalling pathways involved in the co-ordination of goal-directed and habitual behaviour. Concurrent activation of D1 and NMDA receptors in the dorsal striatum activates the extracellular signal-related kinase (ERK) pathway, a sig-

[3] A chatecholaminergic cell-specific neurotoxin usually used in conjunction with a noradrenaline reuptake inhibitor to specifically target populations of dopaminergic cells

nal transduction pathway known to play a central role in corticostriatal plasticity (Valjent et al., 2005), making it an obvious target for researchers interested in the molecular processes regulating instrumental behaviour (Shiflett and Balleine, 2011a).

Indeed, ERK activation in the dorsal striatum increases following instrumental training (Shiflett et al., 2010) and infusions of the ERK-inhibitor, U0126, into the pDMS, both before and after minimal instrumental training, block the expression of goal-directed responding (Shiflett et al., 2010). Surprisingly, the same study found that goal-directed behaviour was also disrupted by post-training infusions of U0126 into the DLS but was unaffected by pre-training infusions targeting this region. Taken together, these results suggest that whilst the acquisition of new A-O associations depends on ERK signalling in the DMS, but not the DLS, the expression of goal-directed requires intact ERK signalling in both of these areas.

1.2.4 Transcriptional changes associated with instrumental learning

The importance of ERK signalling in the acquisition and expression instrumental behaviour is likely due to its role in regulating the expression of plasticity-related transcription factors such as CREB (Xing et al., 1996), yet, despite the wealth of evidence relating to the molecular and cellular mechanisms underlying habit formation, still very little is known about the processes involved in instrumental learning at a transcriptional level. Consequently, one of the main aims of this thesis was to develop approaches to examine training and brain region dependent changes in the transcriptome that may give an insight into key regulatory gene networks operating over the course of instrumental learning.

As putative markers of neural activity, immediate early genes (IEGs) have been the focus of initial investigations and several of these genes have been shown to exhibit learning stage-dependent expression patterns in the brain. Typically, it has been observed that levels of IEG activation peak early on in training and decrease following

extended training (Hess et al., 1995; Bertaina-Anglade et al., 2000; Guzowski et al., 2001). For example, learning-dependent c-fos activation is only observed in the early stages of training; once asymptotic performance levels are reached c-fos levels in the brain return to baseline levels (Bertaina-Anglade et al., 2000). However, Faure et al. (2006) showed that the expression pattern of the IEG Fra-1 in the SNc/VTA remained elevated following over-training, a finding that is consistent with the literature positing a central role for intrastriatal dopamine release in habit formation (Faure et al., 2005). Furthermore, Hernandez et al. (2006) reported changes in the expression of the plasticity-related IEGs Homer 1a and Zif268 in corticostriatal circuits following extensive training. Such studies, although limited in number, have provided valuable insights into the dynamic shifts in corticostriatal function that occur across training and illustrate how the molecular processes underlying habit formation can be explored by mapping behavioural changes on to changes in gene expression.

1.2.5 Summary

To summarize, with extended training instrumental behaviour becomes divorced from its consequences. These habitual behaviours are independent of both the probabilistic contingency between a given response and its outcome and the motivational value of this outcome. The psychological dissociation between purposive, goal-directed actions and inflexible, automatic habits is supported by a wealth of neurobiological evidence indicating that these two types of behaviour are driven by partially dissociable, region-specific, molecular and cellular mechanisms. In particular, the plasticity underlying the shift from associative- to sensorimotor-circuit control of behaviour following repeated training appears to rely on the interaction of dopaminergic and glutamatergic signals within discrete parallel corticostriatal circuits. However, although initial attempts have been made to explore the changes in gene expression that occur over the course instrumental training, the transcrip-

tional correlates of goal-directed and habitual behaviour remain relatively poorly understood.

1.3 Epigenetics

Undoubtedly, instrumental learning involves changes in gene expression and understanding the nature of these changes, and how they are regulated, is an important next step in advancing our understanding of the pathways connecting exogenous experience (i.e. training) with changes in brain function and, ultimately, behaviour. The epigenetic modification of chromatin is a key mechanism by which gene expression is controlled, and, therefore, studying the molecular correlates of instrumental learning may provide important insights into the mechanisms underlying the transition from goal-directed to habitual behaviour.

It has long been known that heritable epigenetic marks play a crucial role in cellular differentiation during development. Indeed, Conrad Waddington, who first coined the term epigenetics, was interested in the processes involved in determining cell fate during embryonic development (Waddington, 1939). Waddington was the first to formally discuss the failings of genetic theory, at that time, in explaining how cells carrying exactly the same genetic material developed to carry out such a vast and diverse range of biological functions. He used the analogy of marbles rolling down a hill to illustrate the idea of the increasing irreversibility of cellular differentiation and defined epiegentics as *"the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being*" (Waddington, 1942, pp 18-20; see Figure 1.3.1).

Waddington's broad ideas about of the interaction between genotype and environment have been extended and refined over the years and, in its current usage, the term epigenetics refers to the processes involved in regulating gene expression, either through direct marking of DNA (e.g. methylation) or changes to chromatin struc-

ture (e.g. post-transcriptional modification of histone proteins). Crucially, these molecular modifications do not involve changes to the genetic sequence itself; rather epigenetic modifications occur, literally, on top of the genome, serving to alter gene expression in terms of what is expressed, how much is expressed, and when and where gene expression occurs. Whilst there is still considerable interest in epigenetics from a developmental (Kiefer, 2007) and pathological perspective (in particular cancer biology e.g. Jones and Baylin 2002), growing evidence of dynamic, experience-driven epigenetic changes in neuronal cells has captured the interest of neuroscientists studying the molecular mechanisms mediating behaviour (see sections 1.3.3 for more detail).

As noted above, there are two major classes of epigenetic mechanisms, those that act directly on DNA by methylating nucleotides and those that act indirectly by altering the interaction between structural histone proteins and their associated regions of DNA and thus altering the accessibility of regions of DNA to transcriptional proteins. A number of post-translational histone modifications (PTHM) have been identified including: acetylation, methylation, phosphorylation, sumoylation and ubiquitylation. Each of these modifications play a different role in gene expression and their regulation by specific enzyme groups has been implicated in an array of cellular processes (Kouzarides, 2007). The current thinking is that these various modifications, along with DNA methylation, act in concert, interacting in a site specific manner to regulate gene expression (Day and Sweatt, 2011; Turner, 2002). It is beyond the scope of this thesis to provide a detailed overview of each of the modifications that constitute the 'epigenome' for (recent reviews see Turner, 2002; Jaenisch and Bird, 2003; Day and Sweatt, 2011); instead the following sections will focus on those mechanisms currently of primary interest to learning and memory processes: DNA methylation and histone acetylation (HA).

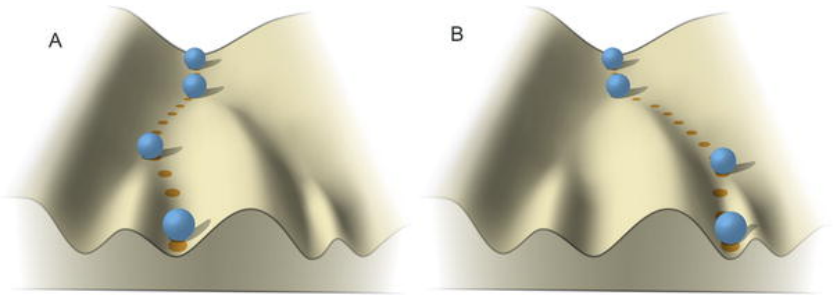


Figure 1.3.1: Waddington's epigenetic landscape (image reproduced from: Mitchell, 2007). The blue ball represents a cell and the topographic environment the genotype. The various phenotypic states possible for the cell given it's genotype are represented by the valleys. Whilst the 'landscape' dictates the possible phenotypes, cells containing the same genetic material can have different the phenotypic end points depending on exposure to different internal and external programs / inputs.

1.3.1 DNA methylation

DNA methylation tends to be associated with transcriptional silencing[4]. The silencing effects of methylation can occur directly, by reducing transcription factor binding at promoter regions, or indirectly, via the recruitment of remodelling proteins (Klose and Bird, 2006). Functional DNA methylation typically occurs at regions dense in CpG dinucleotides[5] (i.e. CpG islands) and such motifs are enriched at promoter regions, pointing to the key role that methylation plays in gene regulation (Illingworth and Bird, 2009).

Both the addition and removal of methyl groups to cytosine residues is catalysed by group of enzymes known as DNA methyltransferases (DNMTs; Métivier et al., 2008). Three DNMTs have been identified as being functional in the mammalian nervous system. The differing expression patterns of these enzymes speaks to their widely differing roles in regulating methylation. DNMT1 is highly expressed in the CNS

[4]Although see for Flintoft (2008) for a more in-depth discussion of the dynamics of DNA methylation

[5]Although non-CpG methylation does occur in embryonic stem cells (Dodge et al., 2002)

across the lifespan and is involved in both maintenance and *de novo* methylation (Goto et al., 1994; Yu et al., 2011). DNMT3a expression peaks just after birth but is also detectable in mature neurones, whilst the expression of DNMT3b is restricted to early embryonic stages (Feng et al., 2005).

Traditionally, it was thought that methylation was specifically involved in cellular differentiation and imprinting and only occurred during cell division. However, the fact that enzymatically active DNMTs are expressed in post-mitotic neurones indicates that the regulation of DNA methylation patterns may be more dynamic than originally believed (Feng et al., 2010). Indeed, there is growing evidence that DNA methylation plays a central role in neuronal and behavioural plasticity (Yu et al. 2011; see section 1.3.3 for more details).

1.3.2 Post-translational histone modifications

Histones are scaffolding proteins that act to organise DNA within the nucleus. Four core histones, H2A, H2B, H3 and H4, form complexes called octamers, consisting of two H2A-H2B dimers and one H3-4 tetramer. DNA strands wrap around these histone complexes organising the DNA into core particles known as nucleosomes. A common and functionally important modification is histone acetylation (HA), which involves the addition of an acetyl group from acetyl coenzyme A (acetyl-CoA) to a lysine residue on the N-terminal tail of a given histone protein. This process causes a relaxation of chromatin structure and allows transcriptional machinery access to DNA at these regions, meaning that HA is typically, although not always, associated with an increase in transcription (Eberharter and Becker, 2002).

HA is bidirectionally regulated by two groups of enzymes: histone acetyl transferases (HATs) catalyse the addition of an acetyl group to a histone protein tail, whilst histone deacetylases (HDACs) catalyse the reverse reaction (Kouzarides, 2007). Both HATs and HDACs also act on non-histone molecules, including receptor proteins

and transcription factors (Popov et al., 2007; Imhof et al., 1997). A number of different protein complexes have been shown to have nucleosomal HAT activity *in vivo*, with each of these complexes displaying a high degree of substrate specificity (Marmorstein, 2001). The HDAC protein superfamily are grouped into four major sub-categories based on their catalytic structure and function (Dokmanovic et al., 2007).

In addition to acetylation, a multitude of other forms of histone modification have been described (Kouzarides, 2007) and appear to act in concert with other epigenetic mechanisms to alter chromatin structure and regulate downstream gene transcription (Miller et al., 2008). A common view is that these modifications form an 'epigenetic code' which acts as a form of cellular memory, translating environmental signals into stable, heritable changes in cell function (Turner, 2002; Graff and Mansuy, 2008).

1.3.3 Epigenetics, plasticity and behaviour

In support of the epigenetic code hypothesis, a number of signal transduction pathways have been identified which translate exogenous signals into tissue-specific epigenetic changes (Jaenisch and Bird, 2003; Caldji et al., 2011), and emerging evidence of dynamic environmentally-mediated epigenetic modification in the adult brain makes this code an attractive mechanism for the cellular plasticity processes underlying learning and memory (Crepaldi and Riccio, 2009; Roth et al., 2010; Day and Sweatt, 2011). Indeed, one of the difficulties faced by researchers in the search for the molecular mechanisms of memory has been the need to reconcile the apparent functional conflict inherent in a system which exhibits the capacity for both flexible, experience-dependent modification and stable, long-term storage of information. Graff and Mansuy (2008) suggest that an epigenetic code for memory may offer a mechanistic solution to this apparent paradox, positing discrete sub-codes mediating the different stages of learning and memory.

Until recently DNA methylation marks were thought to be relatively immutable, and involved mainly in cellular differentiation and genomic imprinting (Reik, 2007). However, emerging evidence indicates that the regulation of DNA methylation in the brain may be involved in certain forms of synaptic plasticity (Day and Sweatt, 2010; Guo et al., 2011). At a molecular level, depolarization-induced increases in the expression of plasticity related genes have been attributed to a reduction in methylation levels at the promoters of these genes (Chen et al., 2003; Martinowich et al., 2003; Miller and Sweatt, 2007), whilst, at a cellular level, DNA methylation has been shown to mediate the effect of cocaine on dendritic spine morphology in the NAc (Laplant et al., 2010). Furthermore, DNMT inhibitors impair the induction of LTP, in both the hippocampus (Levenson et al., 2006) and the lateral amygdala (Monsey et al., 2011), and the induction of LTP in the medial prefrontal cortex is associated with an increase in DNMTs levels in this region (Sui et al., 2012). Given the stability and regenerative capacity of methylation marks, these findings point to a role for DNA methylation in long-term, environmentally-induced changes in neuronal function (Bird, 2002).

A central role of DNA methylation in brain plasticity processes is further evidenced at the behavioural level by studies correlating various learning processes with changes in methylation. For example, Lubin et al. (2008) showed that the methylation status of the bdnf gene promoter was altered following fear conditioning, and that manipulations that impaired fear memory also blocked learning-related regulation of bdnf methylation and the resulting changes in its expression. Interestingly, fear conditioning has also been associated with both increased methylation at the promoter of the memory suppressor gene PP1 and the demethylation of the plasticity gene reelin (Miller and Sweatt, 2007), suggesting that memory formation may involve the bidirectional regulation of DNA methylation (Day and Sweatt, 2010). Although the precise mechanisms driving this bidirectional regulation remain unclear, unsurpris-

ingly, DNMT expression appears to be central to these effects; DNMT knockout mouse models exhibit impaired hippocampal LTP as well as deficits in both spatial learning and contextual fear conditioning (Feng et al., 2010). Consistently, fear consolidation is associated with an up-regulation of DNMT expression in the hippocampus (Miller and Sweatt, 2007) and DNMT inhibitors have been shown to impair memory consolidation (Miller and Sweatt, 2007).

Despite the increasing interest in the role of dynamic methylation marks in regulating neuronal processes, much of the neuroepigenetics literature to date has focused on PTHMs and, in particular, there is a growing body of evidence indicating that enzymatic regulation of histone acetylation (HA) plays a central role in regulating cellular and behavioural plasticity (Crepaldi and Riccio, 2009; Gräff and Tsai, 2013). Both the activation of excitatory neurotransmitter pathways and the induction of LTP have been associated with increases in histone acetylation at the promoters of various plasticity genes in both cortical and sub-cortical regions (Crosio et al., 2003; Sui et al., 2012; Guan et al., 2002). Furthermore, reducing the enzymatic activity of HDAC proteins, with histone deacetylase inhibitors (HDACis), has been shown to enhance synaptic plasticity in the hippocampus (Levenson et al., 2004; Miller et al., 2008) and the amygdala (Yeh et al., 2004), whilst increasing the activity of HDAC2 reduces the magnitude of hippocampal LTP (Guan et al., 2009). Conversely, both genetic and pharmacological manipulations which reduced the HAT activity of CREB-binding protein (CBP), lead to impaired memory-related plasticity (Alarcon et al., 2004; Wood et al., 2005; Barrett and Wood, 2008; Maddox et al., 2013). Taken together these findings indicate that the bidirectional enzymatic regulation of HA in the brain plays a key role in experience-dependent changes in neural activity.

Consistent with these electrophysiological findings, the the regulation of HA has also been implicated in various forms of learning and memory (Crepaldi and Riccio, 2009). Much of this work has been conducted using fear conditioning paradigms and

there is evidence that HA is involved in the acquisition, consolidation, reconsolidation and extinction of long-term fear memories (Levenson et al., 2004; Bredy et al., 2007; Bredy and Barad, 2008; Maddox and Schafe, 2011; Monsey et al., 2011). In addition, HA has also been implicated in spatial learning (Wood et al., 2005; Dash et al., 2009; Reul et al., 2009; Bousiges et al., 2010; Haettig et al., 2011), reward processing and addiction related behaviours (Renthal and Nestler, 2009), and even in some forms of emotional learning (Renthal et al., 2007). Importantly, these studies have employed a number of different experimental approaches and converging evidence from both genetic and pharmacological manipulations, as well as from studies correlating behavioural experience with epigenetic changes, provide compelling support for the involvement of HA in various types of learning and memory. Tables 1.3.1, 1.3.2, and 1.3.3 provide a comprehensive summary of some of the key experimental findings from each of these three different approaches.

Recently, attempts have been made to identify the specific molecular substrates mediating the mnemonic role of HA. In a seminal study Guan et al. (2009) showed that both neural plasticity and memory formation were effected by manipulations targeting the HDAC isoform HDAC2, but that these same processes were unaffected by manipulations targeting a different HDAC isoform, HDAC1. Similarly, McQuown et al. (2011) used a combination of genetic and pharmacological manipulations to show that HDAC3 acts as a negative regulator of hippocampal-dependent long term memory. HDAC5, on the other hand, appears to be specifically involved in cellular and behavioural responses to both chronic drug administration and stress (Renthal et al., 2007). By identifying specific molecular targets, delineating the role of individual HDAC isoforms in cognitive and behavioural processes in this way may prove useful in the development of novel drug compounds for the treatment of various pathological brain disorders (Stahl, 2010; Day and Sweatt, 2012).

Perhaps, indeed undoubtedly, one of the reasons HA has received so much attention

in the behavioural neuroscience literature is because there exist a number of well utilised drug compounds that alter the enzymatic activity of HDACs enabling manipulation of HA *in vivo*. Although currently largely lacking the specificity required to target individual enzyme isoforms (Lattal et al., 2007), these compounds have proved invaluable in exploring the causal link between HA and memory. As already mentioned, HDACis have been widely used in various fields of research and increasingly have clinical applications. To date the majority of these applications have been in experimental studies for novel cancer therapies (Shabason et al., 2010) but HDACis are beginning to be recognised as potential treatments for particular brain disorders, such as bipolar disorder and schizophrenia (Guidotti et al., 2010; Machado-Vieira et al., 2011). The fact that this class of drugs have a proven safety record for use in humans, coupled with evidence that exposure to HDACis can have profound effects on behaviour and brain physiology, makes them attractive to researchers looking at ways to modify and treat pathological behaviours and cognitions (Fischer et al., 2010). Table 1.3.3 summarises some of the keys findings relating to the effects of various HDACis on a number of brain processes and functions.

The limited availability of HAT inhibitors with *in vivo* utility has meant that the majority of the work conducted in this field to date has focused on the effects of indirectly increasing HA by blocking HDAC activity. However, recently it has been shown that, consistent with the genetic manipulation work, pharmacological blockade of the HAT activity of p300/CBP impairs the consolidation and reconsolidation of fear memory (Maddox et al., 2013). Interestingly, however, inhibiting HAT activity in the mPFC has been shown to enhance the extinction of fear memories (Marek et al., 2011). This apparently paradoxical finding highlights the complexity of the role that HA plays in the brain, suggesting as it does, that the effects of HA manipulations are likely to be dependent both on the region in which they occur and the type of brain plasticity, and hence, cognitive process, that is targeted.

Behavioural Correlate	Epigenetic Correlate	Reference
Fear memory reconsolidation	Increased H3 acetylation in amygdala and hippocampus	Bredy and Barad (2008); Maddox and Schafe (2011)
Spatial memory	Increased H2B, H3 & H4 acetylation in hippocampus	Reul et al. (2009); Bousiges et al. (2010)
Object recognition memory	Increased H3acetylation in hippocampus	Fontán-Lozano et al. (2008); Gräff et al. (2012)
Latent inhibition	Increased H4 acetylation in hippocampus	Levenson et al. (2004)
Eye blink conditioning	Increased H3 acetylation in hippocampus	Fontán-Lozano et al. (2008)

Table 1.3.1: Summary of evidence supporting a role of histone acetylation in learning and memory: Correlational studies. HA: histone acetylation.

Consequentially, researchers have recently started using direct microinfusions of HDACis and HAT inhibitors to target specific brain structures thought be involved in certain types of memory. For example, intra-hippocampal infusions of the HDACi Trichostatin A (TSA) significantly enhance hippocampal-dependent extinction learning (Vecsey et al., 2007), whilst infusions of the HAT inhibitor garcinol into the dorsal hippocampus impair object recognition (Zhao et al., 2012). Furthermore, inhibiting HDAC activity in the ventrolateral orbital cortex, using the HDACi Valproic acid (VPA), enhanced learning about stressful events (Zhao et al., 2013).

In addition to these brain-region specific effect, studies using HDACis to manipulate learning and memory in animals have revealed a high degree of temporal specificity in the effects of these drugs (Raybuck et al., 2013; see Chapter 5 for further discussion of

the temporal specificity of HA manipulations) and have, in turn, provided invaluable insights into the dynamic role that the enzymatic regulation of HA plays in the brain (Morris et al., 2010).

Manipulation	Effect on HA	Behavioural Effects	Molecular / Cellular Effects	Reference
CBP+/- mice	↓	Reduced long-term fear memory. Impaired CFC and CuFC.	Reduced DA-regulated hippocampal L-LTP.	Alarcon et al. (2004)
CBPΔ1 mice (Inhibitory CBP; forebrain specific)	↓	Deficits in spatial learning. Impaired long-term CFC.	Reduced DA-regulated hippocampal L-LTP	Wood et al. (2005)
CBP-/- mice (CA1 neuron specific)	↓	Impaired long-term CFC. Impaired long-term NOR.	Loss of CREB:CBP-mediated gene expression. Impaired hippocampal LTP.	Barrett et al. (2011)
CBP(HAT-) mice (hippocampal specific)	↓	Impaired spatial learning. Impaired NOR.	n/a	Korzus et al. (2004)
HDAC2OE mice	↓	Reduced CFC & CuFC. Impaired spatial working memory.	Reduced dendritic spine density, synapse number & synaptic plasticity.	Guan et al. (2009)
HDAC2KO mice	↑	Enhanced CFC & CuFC. Enhanced spatial memory.	Enhanced hippocampal LTP.	Guan et al. (2009)

Table 1.3.2: Summary of evidence supporting a role of histone acetylation in learning and memory: Genetic Manipulations. Abbreviations: CBP: CREB binding protein; CFC: contextual fear conditioning; CuFC: Cued fear conditioning; DA: dopamine; HAT: histone acetyltransferase; HDACi: histone deacetylase inhibitor; (L)LTP: (late) long term potentiation; NOR: novel object recognition; HDAC2OE: HDAC 2 over expression; HDAC2KO: HDAC 2 knock out.

Manipulation	Effect on HA	Behavioural Effects	Molecular/Cellular Effects	Reference
Systemic NaB	↑	Enhanced CFC	Enhanced hippocampal LTP *in vitro*	Levenson et al. (2004)
Intra-hippocampal SAHA	↑	Enhanced CFC	Increase of H4K12 acetylation	Peleg et al. (2010)
Intra-hippocampal TSA	↑	Enhanced consolidation CFC	CREB:CBP-dependent increase in expression of specific genes	Vecsey et al. (2007)
Intra-accumbens SAHA	↑	Antidepressant effects	Increased levels of H3K14 acetylation in NAc	Covington et al. (2009)
Systemic NaB	↑	Increased LMS & CCPP	Enhanced cocaine-induced PAcH3 in striatum	Schroeder et al. (2008)
Systemic NaB	↑	Enhanced extinction of CFC	N/A	Lattal et al. (2007)
Systemic NaB	↑	Enhanced CuFC and spatial learning	Increased levels of synaptic marker proteins	Fischer et al. (2007)
Systemic NaB	↑	Enhanced NOR	N/A	Stefanko et al. (2009)
Intra-CA1 NaB	↑	Rescued DNMTi-induced deficit in memory consolidation	Rescued DNMTi-induced deficit in hippocampal LTP	Miller et al. (2008)
Intra-CA1 TSA	↑	Facilitated shift from cue to place spatial learning	Rescued aging-associated deregulation of H4 acetylation in CA1	Dagnas et al. (2013)
Systemic VPA	↑	Enhanced spatial learning and memory	Enhanced plasticity in DG	Murphy et al. (2001)

Table 1.3.3: Summary of evidence supporting a role of histone acetylation in learning and memory: Pharmacological Manipulations. Abbreviations: CFC: contextual fear conditioning; CCPP: cocaine conditioned place preference; CBP: CREB binding protein; CREB: cAMP response element-binding protein; CuFC: Cued fear conditioning; DG: dentate gyrus; DH: dorsal hippocampus; DNMTi: DNA methyltransferase inhibitor; HDACi: histone deacetylase inhibitor; PAcH3: H3 phosphoacetylation; LMS: locomotor sensitization; LTP: long term potentiation; NOR: novel object recognition; TSA: Trichostatin A; SAHA: Suberoylanilide hydroxamic acid; VPA: Valproic acid.

1.3.4 Summary

The relatively new field of neuroepigenetics has proved fruitful in demonstrating the involvement of a number of epigenetic modifications in various forms of learning and memory and there is a growing consensus that the regulation of epigenetic processes in the brain offers a mechanistic link mediating the interaction between the genome and environmental experience. The marked complexity of these interactions poses both a theoretical and a practical challenge and the aims of a given experiment will determine the best approach to employ. The degree of spatial, temporal and molecular specificity appropriate to a given research question will vary. Relatively new fields of investigation, such as those presented in this thesis, will need to adopt more general, broad-brush approach, whilst researchers interested in more well studied paradigms, such as contextual fear conditioning, are beginning to utilise more and more specific methods to delineate the discrete molecular pathways underlying the various stages of learning. This fact was illustrated in the current work where there was a need to tailor and develop new methods and approaches to address novel research questions concerning the molecular substrates of actions and habits. Moreover because of the novel nature of this research, the experiments presented in this thesis were not constrained by a directional hypothesis. Instead, this body of work was based on the premise that the brain plasticity mechanisms underlying this learning induced behavioural change from goal-directed to habitual behaviour involve gene expression changes which are regulated by epigenetic modification and each set of experiments was designed to to provide initial information, at a fairly general level, about the nature of these changes.

1.4 Aims and objectives

This thesis was motivated by the need for a better understanding of the molecular mechanisms underlying the transition from goal-directed to habitual instrumental

responding that occurs following extensive practice. A series of correlative molecular analyses and pharmacological manipulation studies are presented.

The first experimental chapter (Chapter 3) explores the optimal parameters for measuring goal-directed and habitual instrumental responding in rats and, thereby, establishes the training protocols to be employed in subsequent experiments. The instrumental performance of rats from two different suppliers, Harlan and Charles River, was compared. In addition to this 'Source' comparison, two different devaluation protocols were employed to test the animals' sensitivity to changes in the outcome value. After three sessions of instrumental training, only rats sourced from Charles River and devalued in the training context, significantly reduced instrumental responding for a devalued outcome, indicating that lever pressing behaviour was goal-directed in these animals. The instrumental behaviour of all the other groups was insensitive to the devaluation manipulation. Drawing on the results of Experiment 1, in Experiment 2, rats sourced from Charles River were given 10 sessions of instrumental training. Here, in contrast to the previous experiment, lever pressing was insensitive to outcome devaluation carried out in the training context, suggesting that the instrumental performance of these animals was habitual.

The work presented in Chapter 4 used DNA microarray analysis to compare patterns of gene expression across key brain regions following different amounts instrumental training. Animals assigned to one of three training groups: *minimal*, *moderate* and *extended*, were trained to lever press across three, six or 10 sessions respectively. At the end of training striatal subregions of interest, the DLS and pDMS, were bilaterally dissected from a proportion of animals from each of these training groups, as well as a no training group with no experience of lever pressing. Transcriptome changes were measured and compared across both regions, and across the different training groups, using Rat Gene 2.0 ST Affymetrix arrays. There was evidence of bidirectional shifts in regulation between the training groups in both the DLS and

pDMS. Overall, the DLS showed a higher level of experience-dependent changes in gene expression during the later stages of training, consistent with it's apparent importance in habitual behaviours. Interestingly, the DLS and pDMS exhibited distinct populations of regulated genes, with little overlap between the two regions, suggesting that discrete, region-specific, molecular pathways underlie these training-related expression changes.

In Chapter 5 the causal role of post-translational histone modifications in the transition from goal-directed to habitual responding was investigated more directly using a systemically delivered HDACi, sodium butyrate (NaB), to manipulate endogenous histone acetylation. For each of the three experiments presented in this chapter, the drug manipulation occurred at a different point during training, with the aim of targeting different stages of learning. To test the hypothesis that HA is involved in the *acquisition* phase of learning, in Experiment 1, NaB was administered 30 minutes prior to each of the three RI30 lever press training sessions. Both treatment groups showed comparable levels of sensitivity to outcome devaluation, reducing both their lever press responding and magazine entries for the devalued reinforcer, suggesting that histone acetylation is not involved in the regulation of goal-directed and habitual responding during the acquisition phase of instrumental learning. Following on from this, the aim of Experiment 2 was to investigate whether histone acetylation is involved in the *consolidation* phase of instrumental learning with NaB being administered at the end of each RI30 training session. Again, in extinction, both treatment groups showed a comparable reduction in lever pressing and magazine entries following devaluation. The final experiment in this series explored the role of HA in the *retrieval* of a previously acquired instrumental response. Here, NaB was administered, after all training and devaluation sessions had been completed, 30 minutes prior to extinction testing. In contrast to the previous two experiments, the sensitivity of lever press responding to devaluation was reduced in the NaB-treated

group compared to controls. Although there are a number of caveats that need to be considered when interpreting this finding, which are discussed in detail, one possible explanation for this finding is that histone acetylation plays a necessary role in the retrieval and/or execution of previously learnt action-outcome contingencies.

Chapter 6 presents data from initial, exploratory investigations into the development of a protocol for infusing HDACis directly into functionally important brain regions. With the aim of addressing some of the limitations of systemic drug studies by increasing the spatial specificity of drug administration, microcannula were implanted, using stereotaxic co-ordinates, to target the infralimbic cortex in a cohort of Lister hooded rats. Animals were trained to lever press using the protocol developed in Chapter 3, before receiving two microinfusions of either NaB or aCSF, given 24 hours apart. 30 minutes after the final infusion animals were tested in extinction. Off-line molecular analysis of mPFC tissue samples revealed an increase in histone H4 acetylation in samples treated with NaB relative to vehicle-treated samples. Behavioural data showed an enhanced sensitivity to outcome devaluation in NaB-treated animals relative to controls indicating that NaB may act to reduce the damaging effects of the microinfusions. A number of practical issues were highlighted by these experiments and are discussed in the context of future work, but these preliminary data represent the first step in the development of a microinfusion protocol for studying the role of histone acetylation in instrumental learning.

Chapter 2

General Methods

The following chapter focuses mainly on those behavioural methods and apparatus common to the majority of experiments in this thesis. Specific details of each experimental design can be found in the method sections of the relevant chapters.

2.1 Subjects

All the experiments presented here used cohorts of male Lister hooded rats sourced from either Harlan UK (Bicester, Oxon, UK) or Charles River (Margate, Kent, UK). Lister hooded rats are commonly used in behavioural neuroscience due to their good visual acuity, inquisitive nature and, importantly, their ability to rapidly acquire, retain and utilise spatial information and associative structures (e.g. Broersen and Uylings, 1999). The weight ranges and supplier details of specific experimental cohorts are presented for each chapter individually.

2.1.1 Animal husbandry

Upon arrival at the animal unit rats were always housed, in pairs, in a climate-controlled vivarium (lights: 8:00am - 8:00pm; temperature: 21^{o}C; humidity 55% $\pm$ 5%). Home cages were furnished with a cardboard tube and a wooden chew stick.

Pictures of the home cages are shown in Figure 2.1.1. *Ad libitum* water was available for the duration of every experiment. Any licensed procedures, including food deprivation, were started after at least a week-long acclimatisation period in which animals has *ad libitum* access a basic lab chow diet. All animals undergoing surgical procedures (i.e. cannulation) were singly housed following cannula implantation and remained so for the rest of the experiment. All procedures involving the use and care of live animals complied with international (Directive 86-609, 24 November 1986, European Community and Directive 2010/63/EU) and national (Animals (Scientific Procedures) Act 1986) regulatory legislation and policy and were subject to Home Office approval (project license: 30/2673).

Figure 2.1.1: Images of a home cage. Animals were housed in pairs (except after surgery) and all homes cages contained sawdust and cardboard tube and a wooden chew stick (not shown).

2.2 Behavioural Apparatus

2.2.1 Operant boxes

All instrumental training was conducted in one of two sets of eight operant boxes (Med Associates Inc., St Albans, VT), measuring: 240mm high x 240mm deep x 300mm wide and arranged in two rows of four (Figure 2.2.1). All the boxes consisted of two plate aluminum walls and a clear Perspex front and back wall and ceiling.

A grid floor, made up of 19 parallel stainless steel bars 4.8mm in diameter and spaced 16mm apart, was inserted 30mm above the base of the operant chamber. Each operant box was housed in it's own sound and light attenuating chamber. The chambers housing the boxes in one set of boxes also had a small perspex front-facing viewing window.

During training, sucrose pellet reinforcers (45 mg; P. J. Noyes, Lancaster, NH) were delivered into a recessed food magazine situated in the center of the right-hand wall of the operant box. The magazines were fitted with pair of infra-red detectors which recorded magazine entry and exit. A retractable flat panel lever was inserted to the left of the magazine at the start of each lever press training session and retracted when the session ended. Equipment control and data recording was conducted on an IBM-compatible microcomputer equipped with MED-PC software (Med Associates Inc., St Albans, VT).

Figure 2.2.1: Images of the operant chambers: a) view inside the operant box; b) view of recess magazine (lever slots are visible on either side of the magazine); c) front view of the chambers housing the boxes in set A; d) front view of the chambers housing the boxes in set B.

2.2.2 Activity boxes

Locomotor activity levels were assessed using a set of eight opaque plastic chambers measuring: 175mm high, 510mm deep and 320mm wide, with a metal grid floor and ceiling. Each chamber was fitted with two pairs of photobeam projectors, positioned along the long wall of the chamber and spaced 20cm apart (see Figure 2.2.2). A detection interface system (Paul Fray Ltd, Cambridge, England) registered each beam interruption made during a session and an Acorn A500 computer recorded and stored the session data for each chamber. Beam break data was recorded across 10 minute bins for each session and presented in the following categories: total beam breaks, sames (same beam broken sequentially) and runs (the two different beams broken sequentially). All analysis of activity data presented in the following

experiments was conducted on the total number of beam breaks, across time-bin, for the entire session. Sessions lasted either 30 minutes or one hour depending the experimental design.

Figure 2.2.2: Images of the activity chambers: a) view from above; b) a view from the side; c) front view of the chambers.

2.3 Behavioural procedures

All behavioural procedures were conducted during the light phase of the light-dark cycle. The basic instrumental training protocol is summarised in Figure 2.3.1.

2.3.1 Food restriction

In order to increase motivation to work for food, all animals were food restricted prior to the start of behavioural training. Animals were fed once a day, after training, with

enough food to maintain their weights at above 80% of their *ad libitum* feeding weight for the duration of training.

2.3.2 Behavioural training

At the start of training each animal was assigned to one of the eight operant chambers and was trained in the same chamber for the duration of the experiment. The overhead light in the testing room remained off for the duration of each training session. Animals were trained to lever press for sucrose pellet reinforcers (45 mg; P. J. Noyes, Lancaster, NH) across a given number of sessions. The majority of sessions were run on separate days, although occasionally an animal received two sessions on the same day, such as when they failed to complete the first CRF session.

Pre-training. All experiments began with a pre-training phase in which animals were trained to approach the recess food magazine during a single session in which the lever remained retracted and response-independent reinforcers were delivered on a random interval 60-second (RI60) schedule, with a reinforcer being delivered, on average, every 60 seconds. This session was terminated after 20 reinforcers had been delivered. Following magazine training animals learnt to lever press during a session in which the left lever was inserted into the box and lever presses were reinforced on a continuous reinforcement schedule (CRF), with each presses resulting in contingent reinforcer delivery. Again, this session was terminated after 20 reinforcers had been earned. Occasionally, if an animal failed to learn to lever press during the first CRF session, they were given a repeat session.

Figure 2.3.1: Summary of the behavioural details common to all experiments

Instrumental training. Lever press training then continued on an RI30 reinforcement schedule, with a reward becoming available, on average, every 30 seconds. As explained in more detail in Chapter 3, *minimal training* (i.e. training which gives rise to goal-directed behaviour) was defined as three sessions of RI30 training, whilst *extended training* (i.e. training which gives rise to habitual behaviour) was defined as 10 sessions of RI30 training. Although interval reinforcement schedules, in contrast to ratio schedules, bias instrumental behaviour towards S-R responding (i.e. they favour habit formation; Dickinson et al., 1983), more recent research has shown that, if training is sufficiently brief, RI schedules can give rise to goal-directed behaviour (Dickinson et al., 1995; Nelson and Killcross, 2006). Furthermore, since a critical determinant of sensitivity to outcome devaluation appears to be the level of exposure to the instrumental contingency (Adams, 1982; Dickinson et al., 1983), session duration was determined by reward delivery, rather than by time or by number of responses made (Nelson and Killcross, 2006), and each RI30 training session was terminated after an animal had earned 40 rewards. If an animal failed to earn 40 rewards within 90 minutes the session was closed manually by the experimenter.

2.3.3 Outcome devaluation

In the experiments presented in this thesis, the relative sensitivity/insensitivity of lever press responding to outcome devaluation was used as an index of goal-directed or habitual instrumental behaviour respectively. Outcome devaluation was achieved by pairing sucrose pellet consumption with lithium chloride-induced nausea in order to establish a conditioned taste aversion (CTA) to the pellets. More specifically, the animals were given access to sucrose pellets either in a cage similar to their home cage or in the operant box they had previously be trained to lever press in. Further details regarding this pre-feeding phase can can be found in Chapter 3.

Following pre-feeding, animals were given an intraperitoneal injection of either LiCl (Sigma; 0.15M; 10ml/kg), dissolved in distilled water, or an equivalent volume of physiological saline (0.9%), before being returned to their home cages. To avoid the aversive effects of the LiCl transferring to their standard diet, all animals were fed at least an hour after injection. This whole procedure was repeated the following day to ensure a robust taste aversion was established.

2.3.4 Test phase

Extinction test. The day after the final devaluation session, all animals were returned to the same operant boxes in which they had been trained for a 10 minute extinction test during which the left lever was extended and lever press and magazine behaviour were recorded in the absence of reward delivery. Measuring behaviour in extinction allowed the influence of expected outcome value over responding to be assessed, independent of any potential confounding due to the punishing effects of direct exposure to the aversive outcome (Nelson and Killcross, 2006).

Consumption test. Finally, to confirm that the devaluation manipulation was successful in reducing the motivational value of the sucrose pellets, and, in animals that were pre-fed in the operant chambers, to test for contextual generalisation of the CTA, all animals were given a 30 minute consumption test. At the end of the extinction test animals were moved to a different testing room and placed into individual cages, similar to their home cages, containing 10-15g of sucrose pellets and left, in the dark, for 30 minutes. At the end of testing, animals were returned to their home cages and the weight of the pellets (in grams) left in the consumption test cages was recorded.

2.4 Brain dissections

For the experiments where brain tissue samples were taken, animals were culled by exposure to a rising concentration of CO_2 and decapitated. Brains were removed immediately post mortem and placed on a chilled aluminum plate, which was kept on wet ice for the duration of the dissection. Regions of interest were then hand dissected, bilaterally, using a razor blade and placed in an eppendorf tube before being flash frozen on dry ice, and stored at $-80°$. The co-ordinates for and dissections of the specific brain regions are described in the methods sections of the relevant chapters.

2.5 Data analysis

Those aspects of data analysis specific to individual experiments will be described fully in the relevant experimental chapter.

2.5.1 Behavioural data

All behavioural data analysis was conducted using either ALAS or SPSS 18 software. Significant effects were defined as those with an *alpha* value of 95% or above ($p \leq .05$). All significant interactions were subjected to simple effects analysis in which the condition means were compared for each factor independently.

For the training and test phases of each experiment, analysis was conducted on lever press and magazine entry data. For training data, the total number of responses per session was analysed across the training sessions to show patterns of learning-related changes in response rate. In experiments in which one or more drug manipulations were conducted a mixed ANOVA model was used, including the factors (to be) DEVALUATION group and (to be) HDACi group, in order to confirm that there were no pre-existing differences between the treatment groups. The same analysis

was used to look at magazine entry behaviour across the 2 devaluation pre-feeding sessions. Test phase data were analysed using a between subjects ANOVA model comparing overall response levels for the entire 10 minute extinction test between the various treatment groups. Consumption test data (i.e. quantity of sucrose pellets consumed [g]) were analysed in the same way. Analysis of locomotor activity data was conducted on total number of beam breaks across 10 minute time-bins and across session, using a mixed model ANOVA with the within-subjects factor HDACi group and the between-subjects factors SESSION and BIN. Table 2.5.1 summarises the various experimental factors in general terms.

	Factor	**Levels / Description**
Within-subjects	SESSION	RI30 training session number (incremental)
	BIN	10 minute time-bins across activity sessions (incremental)
Between-subjects	HDACi group	*NaB* (sodium butyrate)
		Vehicle: *saline* (SAL) or *artificial cerebro-spinal fluid* (aCSF) treated
	DEVALUATION group	*Devalued* (DV): LiCl-treated
		Non-Devalued (NDV): Saline-treated
	DEVALUATION TYPE group	*Box*: pre-fed in operant box
		Cage: pre-fed in home cage-like environment
	TRAINING group	*Minimum*: 3 RI30 sessions
		Moderate: 6 RI30 sessions
		Extensive: 10 RI30 session

Table 2.5.1: Summary of experimental factors

2.5.2 Molecular data

Analysis of molecular data was conducted in R. All details regarding the specific analyses applied to the microarray and qPCR data can be found in Chapter 4.

2.5.3 Exclusion criteria

As noted earlier, it was important to keep instrumental contingency exposure tightly constrained across the experimental groups and each session was terminated once 40 reinforcers had be earned. The majority of animals acquired lever press behaviour quickly and reached 40 reinforcers within 30-40 minutes. However, occasionally certain animals failed to earn the full amount of sucrose pellets within the maximum time frame allowed for time (~ 90 minutes). Therefore, any animal earning a total number of reinforcers less than four standard deviations (<4SD) from the mean at the end of training was excluded from the final analysis. For infusion experiments, animals were also excluded from the final analysis if histological analysis revealed cannula placement to be in outside of the target area. The number of animals excluded from each experimental cohort, and the justification for their exclusion, will be specified in the method section of specific chapters.

Chapter 3

Development of experimental parameters for modelling goal-directed and habitual behaviour in rats

3.1 Introduction

Although, as described in 1.1, there have been numerous studies describing goal-directed and habitual behaviour in rats, there is a large degree of variability in the details of the experimental design between studies. Therefore, in order to begin to address the issue of the role of molecular events in the transition from goal-directed to habitual behaviour, it was necessary to first establish a behavioural paradigm that allowed these two types of behaviour to be modelled in our own hands. The main aim of the current set of initial experiments, therefore, was to develop a robust training protocol with which to model goal-directed and habitual behaviour in rats, as indexed by the relative sensitivity and insensitivity of instrumental behaviour to changes in outcome value respectively. A particular focus was the issue of behavioural variability introduced both by differences in the supplier and background of the animals used, and the method by which the outcome is devalued.

Perhaps the most important task parameter which needs to be defined in studies of goal-directed and habitual behaviour is the reinforcement schedule used during instrumental training (Dickinson, 1985). As noted in section 1.1, ratio schedules (where the reinforcement schedule is determined by the number of responses) have traditionally been used to model goal-directed behaviour whilst interval schedules (i.e. where the reinforcement schedule is determined by *time)* have been used to model habitual behaviour. However, there is evidence that, following low levels of training on interval schedules, rats do in fact display goal-directed behaviour (as indexed by sensitivity to outcome devaluation; e.g. Dickinson et al., 1995). Since here we wanted to be able to use essentially the same behavioural training protocol to model both types of responding, manipulating only the number of training sessions, we used a random interval schedule. Specifically, a 30 second random interval (RI30) was used because it has been shown, in previous studies conducted in our laboratory, that this reinforcement schedule maintains a high rate of lever pressing in extinction (Nelson and Killcross, 2006).

As described in section 2.1, Lister hooded rats are an appropriate and commonly used strain in behavioural neuroscience research and previous work in our laboratory, using male Lister hooded rats supplied by Harlan UK (Bicester, Oxon, UK) to successfully model both goal-directed and habitual behaviours, provided an obvious starting point for the current series of experiments (Nelson and Killcross, 2006; Coutureau and Killcross, 2003; Killcross and Coutureau, 2003). However, our own preliminary investigations, as well as concurrent work by independent groups in our laboratory and wider communications from colleagues reporting increasing variability in behavioural data, suggested a degree of caution was needed in choosing the source of our experimental animals. Notably, in initial pilot studies conducted by the experimenter, using rats from Harlan, animals' lever press responding in extinction did not show the anticipated sensitivity to outcome devaluation after three sessions

of training. Given these issues, we decided to try to develop the task in rats from a different supplier and compare their performance, within the same experiment, to that of Harlan rats. Thus, the current experiment systematically compared the acquisition and expression of instrumental behaviour of two out-bred sub-strains of Lister hooded rats from different suppliers (i.e. Harlan and Charles River).

Finally, different groups have employed different techniques to establish outcome devaluation. Rescorla (1999) demonstrated that the incentive value of a reinforcer is determined not only by its absolute motivational value, but also by its specific sensory features. Thus, allowing an animal to eat a specific food to satiety not only has the effect of reducing the motivation to work for food generally, but also reduces the incentive value of that specific food relative to other equally palatable foods. Similarly, it is possible to selectively devalue a particular food by pairing the experience of eating it with the experience of malaise. Conditioning a taste aversion to a given food by pairing its consumption with nausea, commonly induced by an injection of lithium chloride (LiCl), will result in a selective avoidance of that particular food without affecting the incentive value of other foods with different sensory properties (Vogel et al., 2007). As outlined in section 1.1, the sensory-specific selectivity of these effects has been used as a way to probe instrumental behaviour in order to establish whether responding is goal-directed or habitual and both sensory-specific satiety and conditioned taste aversion have been used to devalue instrumental outcomes in a number of studies (Balleine and Dickinson, 1998; Yin and Knowlton, 2006).

Each method has its own strengths and weaknesses; sensory specific satiety has the advantage of being a transient manipulation, allowing for within-subjects comparisons and has the added benefit that it doesn't require the administration of a psychoactive compound (i.e. lithium), which may interact with other pharmacological manipulations. Conversely, the enduring effects of LiCl devaluation allow the

effectiveness of the devaluation procedure to be assessed independently of instrumental learning requirements in a subsequent consumption test where animals are simply given free access to the reinforcer and consumption is compared between LiCl-treated animals and controls. Furthermore, the behavioural effects of LiCl-induced taste aversion tend to be much larger and more robust (Nelson and Killcross, 2006). Given the need to detect the behavioural effects of taste aversion on top of neurophysiological manipulations, for the novel experiments conducted here it was decided that the large, robust effect-size produced by LiCl-induced conditioned aversion made this the most appropriate method for use here.

3.2 Experiment 1: Development of a training protocol for modelling goal-directed responding

As outlined above, the primary aim of this experiment was to establish a robust and effective method for observing goal-directed instrumental responding in Lister hooded rats. The sensitivity of instrumental responding to LiCl-induced outcome devaluation was compared between groups of Lister hooded rats sourced from two different suppliers following minimal training[1].

Since the effects of LiCl-induced conditioned aversion extend beyond the sensory properties of the ingested food to the contextual features of the environment in which the conditioning occurred the context in which devaluation is conducted is an important feature to consider in experiments using this as a method by which to devalue an instrumental outcome (Rodriguez et al., 2000). Whilst some experiments conduct pre-injection feeding in a different context to the training context, for example in the home cage (e.g. Pittenger and Bevins, 2013), others have pre-fed animals in the operant chambers in which animals were trained (e.g. Nelson and Killcross,

[1] As noted above this is expected to give rise to goal directed behaviour

2006). This is an important methodological distinction since the latter approach has been shown to produce a general aversion to the training context in addition to outcome-specific devaluation effects (Dickinson et al., 2002). However, because in the present experiments we were only interested in devaluing one reinforcer context, contextual aversion effects are not as much of an issue as they are when comparing responding for two different reinforcers following devaluation (Rodriguez et al., 2000). Instead, because the primary concern here was to establish the most robust and appropriate method for studying goal-directed and habitual behaviour in rodents, here the behavioural effects of these two different devaluation methods were compared. One group of animals was pre-fed in the same context in which they were trained to lever press prior to LiCl-induced devaluation, whilst another group was pre-fed in a context similar to their home cage environment.

3.2.1 Methods

Subjects. Two cohorts of animals, each from a different supplier, were used. One cohort was sourced from Harlan UK (Bicester, Oxon, UK) and the other from Charles River (Margate, Kent, UK). Each of these cohorts consisted of 32 naïve male hooded Lister rats. Prior to the start of training animals were food restricted to above 80% of their ad libitum feeding weights, as described in section 2.3.1. Table 3.2.1 shows the mean weights and weight ranges for each cohort immediately prior to food restriction (*Ad lib)* and at the end of the experiment (Restricted). Animal husbandry details were as described in section 2.1.1.

		C. River	Harlan
Ad lib	Mean	282.3	276.3
	Range	263-305	248-296
Restricted	Mean	251.6	250.9
	Range	225-272	223-269

Table 3.2.1: The ranges and mean *ad libitum* and restricted feeding weights for the two cohorts in grams. *Ad libitum* weights were taken immediately prior to food deprivation. Restricted weights are those recorded at the end of the experiment.

Behavioural training. The main stages of training are displayed in Table 3.2.2. Initially, animals were trained to approach the food magazine and press the left lever for sucrose pellets as described in section 2.3.2. Following this pre-training phase, all rats completed three RI30 sessions (i.e. minimal training), earning a total of 120 pellets each. Previous work in our laboratory has shown this level of training, on this reinforcement schedule, produces patterns of goal-directed responding (Nelson and Killcross, 2006).

Day 1	Day 2	Day 3-5	Day 6	Day 7
Magazine training session (20 rewards)	CRF session (20 rewards)	RI30 sessions (40 rewards)	Devaluation sessions	Extinction & Consumption test

Table 3.2.2: Summary of the experimental design for minimal training

Outcome devaluation. Half of each experimental cohort was assigned to one of the two devaluation TYPE conditions (i.e. prefeeding conducted either in homecage-like environment or in training conxext; n = 32). In the *Cage* condition animals were placed in a cage similar to their home cage and given free access to sucrose for one hour. At the end of this hour animals were immediately removed from the cage and given an injection of either LiCl or an equivalent volume of saline (*Devalued* and *Non-Devalued* groups respectively). The LiCl dosage, injection volume and route of

administration were as described in section 2.3.3. After they had been injected the animals were returned to their home cages and the amount of sucrose pellets they had eaten during the session was recorded. In the *Box* condition the remaining animals were placed in the operant chambers in which they had been trained, with the lever retracted, and sucrose pellets were delivered, independent of any response, on an RI60 schedule. This session was terminated after 40 reinforcers had been delivered. The animals were then injected with either LiCl or saline as soon as the session had ended. This procedure was repeated once more after 24 hours for all groups.

Extinction and consumption tests. The test phase was conducted as described in section 2.3.4. All groups were given a 30 minute consumption test at the end of the extinction phase in which they were given free access to sucrose pellets in a cage similar to their home cage[2] and the amount of pellets eaten was recorded.

Measures and data analysis. One animal was excluded from the analysis because of poor training performance (i.e. they earned < 40 rewards during the second session of RI30 training; as described in section 2.5.3). The final group sizes of the experimental groups, after exclusions, are shown in Table 3.2.3. In order to ascertain whether there were any pre-existing differences in baseline performance between the groups, lever press and magazine entry data during training were analysed using a 2x2x2x3 mixed factorial ANOVA with the between-group factors SOURCE (Harlan vs. Charles River), devaluation TYPE (*Box* vs. *Cage*) and DEVALUATION group (*Devalued* vs. *Non-devalued*) and the within-subjects factor SESSION (1-3). A 2x2x2 independent measures ANOVA, with the same between-subjects factors described above, was conducted on the lever press and magazine entry data for the entire 10 minute extinction test and the total amount sucrose pellets (in grams) eaten during the consumption test.

[2]For animals in the *cage* condition this was the same box in which they were pre-fed during the devaluation sessions

		Devalued	Non-devalued
C.River	Box	8	8
	Cage	7	8
Harlan	Box	8	8
	Cage	8	8

Table 3.2.3: Final groups sizes of all the experimental groups after exclusions

3.2.2 Results

Training. Figure 3.2.1 a shows the lever press responding across the three RI30 training sessions for the two SOURCE groups. Lever presses increased across the sessions in both groups (Main effect of SESSION: $F[2, 110] = 82.583$, $p < .001$), with Charles River animals pressing the lever, on average, more than Harlan animals for all three sessions (Main effect of SOURCE: $F[1,55] = 5.495$, $p = .023$). Conversely, as shown in figure 3.2.1 b, magazine entries decreased across the training sessions (Main effect of SESSION: $F[2, 110] = 57.506$, $p < .001$), with Harlan animals entering the magazine more frequently than Charles River animals. However, this difference did not reach statistical significance (i.e. there were no main effects or interactions involving factor SOURCE for this measure [$p > .05$, ns]). Taken together, these data indicated that all animals acquired the basic lever press response and learnt about the instrumental contingency as expected, reducing their magazine approach behaviour in favor of lever pressing, and also that animals from different suppliers showed inherent differences in their propensity to work for food.

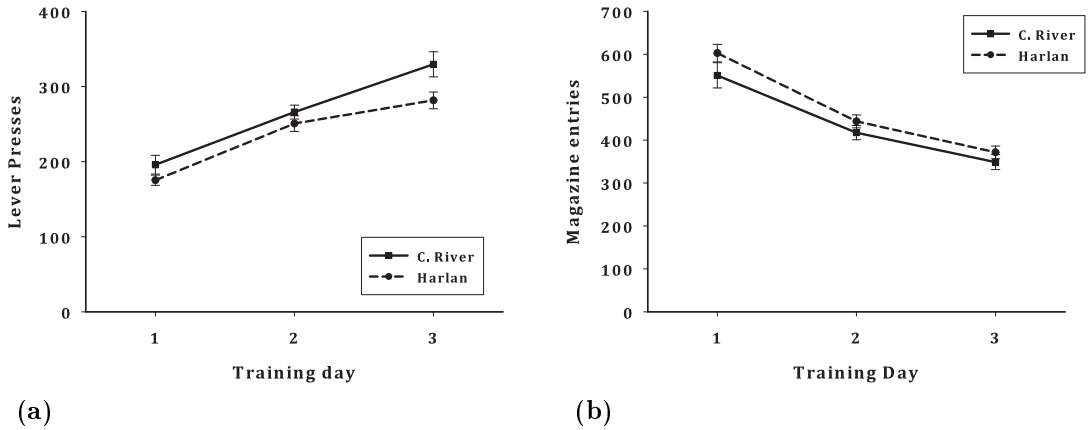
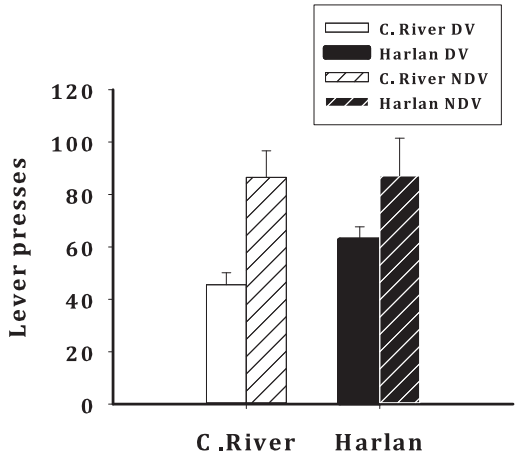


Figure 3.2.1: Mean lever press responses (a) and magazine entries (b) across the three training sessions. Total N = 63; error bars show standard error.

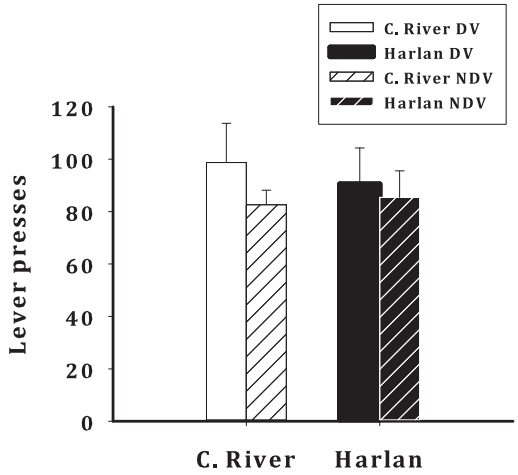
Importantly, prior to reinforcer devaluation with LiCl there were no pre-exsisting differences in either lever press responding or magazine entry behaviour between the "to be" devaluation TYPE and DEVALUATION groups ($p > .05$, ns), meaning that any post-devaluation difference in devaluation sensitivity between the groups were not due to pre-exsisting differences in response rates. For magazine entries there was a significant SESSION x DEVALUATION group interaction ($F(2, 110) = 3.823$, $p = .025$) but this was explained by a initial difference in response rates between the DEVALUATION groups (*Devalued* > *Non-devalued*) at session one, which was no longer evident at sessions two and three (see Appendix A for mean values).

Extinction test. As shown in Figure 3.2.2, reinforcer devaluation reduced lever press responding in extinction in the animals where devaluation occurred in the operant box (i.e. the *Box* condition) only, and not where it occurred in the home-cage like environment (i.e. the *Cage* condition) resulting in a devaluation TYPE x DEVALUATION group interaction ($F[1,55] = 8.839$, $p = .004$). Furthermore, simple effects analysis revealed that the devaluation effects were much greater in those animals sourced from Charles River in the *Box* condition (Simple effect of DEVALUATION group [Charles River, *Box*]: $F[1,55] = 7.881$, $p = 0.007$). In contrast, whilst there

was a trend for a devaluation effect in the animals sourced from Harlan and in the *Box* condition, this trend failed to reach significance ($p = 0.098$).



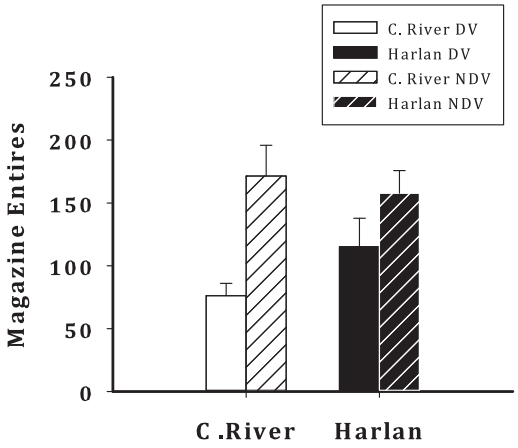
(a) *Box* condition



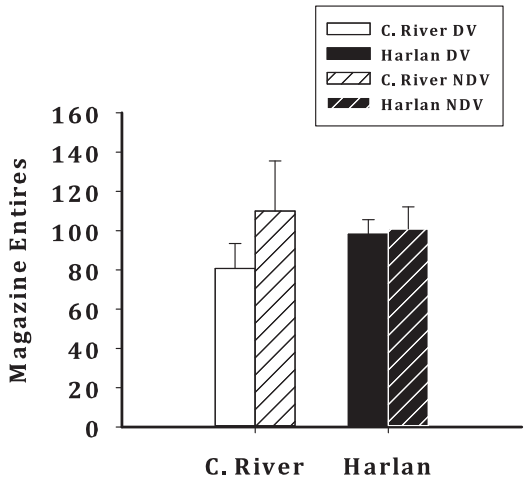
(b) *Cage* condition

Figure 3.2.2: Mean number of lever presses during extinction test for the different devaluation methods. N = 63; error bars show standard error.

The magazine entry data during extinction followed a similar qualitative pattern to the lever press data (see Figure 3.2.3). Again, a significant devaluation TYPE x DEVALUATION group interaction ($F[1,55] = 4.33$, $p = .042$) was found to be driven by a simple main effect of DEVALUATION group in the *Box* condition in animals sourced from Charles River only ($F[1,55] = 14.035$, $p <. 001$).



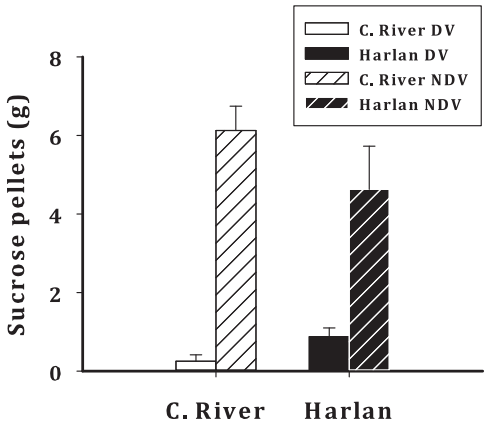
(a) *Box* condition



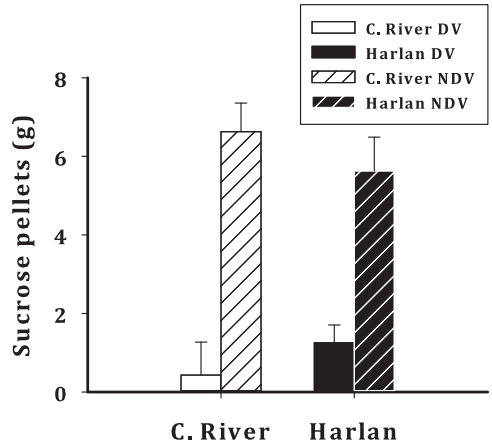
(b) *Cage* condition

Figure 3.2.3: Mean number of magazine entries during extinction test for the different devaluation methods. Total N = 31; error bars show standard error.

A significant main effect of DEVALUATION group ($F[1,55] = 126.606$, $p < .001$) confirmed that devaluation successfully reduced the appetative value of the sucrose pellets (see Figure 3.2.4).



(a) *Box* condition



(b) *Cage* condition

Figure 3.2.4: Mean amount of sucrose pellets eaten during the consumption test for the different devaluation conditions (in grams). N = 63; error bars show standard error.

3.2.3 Summary

All animals acquired the instrumental response, increasing their lever press performance, and decreasing their magazine entries correspondingly, as training progressed. Animals sourced from Charles River pressed the lever significantly more overall during training than Harlan animals, but there was no difference in magazine entries between the two SOURCE groups. At test, Charles River animals, devalued in the training context (i.e. *Box* condition), were the only group to significantly reduce instrumental responding relative to their equivalent non-devalued group. These were

also the only animals to exhibit sensitivity to outcome devaluation in their magazine entry behaviour. Consumption test data suggest the insensitivity of operant behaviour to outcome devaluation displayed by the other groups was not caused by a failure of the LiCl injection to induce a conditioned taste aversion to the instrumental outcome; all devalued groups consumed significantly less than their equivalent non-devalued groups during the consumption test and there were no significant interactions. These results suggest that devaluing animals from Charles River in the training context is the optimal method (from those tested here) by which to demonstrate sensitivity to outcome devaluation, and, therefore, the most appropriate method for modelling goal-directed behaviour.

3.3 Experiment 2: Development of a training protocol for modelling habitual responding

The aim of this experiment was to develop a protocol which produced patterns of habitual responding in rats by extending the period of instrumental training from that used in Experiment 1. A new cohort of animals, sourced from Charles River, were trained on the same reinforcement schedule used in Experiment 1 but the number of training sessions was increased to from three to 10. Based on the results of Experiment 1, devaluation was achieved in the same way as in the *Box* condition described previously.

3.3.1 Methods

Subjects. 16 naive male Lister hooded rats (Charles River, Margate, Kent, UK) with an overall mean *ad libitum* feeding weight of 263.4g (range 249-283g) prior to food restriction and a mean weight of 217.9g (range 200-235g) at the end of training, were used in the experiment. Prior to the start of training animals were food restricted to

above 80% of their ad libitum feeding weights, as described in section 2.3.1. After training all subjects were assigned to one of two experimental groups (*Devalued* or *Non-devalued*). Animal husbandry details were as described in section 2.1.1.

Behavioural training. A summary of the basic experimental design is shown in Table 3.3.1. All other experimental details were the same as for the Charles River animals in the *Box* condition described above in Experiment 1.

Data analysis. Analysis was conducted on lever press, magazine entry and consumption test data. For the training data, lever press responses and magazine entries were analysed using a mixed ANOVA with the within-subjects factor of Session and the between subject factor Devaluation Group. All test data were analysed using independent t-tests to compare the performance of *Devalued* and *Non-devalued* groups.

Day 1	Day 2	Day 3-5	Day 6	Day 7
Magazine training session (20 rewards)	CRF session (20 rewards)	RI30 sessions (40 rewards)	Devaluation sessions	Extinction & Consumption test

Table 3.3.1: Summary of experimental design

3.3.2 Results

Training. A mixed ANOVA revealed a significant main effect of SESSION ($F(9, 126) = 30.675$, $p < .001$; see Figure 3.3.1) with lever presses increasing from session one to session 10. There was also a significant main effect of SESSION ($F(9, 126) = 21.313$, $p < .001$) for magazine entries, with entries decreasing from session one to session 10 (see Figure 3.3.1 b).

Again, there were no pre-exsisting differences between the "to be" *Devalued* and *Non-devlaued* groups for either lever presses or magazine entries ($p > .05$; see Appendix A, Table A.2.1).

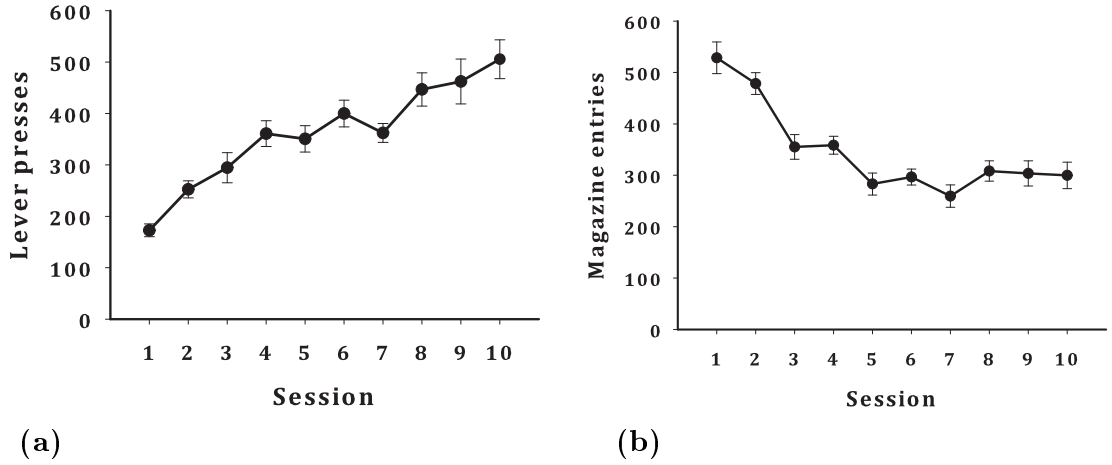


Figure 3.3.1: Mean lever press responses (a) and magazine entries (b) across the 10 training sessions. N = 32 (group n = 8); error bars show standard error.

Test. In contrast to Experiment 1, here there was no difference between the *Devalued* and *Non-devalued* groups in lever press responding (t(14)=-1.169, p = .262; see Figure 3.3.2 a), although there was a trend towards a devaluation effect in magazine entries (t(14)= -2.009, p =.064; see Figure 3.3.2 b).

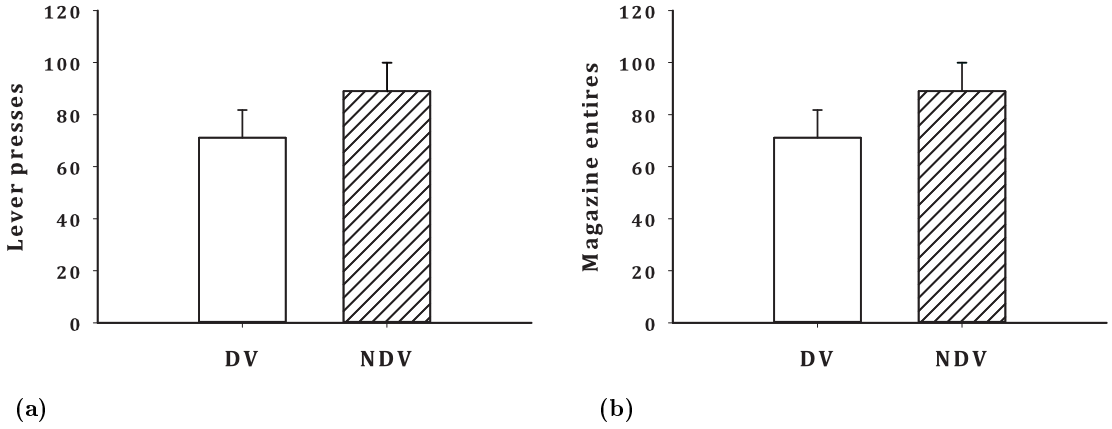


Figure 3.3.2: Mean number of lever press (a) responses and magazine entries (b) during extinction test. N = 32 (group n = 8); error bars show standard error.

Consumption Test. There was a significant effect of DEVALUATION group on the amount of sucrose pellets consumed during the consumption test (t(14) = -6.416, p

$< .001$; see Figure 3.3.3) with *Devalued* animals consuming less than *Non-devalued* animals.

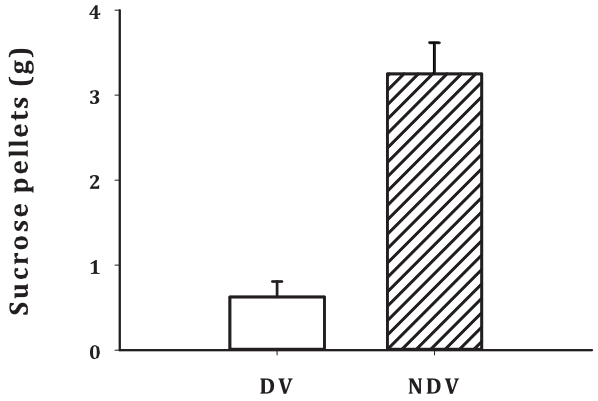


Figure 3.3.3: Mean amount of sucrose pellets eaten during consumption test. N = 32 (group n = 8); error bars show standard error.

3.3.3 Summary

All animals acquired the instrumental response, increasing their lever press performance and decreasing their magazine entries over the course of training. When tested in extinction after, 10 sessions of training, there was no significant difference in lever press responding between the devalued and non-devalued groups. Conversely, there was a trend towards lower levels of magazine entry behaviour in *devalued* animals relative to *non-devalued* animals and sucrose pellet consumption during the consumption test was sensitive to the devaluation manipulation.

3.4 Discussion

Experiment 1 established a reliable method for modelling goal-directed behaviour in rats. In this study the sensitivity of instrumental responding to outcome devaluation of groups of rats, sourced from two different suppliers, was tested following minimal instrumental training. Two different devaluation techniques were also compared; one

group of animals was devalued in the same operant box in which they were trained to lever press (i.e. *Box* condition), whilst another group was devalued in a environment similar to their home cage environment (i.e. *Cage* condition). Only one of the experimental groups displayed sensitivity to outcome devaluation. Charles River animals, devalued in the training context (i.e. *Box* condition), significantly reduced instrumental responding relative to the equivalent non-devalued group. These were also the only animals to exhibit sensitivity to outcome devaluation in their magazine entry behaviour. These results suggest that using animals from Charles River and devaluing them in the training context is the optimal method by which to demonstrate sensitivity to outcome devaluation, and, therefore, the most appropriate method for modelling goal-directed behaviour.

It is not possible to draw firm conclusions about why the instrumental responding of this group was sensitive to outcome devaluation whilst the responding of the other three groups was not, but one can consider a number of possible explanations. Firstly, it is important to note that all devalued groups consumed significantly less than their equivalent non-devalued groups during the consumption test phase of the experiment and that there were no interactions between DEVALUATION and either SOURCE or devaluation TYPE factors. Therefore, a lack of sensitivity of instrumental responding to outcome devaluation was not caused by a failure of the LiCl + sucrose pellet pairings to induce a conditioned taste aversion towards the instrumental outcome. Instead, it appears that these animals failed to modify their instrumental responding despite a change in the motivation value of the outcome. In order to attempt to understand the reasons for this it is necessary to look at the possible effects of the SOURCE manipulation and the devaluation TYPE manipulation in turn.

If we look first at the SOURCE comparison: here, the instrumental responding of groups of animals from two different suppliers, devalued in the same way, showed differing sensitivity to outcome devaluation. One possible explanation for this is that

the instrumental responses of animals from Harlan became habitual sooner (i.e. earlier on in training) than that of animals from Charles River and that three sessions of training was sufficient to produce habitual responding in these animals. Although Charles River animals pressed the lever significantly more than Harlan animals during training, the difference in response rate between the second and the third training session is smaller for Harlan animals than it is for Charles River animals. Dickinson (1985) argues that it is the experienced correlation between response rate and reward rate that is the critical factor in determining the associative structure underlying instrumental responding and that awareness of the instrumental contingency is critically dependent on the experience of a variation in behaviour giving rise to a variation in reward rate. Therefore, the reduced variability in responding in Harlan animals may have lead to a decrease in the perceived correlation between action and outcome and meant that responding was driven by S-R associations (i.e. was habitual). Whilst it is not possible to ascertain a mechanistic explanation for why it may be that animals from different suppliers exhibit differences in instrumental learning based on the data presented here, it is interesting to note that there is experimental evidence of a number of behavioural differences between substrains of mice maintained by different suppliers (Bryant et al., 2008) and Lederle et al. (2011) have even reported strain differences, although not substrain differences, in sensitivity to outcome devaluation. Thus it seems plausible, *a priori*, that genetic differences, caused by genetic drift, for example, may explain at least some of the behavioural variation observed here. Alternatively, and not exclusively, environmental factors may also contribute to behavioural differences in instrumental learning. Animal breeding facilities differ in a number ways. Animal breeding facilities differ greatly in the breeding strategies they adopt (e.g. cross-fostering vs. conventional rearing), colony size, weaning age etc, and all of these factors may potentially have effects on brain development and/or behaviour. Indeed, it is widely recognised in behavioural neuroscience research that experimental outcomes can vary greatly de-

pending on the source/supplier and genetic background of the animals used (Isles et al., 2004; Kiselycznyk and Holmes, 2011; Lederle et al., 2011). Aside from this variation being of interest in itself, in terms of the insight it gives into the effects of environmental and genetic influences on behaviour, as clearly demonstrated by the current set of experiments, it is an issue which needs to be carefully considered when employing tasks, such as those used in this thesis, that rely on a particular pattern of behavioural effects in control groups in order to be informative (Bryant et al., 2008).

It is possible to make stronger inferences about the reasons for the devaluation TYPE effects. Here, whilst only the Charles River animals devalued in the training context showed a significant reduction in responding for a devalued outcome, Harlan animals devalued in the same way showed a slight trend towards a devaluation effect in both lever presses and magazine entries. In contrast, the behaviour of animals from both suppliers, devalued in the home cage-like context, was entirely insensitive to outcome devaluation. This was likely due to a failure of these animals to transfer the learned aversive association between the ingestion of the sucrose pellets and the feeling of malaise from the context in which they were pre-fed to the context in which they were trained and tested. It has been suggested that such apparent context-specific conditioning effects are in fact a reflection of familiarity rather than the associative value attached to a given context (Lovibond et al., 1984), which could indeed be the case in the current set of experiments given the lack of prior exposure to the environment in which pre-feeding was conducted in the *Cage* condition. However, Rosas and Bouton (1997) have argued that conditioned taste aversion shows a degree of contextual sensitivity not seen in other conditioning paradigms. There has been considerable debate regarding the implications of such transfer effects (Jonkman et al., 2010) and it is beyond the scope of this chapter to discuss these in depth. Suffice to say that, for our practical purposes, devaluing the animals in the context in which they were trained appears to be the most appropriate for modelling goal-directed

behaviour.

The aim of Experiment 2 was to investigate whether it was possible to model habitual responding using essentially the same design as that used in Experiment 1 but increasing the period of training from three sessions to 10 sessions. At test there was no significant difference in lever press responding between the devalued and non-devalued groups. Given the significant effect of devaluation on sucrose pellet consumption during the consumption test, this finding indicates that the animals were pressing the lever habitually after 10 sessions of training.

Interestingly, in contrast to lever press responding, there was a trend towards a devaluation effect in magazine entry behaviour. This is consistent with previous findings and supports the view that magazine entry is under the control of a separate behavioural system. Balleine et al. (1995) and others have suggested that magazine entry, being more proximal to reward delivery than lever-pressing, is more sensitive to changes in outcome value. Conditions that result in habitual instrumental responses, such as over-training(Holland, 1998) and pre-training amphetamine sensitization (Nelson and Killcross, 2006), do not produce habitual magazine approach behaviour.

In conclusion, the current set of experiments has successfully established a method for modelling both goal-directed and habitual instrumental responding in rats. This design will be utilized, in conjunction with molecular analysis and pharmacological manipulations, to help advance understanding of the neurobiological mechanisms underlying the transition from goal-directed to habitual behaviour.

3.5 Chapter summary

- The experiments presented in this chapter aimed to design and develop a training protocol for modelling goal-directed and habitual behaviour in rats.

- In Experiment 1 two cohorts of rats, each from different suppliers, were trained to lever press across three sessions on an RI30 schedule of reinforcement.
- Outcome devaluation by LiCl-induced conditioned taste aversion was conducted either in the training context (i.e. animals were pre-fed in the operant boxes in which they were trained) or in a separate context (i.e. animals were pre-fed in an environment similar to their home cages).
- Only animals from Charles River, in which outcome devaluation was conducted in the training context, showed behavioural sensitivity to outcome devaluation in extinction, used here as an index of goal-directed behaviour.
- In Experiment 2, rats from Charles River were trained to lever press across 10 sessions on an RI30 schedule of reinforcement.
- In contrast to Experiment 1, here behaviour, in extinction, was insensitive to outcome devaluation carried out in the training context.
- Taken together, these findings indicated that extending training from three sessions to 10 caused a shift in the nature of instrumental behaviour away from goal-directed actions, controlled by A-O associations, towards habitual responding, controlled by S-R associations. These experiments were used to inform the training protocol for the other studies in this thesis.

Chapter 4

Molecular characterisation of the effects of minimal and extended instrumental training

4.1 Introduction

The experiments presented in Chapter 3 established a method for modelling both goal-directed (i.e. sensitivity to outcome devaluation) and habitual (i.e. insensitivity to outcome devaluation) instrumental lever press behaviour, in separate cohorts of rats, by increasing the number of training sessions from three (*minimal* training) to 10 (*extended* training) respectively. This switch from flexible, purposive actions to automatic, relatively inflexible habits following the extensive practice of an instrumental response has been well documented in both animal models (Dickinson et al., 1995) and human participants (Tricomi et al. 2009; see section 1.1). However, despite the potential utility of understanding the physiological mechanisms involved in the regulation of instrumental behaviours, for example in the understanding the biological basis of disorders of action control (Fontenelle et al., 2011), we still know very little about the molecular processes underlying this transition.

Initial attempts have been made to delineate the specific molecular correlates of goal-directed and habitual behaviours. For example, Shiflett et al. (2010) have shown that Extracellular signal-regulated kinase 2 (ERK2) is activated in the dorsal striatum following the acquisition of a novel response-outcome association. Interestingly, whilst extended training resulted in further increases in ERK2 activation in the pDMS, activation of the same molecule was reduced in the DLS following extended training. Furthermore, the immediate early genes (IEGs), Homer1a and EGR1 (also known as Zif268) have been shown to exhibit training dependent expression patterns across a number of corticostriatal regions (Hernandez et al., 2006), giving an insight in to the spatial and temporal localisation of the up-stream molecular correlates of early- and late-phase instrumental learning. Similar, dissociable, brain region-specific IEG expression patterns have also been observed in the hippocampus and ventral tegmental area (VTA) following instrumental training (Faure et al., 2006). Here, expression of the IEG Fra-1 increased in both the ventral hippocampus and the VTA/ substantia nigra core (SNc) during the early stages of training. Whilst this increase persisted in the VTA/SNc following extended training, the number of Fra-1 expressing neurones in Cornu Ammonis 1 (CA1) was found to decrease in extensively trained animals. These studies are amongst the first to explore the molecular changes that occur in the brain following both the acquisition of a learnt response and its continued practice, and highlight how focusing on brain region-specific molecular changes can inform us about the dynamic nature of the neurofunctional architecture underlying complex behaviours. Furthermore, the bidirectional nature of these reported changes provides an initial insight into the complexity of the regulatory pathways mediating the control of these higher-level behaviours.

Nevertheless, the work which has been done in this area so far has been entirely candidate-driven and, therefore, reliant on *a priori* knowledge and educated guesswork about the mechanisms involved in these behaviours. In contrast, the current

set of experiments used Affymetrix arrays (Rat Gene 2.0 ST Affymetrix) to measure and compare the gene expression profiles of samples of brain tissue taken from groups of non-drug treated animals, at different stages of instrumental training. This approach allowed us to conduct a hypothesis-free analysis of the molecular changes that occur in the brain following different amounts of instrumental learning. By enabling the high-throughput measurement of genome-wide expression profiles, Affymetrix arrays offer a powerful tool for initial investigations into the molecular correlates of behaviour (Mirnics and Pevsner, 2004), and the whole transcript coverage provided by this technique has the potential to highlight novel candidate genes involved in the co-ordination of goal-directed and habitual behaviour.

Specifically, Affymetrix array data was used to identify differences in gene expression between three groups of animals trained to lever press across incremental numbers of sessions. The training manipulation was designed, based on the results presented in the previous chapter, to reproduce the conditions favouring goal-directed and habitual behaviours respectively. However, for both practical and scientific reasons, the effectiveness of the training manipulation in altering sensitivity to reinforcer devaluation was not explicitly tested. This was partly due to concern over the fact that the inclusion of a devaluation manipulation and an extinction test would introduce too much noise in to the data, reducing the probability of detecting specific, training-related changes in gene expression by increasing the temporal distance between behavioural training and dissection. Furthermore, practical constraints limited the number of samples it was possible to include in the microarray analysis, preventing the inclusion of 'Devaluation Group' as a factor. Nevertheless, the two training regimes developed in 3 were used to inform the behavioural design and, in addition to the *minimal* (i.e. three training sessions) and *extended* (i.e. 10 training sessions) training groups described previously, a *moderate* training group, that received six training sessions, was introduced. As well as increasing the power of the microar-

ray analysis to detect any incremental, training-related effects by providing an extra point on a curve of increasing experience of the instrumental contingency, the inclusion of this group also provided additional information regarding the nature of any detected patterns in gene expression changes; for example whether or not the switch in response style is driven by step-like qualitative expression changes, perhaps via some kind of threshold mechanism (Molfese, 2011), or via more linear, quantitative changes.

As alluded to above, cross-brain region comparisons have the potential to reveal highly informative dissociations. Moreover, by including a within-subjects comparison, such designs have the added advantage of providing a level of control not afforded by single region or whole brain analyses. Since lesions studies have strongly implicated the posterior dorsomedial striatum (pDMS) and dorsolateral striatum (DLS) in the acquisition and storage of the associative structures underlying goal-directed and habitual behaviours respectively (Yin and Knowlton, 2006; see Section 1.2), the current experiments compared the expression profiles of these two regions. Furthermore, given the evidence of training induced molecular changes reviewed above, and the functional dissociation of the DLS and pDMS evidenced in the behavioural and electrophysiological habit formation literature (see Section 1.2), it was anticipated that the array data would reveal dissociable, training-related expression changes between the brain regions, indicative of a shift in the involvement of these two striatal subregions in instrumental behaviour.

4.2 Materials and methods: Behavioural

4.2.1 Subjects

A total of 72 naive male Lister hooded rats, from Charles River (Margate, Kent, UK), were used in this experiment. After the week-long acclimatisation period all,

animals were food restricted to above 80% of their *ad lib* feeding weights for the duration of the experiment. All experimental groups were placed on a restricted diet on the same day, meaning that, across all the subgroups, there was a range of between 24 hours and nine days between the start of food restriction and the first session of training. Although this meant that the animals' motivational state at the start of training may have differed across the various subgroups, this design was necessary in order to keep the overall period of food restriction the same for all animals (this issue is discussed at length later in this chapter). Table 4.2.1 summarises the mean weights and ranges for each cohort immediately prior to food restriction (*Ad lib)* and at the end of the experiment, after a period of food restriction (Restricted). All other animal husbandry details were as described in section 2.1.1.

Trained Groups	*Ad Lib*	Restricted
Mean	258.792 (258.167)	214.764 (214.833)
Range	195-244 (197-244)	235-294 (235-291)

(a) Ranges and mean *Ad lib* and Restricted weight values for whole *trained* cohort

No training group	*Ad Lib*	Restricted
Mean	241.917 (239.833)	223.833 (220.667)
Range	235-252 (235-249)	216-234 (216-255)

(b) Ranges and mean *Ad lib* and Restricted weight values for *no training* control group

Table 4.2.1: Mean weights and ranges (in grams) for the *instrumental training* group (a) and *no training* group (b). Weight values for those animals included in the microarray analysis are presented in brackets.

4.2.2 Design

As described above, at the start of the experiment animals were assigned to one of three training GROUPS (*minimal* [three training sessions], *moderate* [six training sessions] and *extended* [10 training session] training). Limitations due to availability of behavioural apparatus and the time required for each brain dissection meant that it was necessary to use a 'staggered' design: eight rats from each of the training groups were assigned to one of three 'squads' (A, B and C) and the start of training for each of these squads staggered across three days. This meant that all animals

within an individual squad finished behavioural training on the same day, with the three squads finishing training on separate consecutive days, as shown in Figure 4.2.1. This pseudo-randomised design minimised the risk of confounding posed by potential sources of systematic bias, such as the time of day of behavioural training and the day of brain dissection. Table 4.2.2 summarises the numbers of animals in each of the various training groups and squads.

	Minimal	Moderate	Extended	*Total*
Squad A	8 *(2)*	8 *(2)*	8 *(2)*	24 *(6)*
Squad B	8 *(2)*	8 *(2)*	8 *(2)*	24 *(6)*
Squad C	8 *(2)*	8 *(2)*	8 *(2)*	24 *(6)*
Total	24 *(6)*	24 *(6)*	24 *(6)*	**72 *(18)***

Table 4.2.2: Summary of experimental group sizes (values in brackets refer to the number of animals from which tissue was used in the microarray experiment, see below for criteria used for selection of animals for molecular analysis).

Within a squad, animals belonging to the same training group were trained simultaneously in one of eight operant chambers (details as described in section 2.2.1), so that, ultimately, there were three training 'runs'[1] per squad, per training session (see Figure 4.2.1). Obviously, to ensure all animals within a squad completed their last training session on the same day, it was necessary for the *extended* training groups to start training before the *moderate* and *minimal* training groups. However, to control for any potential order effects, either during training or at the point of dissection, the eventual order of runs within a squad was partially counterbalanced across the training groups in the following way: Squad A: *extended, moderate, minimal;* Squad B: *moderate, minimal, extended;* Squad C: *minimal, extended, moderate* (as shown in Figure *4.2.1*). For each training day the three squads were run in the following

[1] A run being a subgroup of animals that were trained at the same time.

order: Squad C: morning, Squad B: middle of the day, Squad A: afternoon. Again, any possible confounding effects due to time-of-day effects were minimised by the staggered design.

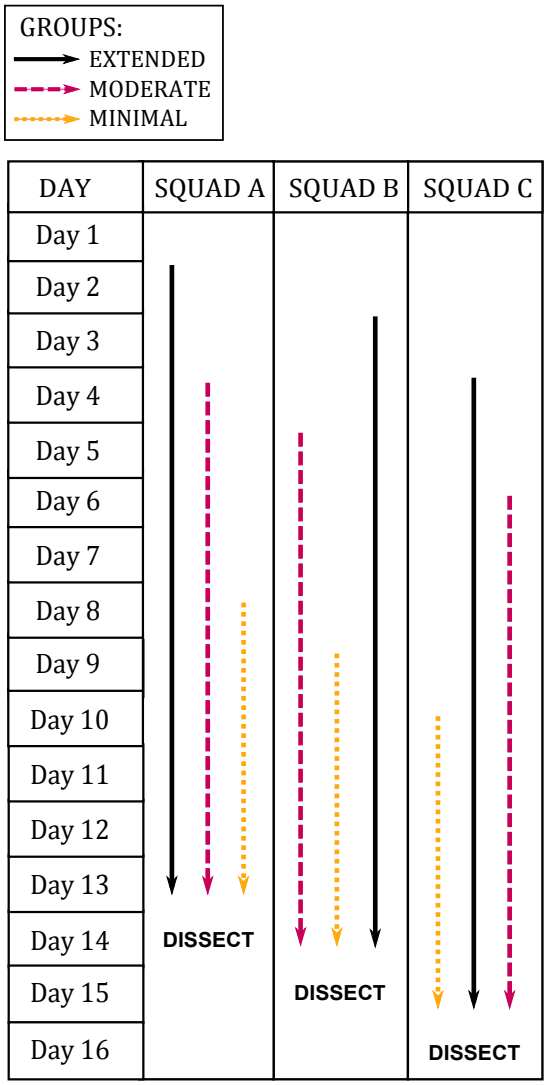


Figure 4.2.1: Summary of the basic experimental design showing the time course of behavioural training for the three squads.

4.2.3 Instrumental training

All animals received two pre-training sessions at the start of training, as described in section 2.3.2, after which lever press training commenced on an random interval 30 second (RI30) reinforcement schedule, as described in section 2.3.2. Animals in the *minimal*, *moderate* and *extended* training groups completed three, six and 10

sessions of RI training respectively. As explained above, to enhance experimental control, animals were trained in squads of 24, consisting of eight animals from each training group. Given the issues outlined at the start of this chapter, the experiment did not include an outcome devaluation stage or extinction test, and all dissections took place approximately 24 hours after the final lever press training session, with the animals spending this time in their home cages.

4.2.4 Brain dissections

Brain tissue was collected from total of 12 animals from each squad, four from each training group. These subgroups were selected to be as representative of the entire cohort as possible and specifically did not to include animals with abnormal levels of lever press responding (see 2.5.3 for details of how outliers were identified)[2]. As described above, dissections were spread across three days, with four animals from each training group being culled on each day. Given that each dissection lasted approximately 30 minutes from start to finish (with the brain being exposed for $\sim$ 20 minutes), the start of each dissection was scheduled to fall within one hour (+/-) of the start time of the final training session on the previous day. Animals were killed by exposure to rising concentration of CO_2 before their brains were rapidly removed and dissected by hand (see section 4.2.4 for further details).

The co-ordinates (from bregma) for the center volume of each region were as follows: DLS (from Yin et al. 2004): +0.7 Anterior-Posterior (A-P), $\pm$3.6mm Medial-Lateral (M-L) and -5mm Dorsal-Ventral (DV) and pDMS (from Yin et al. 2005): -0.4mm A-P, $\pm$2.6mm M-L and -4.5mm D-V. Figure 4.2.2 details the regions of interest dissected. All samples were then stored at -80^{o} until RNA extraction[3].

[2]More animals were trained than were eventually dissected to ensure that the molecular analysis was conducted on tissue from animals whose behaviour was consistent with that of a larger group.

[3]Note that RNA extraction and all proceeding steps in the microarray analysis were conducted in a laboratory in the Department of Pharmaceutical Bioscience at Uppsala University. Tissue samples were shipped from Cardiff University on dry-ice by specialist courier company.

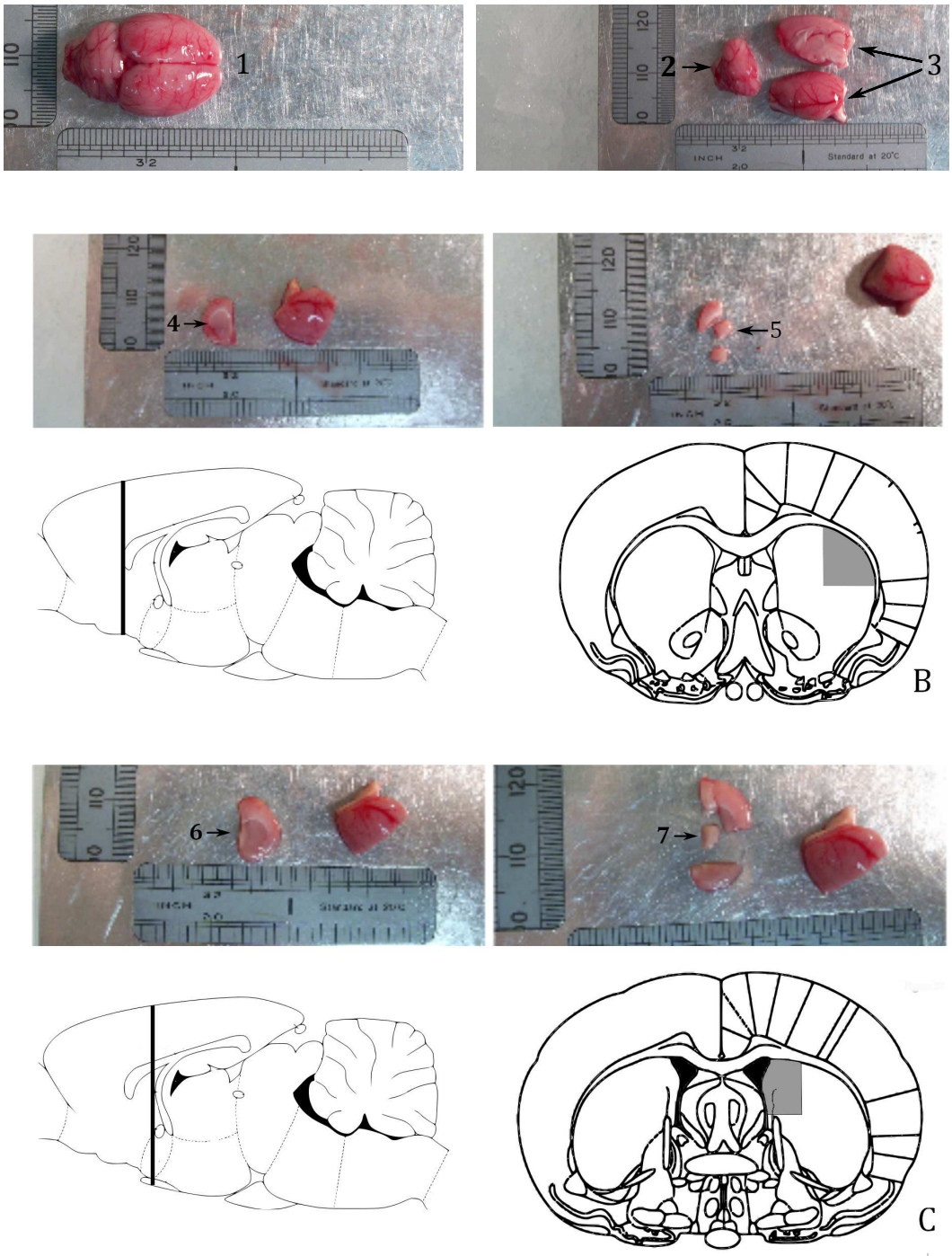


Figure 4.2.2: Figures describing the initial stages of dissection (A), from whole brain (1) to removal of the cerebellum (2) and hemispheric separation (3) and subsequent stages for the dissection of the DLS (B) and pDMS (C) tissue samples. Top panels of figures B and C show photographs of representative dissections for striatal subregion. Labels refer to: (A)4. Whole brain slice from which DLS samples were dissected, (A)5. DLS tissue sample, (B)6. Whole brain slice from which pDMS samples were dissected, (B)7. pDMS tissue sample. Bottom panels of each figure show Rat Brain Atlas images of the respective regions of interest based on stereotaxic co-ordinates taken from bregma (based on Paxinos and Watson, 1998).

4.2.5 Measures and data analysis

As discussed above, it was essential to ensure that the tissue used in the molecular analysis was taken from animals that were behaviourally comparable to the larger cohort. Therefore, the total number of lever press responses and magazine entries made by each animal were averaged across the RI30 training sessions to find an overall mean response rate on each of these measures for those subjects included in the molecular analysis and those that were not. These values were then compared using a two-factor ANOVA, with the between-subjects factors: TRAINING (*minimal, moderate* and *extended*) and ANALYSIS (*array, qPCR* and *other*). These analyses also allowed us to compare relative response levels between the three training groups. A further set of single-factor, within-subjects ANOVAs, comparing mean responses rates (lever presses and magazine entries) across the training SESSIONS, provided information about patterns of learning for each of the training groups individually. Finally, a single-factor ANOVA was used to compare response levels during the last session of training between the three training groups.

4.3 Materials and methods: Molecular

The microarray, qPCR and statistical analyses were conducted in collaboration with Dr Birger Scholtz at the Department of Pharmaceutical Bioscience, Uppsala University. Striatal tissue samples selected for microarray analysis were transported from Cardiff University to Uppsala, on dry ice, by a specialised courier company. Upon arrival at Uppsala University all samples were stored at -80° prior to RNA extraction.

4.3.1 RNA preparation

Extraction, isolation and purification of total RNA was conducted with RNeasy (Quiagen Ltd,), according to the manufacturers specifications. Sample integrity

was assessed using a Agilent Bioanalyser (Agilent Technologies, Amsterdam, The Netherlands) and any samples with an RNA Integrity Number (RIN)[4] below eight were excluded.

Feature	Values / specifications
Total probes	>610,400
Exon-level probe sets	>214,300
Gene-level probe sets	>29,400
ERCC probe sets1	92
Background probes	Antigenomic set
Poly-A controls	dap, lys, phe, thr
Hybridization controls	bioB, bioc, bioD, creX
Total RNA input required	50–500 ng
Probe feature size	5 μm
Probe length	25-mer
Probes per gene (median)	22
Target RNA orientation	Sense target

Table 4.3.1: Summary of array chip details (taken from Affymetirx Data Sheet for GeneChip® Rat Gene 2.0 ST Array)

4.3.2 Microarray details

Those samples with sufficient integrity were hybridized to Affymetrix Rat 2.0 Gene Arrays (29489 probe sets/gene representations; further details for array chips can be found in Table 4.3.1). Table 4.3.2 provides a summary of the experimental conditions and the number of samples included in each condition. Hybridization of individual arrays (n = 40) was conducted simultaneously, in a single batch, before being scanned in an Agilent microarray scanner at the Uppsala Array Platform.

[4]Individual RINs are determined by the application a regression algorithm to spectrophotometric data to produce an reliable, user-independent measure of RNA sample integrity Schroeder et al. (2006).

	DLS	pDMS	Total
No training	DLS00 (n=5)	pDMS00 (n =5)	n = 10
Minimum	DLS03 (n = 5)	pDMS03 (n = 5)	n = 10
Moderate	DLS06 (n = 5)	pDMS06 (n = 5)	n = 10
Extended	DLS10 (n = 5)	pDMS10 (n = 5)	n = 10
Total	n = 20	n = 20	n = 40

Table 4.3.2: Summary of experimental conditions and sample sizes submitted for microarray analysis for the three training groups and the *no training* control group. DLS: dorsolateral striatum; pDMS: posterior dorsomedial striatum; DLS00 / pDMS00: tissue samples from the *no training* group; DLS03 / pDMS03: tissue samples from the *minimal* training group DLS06 / pDMS06: tissues samples from the *moderate* training group; DLS10 / pDMS10: samples from the *extended* training group.

4.3.3 Data analysis

As described below, a number of approaches were employed during the analysis of the array data. This multifaceted approach provided a number of levels of data description and was designed to avoid any analysis-driven bias.

Pre-processing and quality control. Initially all arrays were subject to standard Affymetrix microarray quality control (QC) assessment using the R package 'simpleaffy' (v.2.36.1). Only arrays which passed the stringent QC measures were included in data analysis. A basic summary of the QC work flow is shown in Figure 4.3.1. Background correction and quantile normalization of raw intensity values and median polish summarisation of log2 expression intensities was performed using the Robust Multi-array Average (RMA) algorithm (Irizarry et al., 2003).

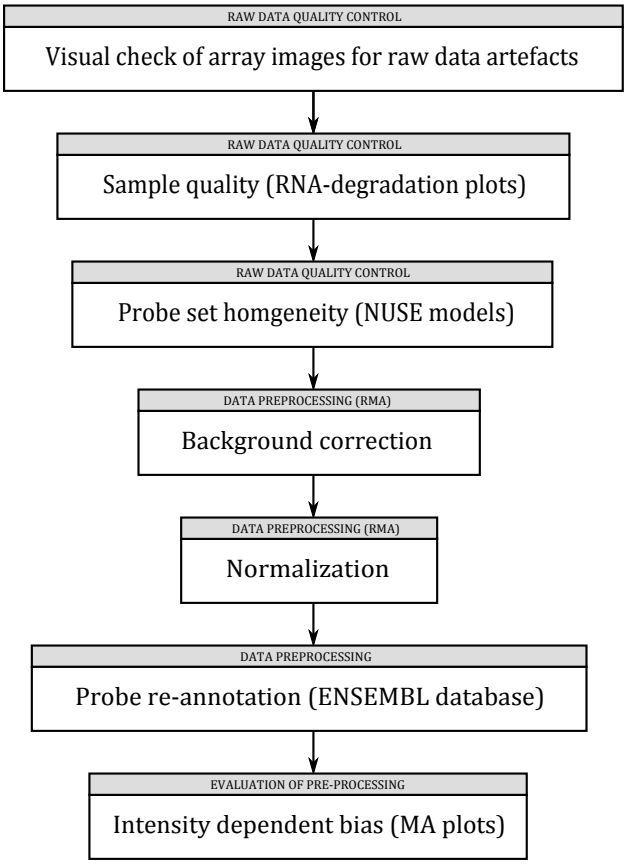


Figure 4.3.1: Summary of the quality control work flow (adapted from www.arrayanalysis.org/main.html)

Multivariate analysis: Principal component analysis (PCA). Initial multivariate (MV) analysis of pre-processed log2 intensity values, for all probes, provided a hypothesis-free overview of the structure of the overall data space. An unsupervised Pareto-scaled principal component model (PCA; Eriksson et al., 1999), extracted using SIMCA-P v.13 software (Umetrics, Sweden), was generated and used to visualize any brain-region and training-related clusters in the data set as a whole, as well as to check for systematic variation indicative of technical artifacts and potential design confounds.

Univariate analysis: pairwise comparisons. Univariate analyses provided information about cumulative changes in gene expression across the training sessions. The

LIMMA (linear models for microarray data) function 'lmFit' was used to fit a linear model to the log2 intensity values for each probe. Since there was insufficient power to detect interaction contrasts (i.e. region x training group), a contrast matrix was generated to extract between-training group, within-brain region, paired contrasts. Table 4.3.3 summarises the pairwise comparisons defined by this matrix. Differential expression estimates were derived from moderated t-statistics using an empirical Bayes method, which produces more stable standard errors for log2 fold change estimates by moderating error terms across the entire probes set (Smyth, 2004). The results of the pairwise comparisons were displayed in Venn diagrams showing absolute numbers of unique and shared significantly regulated probes (defined as $p < .05$) for each comparison. Finally, initial information regarding the biological characteristics of the data set was obtained by comparing numbers of significantly regulated protein-coding and non-coding (nc) probes for each pairwise comparison.

	DLS03	**DLS06**	**DLS10**
DLS00	DLS1		
DLS03		DLS2	
DLS06			DLS3

(a) Between-training group comparisons for DLS samples

	DMS03	**DMS06**	**DMS10**
DMS00	DMS1		
DMS03		DMS2	
DMS06			DMS3

(b) Between-training group comparisons for pDMS samples

Table 4.3.3: Summary of the pairwise comparisons defined in the LIMMA design matrix

Biological interpretation. Any meaningful biological interpretation of microarray data sets requires researchers to move beyond approaches based purely on statistical significance testing. Therefore, a number of functional analyses were applied to the current data set to provide information about the biological relevance of the differentially regulated probes identified by the pairwise comparisons.

Functional annotation. Functional annotation and clustering analysis were conducted using the DAVID (Database for Annotation, Visualization and Integrated

Discovery) platform (Huang et al., 2009). Initial annotation terms were sourced through the Gene Ontology (GO) website (www.geneontology.org), which provides publicly available controlled and structured vocabularies (i.e. ontologies) that summarise functional information about genes and gene products and, importantly, describe biologically relevant relationships and interactions between sets of functionally related genes (Ashburner et al., 2000). Annotating probes sets of interest with defined GO terms, representing the biological attributes of a particular gene product, enables researchers to extract standardized gene lists which can then be interrogated at different levels to obtain information about the biological processes underlying the observed expression differences between experimental conditions. Here, GO annotation required probes to have at least one annotation term in the biological processes (GOBP), cellular component (GOCC) or molecular function (GOMF) ontology in the Affymetrix array annotation.

Ontology enrichment: Over-representation analysis (ORA). P-values, derived from the modified t-statistics, were used to rank up- and down-regulated probes in order of significance. From this ranking, candidate sets of the top (i.e. most significant) differentially expressed, GO-annotated probes for each pairwise comparison were defined at multiple p-value cut-off points (see Table 4.3.4). The gene lists generated within each of these ranking sets were then submitted for over-representation analysis (ORA).

Probe set label	P-value ranking label	Ranked p-value cut-off (Number of "top" GO annotated probes included in gene list)	Up-Regulation (↑) / Down-regulation (↓)
$\mathbf{300_p}$	n_{1u}	300	↑
$\mathbf{300_m}$	n_{1d}	300	↓
$\mathbf{400_p}$	n_{2u}	400	↑
$\mathbf{400_m}$	n_{2d}	400	↓
$\mathbf{500_p}$	n_{3u}	500	↑
$\mathbf{500_m}$	n_{3d}	500	↓

Table 4.3.4: Summary of p-value ranking sets defined for ORA

ORA was conducted in EASE (Expression Analysis Systematic Explorer; Hosack et al. 2003) against six different functional ontologies (described in Table 4.3.5). To identify statistically over-represented ontology classes[5] in each gene list, a modified Fisher's exact test was used to estimate the probability of observing the number of listed genes belonging to the same ontology class (i.e. 'list hits'), given the number genes from the entire set of Affymetrix Rat 2.0 Gene Array annotated probes (i.e. the background 'population total') contained within that class (i.e. 'population hits'). Only those classes containing ≥ 5 listed genes, with an EASE score[6] of $p < 0.01$ and an enrichment score of > 2[7], were included. Clusters of functionally related classes were identified in using DAVID's Functional Classification Tool. To maximise the biological relevance of class clusters, only those classes functionally related to > 3 other significant classes, and with a Kappa similarity threshold of ≥ 0.85[8], were selected (Huang et al., 2007). Finally, to ensure that each ranking set

[5]Ontology classes model *types* of functional attributes (i.e processes or components) rather than specific *instances* (i.e. individual genes).

[6]The EASE score calculated in DAVID as a conservative one-tailed Fisher's exact p-value which penalizes smaller clusters relative to larger functional classes by subtracting 1 gene from within a given classification category prior to analysis. This biases the p-value in favor of

[7]The Enrichment score for a functional class is calculated in DAVID as the geometric mean of the EASE scores for all significantly enriched annotation terms within the class.

[8]Kappa statistics were used to estimate the degree of agreement between all significant classes.

contained relatively stable numbers of genes, only those classes showing a continuous proportional difference in gene counts (e.g. n300m < n400m < n500m etc) with no reversal ratio differences (e.g. n300m / n300p $\propto$ n400m / n400p) across the ranking sets were selected[9].

Functional Class	Ontology Database	Description
Gene Ontology Biological Processes (GOBP)	GO	A biological *objective* to which the gene or gene product contributes often involves a chemical or physical transformation. Functional terms arranged on a spectrum of specificity from broad (e.g.'signal transduction) to specific (e.g. 'translation').
Gene Ontology Molecular Function (GOMF)	GO	The biochemical activity (or potential activity) of a gene product. Specifies *what* is done not where or when the event actually occurs. Functional terms are arranged on a spectrum of specificity from broad (e.g. 'enzyme') to specific (e.g. 'adenylate cyclase')
Gene Ontology Cellular Component (GOCC)	GO	Refers to *where* in the cell a gene product is active (e.g 'ribosome' or 'proteasome').
Panther biological process (PBP)	PANTHER	Used to classify proteins based on their biochemical properties (e.g. receptor, cell adhesion molecule, or kinase).
Panther Molecular function (PMF)	PANTHER	Used to classify proteins based on their *cellular role* or the *process* in which they are involved (e.g. signal transduction [cellular role], TCA cycle [pathway]).
KEGG	KEGG (Kyoto Encyclopedia of Genes and Genomes)	Database links genomic information with higher order functional information. Functional assignment involves linking gene sets with molecular networks, such as a pathway or a complex, representing a higher order biological function.

Table 4.3.5: Summary and description of ORA candidate sets. GO domain definitions taken from Ashburner et al. (2000). PANTHER definition taken from Thomas et al. (2003). KEGG definition taken from Kanehisa and Goto (2000).

[9]The stipulation of a proportional difference in GO class counts between the p-value ranking sets ensures that the relative cluster size remains stable across an arbitrary range of the top-ranked gene candidates.

Heat maps were generated to visualize the ORA output. The raw gene counts for each ranking set were normalized by dividing the set gene count (n_i) by the mean gene count across all sets for each significant class ($n_i / \{\{n300_m + n400....500_p\} /6\}$). The magnitude of the normalized gene count values was coded from high to low according to a colour scale (red to green respectively). Dendrograms were used to represent the degree of similarity between the different classes based on the results of agglomerative hierarchical clustering using complete linkage [10] with a euclidean distance matrix.

Between group expression profiles: Short Time-series Expression Miner (STEM). Short Time-series Expression Miner (STEM) software (Ernst and Bar-Joseph, 2006)[11] was used to explore patterns of between region gene expression changes across all training groups. In contrast to the t-test statistics, this analysis allows one to move beyond simple pairwise comparisons and interrogate the data for patterns across the training groups. In addition, by using log2 fold change data, the analysis is more closely based on actual expression changes rather than relative p-values.

For each region, three categories of data were analysed: a set including all probes, a set containing annotated probes (i.e. probes with official gene names or GO-terms), and a set containing ncRNA probes only. Log2 fold change data from each pairwise comparison were ordered according to training group (i.e. DLS1»DLS2»DLS3). The STEM algorithm was used to derive clustered expression profiles according to a pre-specified set of profile models, optimized to capture patterns characteristic of experiments containing a small number of meaningfully ordered groups (Ernst and Bar-Joseph, 2006). A significance level was generated for each selected model profile,

[10] Relationships between clusters are derived from the furthest distance between data points within each cluster

[11] STEM software uses a unique set algorithms especially designed for analysing short-time series microarray experiments to cluster expression data and identify statistically significant profiles to capture temporal, between-group expression changes.

based on the of number of user-specified probes assigned to the model (i.e. those probes showing patterns of regulation that matched the model profile) compared to the expected number of genes assigned to the same model.

The three sets of ordered data were analysed separately according to the following parameters: maximum number of candidate model profiles: 100; minimal difference between groups for each comparison: > 0.5; permutations per gene: 30000; and a multiple-comparison adjusted false discovery rate (FDR): $p < 0.001$. Significant profiles with a correlation factor ≥ 0.85 were meta-clustered.

Genes belonging to significantly enriched profiles were submitted for further ORA, conducted as described above. This enabled the extraction of information regarding across-group expression changes for STEM-specific enriched GO classes. The work flow for these analyses is summarised below in Figure 4.3.2.

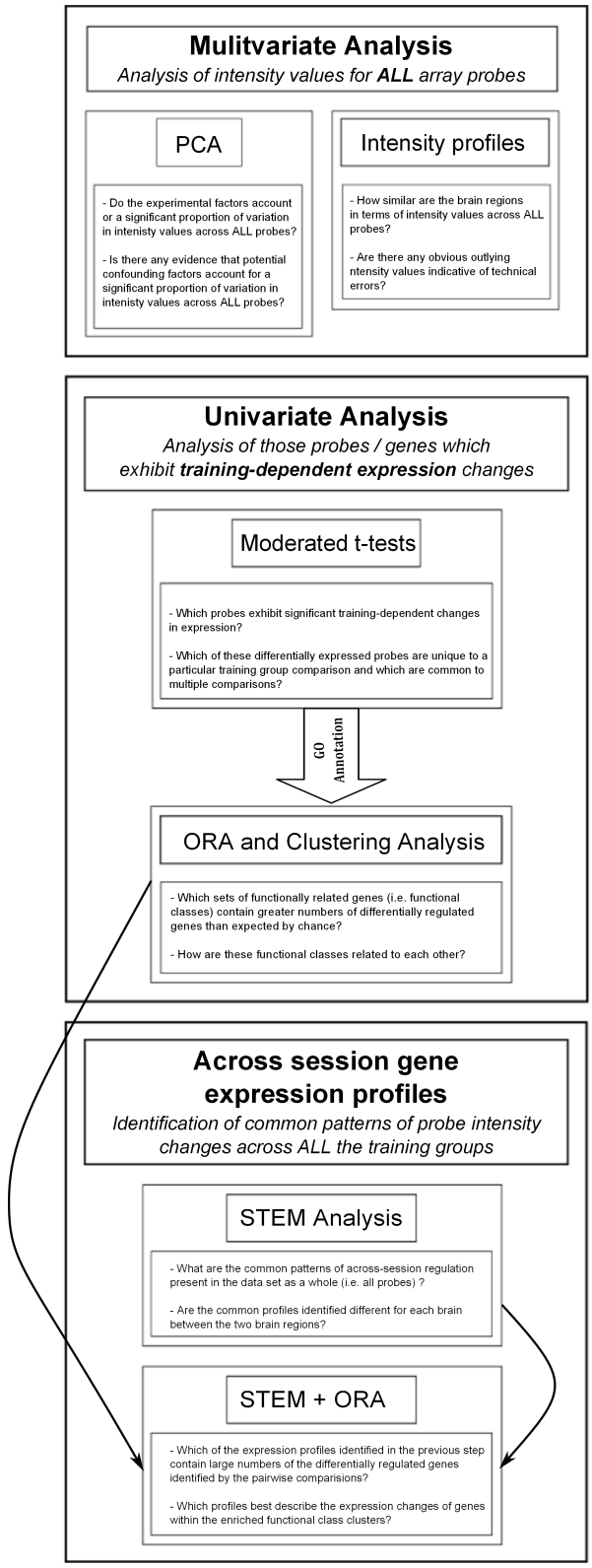


Figure 4.3.2: Work flow summarising the major stages of analysis of normalised probe intensity data. GO: gene ontology; ORA: over representation analysis; PCA: principle components analysis; STEM: short time series expression miner.

Fold change dependent enrichment of RNA classes. Expression of ncRNA is enriched in the nervous system and there is an increasing recognition of the important and complex role that ncRNA transcripts play in a number of structural, functional and regulatory neuromolecular processes (Qureshi and Mehler, 2012). Increasingly researchers are starting to explore how the expression of these transcripts is altered by experience and there is evidence that subclasses of ncRNA are involved in the epigenetic regulation of memory (Mercer et al., 2008), making them of particular relevance here. However, ontology databases have yet to catch-up with this growing interest and the majority of ncRNA transcripts do not yet have functional annotations. Therefore, for the current data set, probes were manually filtered and grouped into categories based on Affymetrix accession numbers[12], transcript name or description. The categories, and the number of probes classified as belonging to each, are shown in Tables 4.3.6 and 4.3.7. Whilst ncRNA probes were further categorised into sub-classes to allow for more detailed analysis, for clarity and brevity, the results presented here will focus only on the comparison of mRNA and general ncRNA probe sets. The probes were further categorised according to their direction of regulation (i.e. up/down) and ranked, within their respective class categories, in order of significance (i.e. high to low) based on the pairwise moderated p-values. As for the ORA ranking, sets of probes were defined at different p-value rankings ranging from the top 200-1000 ranked probes, resulting in 10 ranking sets (200p, 400p, 600p, 800p, 1000p and 200m, 400m, 600m, 800m, 1000m). Fisher's exact test (two-tailed) was then used to test for a significant difference between the number of up- and down-regulated ncRNA probes in each of the ranking sets.

[12] A unique identifier for each probe

Class	Description	Number of probes
Messenger RNA (mRNA)	Protein coding transcripts	23959
Complimentary DNA (cDNA)	DNA sequences synthesized by reverse transcription of mRNA molecular	156
Miscellaneous RNA (miscRNA)	n/a	992
Pseudogenes	Non-functional genomic sequences, some of which are transcribed but which no longer have protein coding function.	77
Non-coding RNA (ncRNA)	See table b below for details of ncRNA subclasses	3792

Table 4.3.6: Summary RNA class categories and numbers of Affymetrix probes for each on the Rat Gene 2.0 ST Affymetrix arrays.

Subclass of ncRNA	Description	Number of probes
Long non-coding RNAs (lncRNA)	Non protein coding (NPC) transcripts >200 nucleotides in length.	109
MicroRNAs (miRNA)	Small NPC transcripts (~20 nucleotides) with translational and post-translational regulatory functions.	712
Ribosomal RNAs (rRNA)	The RNA component of ribosomes	277
Small nucleolar RNAs (snoRNAs)	Involved in post-transcriptional regulation of other RNA molecules	1534
Small nuclear RNAs (snRNA)	Involved in the processing of pre-mRNA into mature mRNA within the nucleus	1144
Transfer RNAs (tRNA)	Involved in the transport of amino acids to the ribosome during protein synthesis	16

Table 4.3.7: Summary ncRNA subclass categories and numbers of Affymetrix probes for each on the Rat Gene 2.0 ST Affymetrix arrays.

4.3.4 Technical verification of microarray data by quantitative polymerase chain reaction (qPCR)

RNA samples were reused for technical verification of the Affymetrix microarray results using qPCR analysis. A number of top ranked genes (based on the ranking of limma p-values) for each pairwise comparison (DLS0...DLS10 / pDMS0...pDMS10) were selected for as candidates for qPCR. Candidate selection was based primarily on primer availability and quality, however, since Affymetrix data for the pDMS revealed a down-regulation of a number of immediate early genes (IEGs) between "*no training*" controls and the *minimal* training group (this feature of the data is discussed in more detail below) qPCR analysis for this particular comparison (pDMS1) focused on three IEG candidates (Arc, Erg1 and Erg2) to enable us to investigate the reliability of this apparent trend towards reduced IEG expression. Candidates and primer sequences are shown in Table 4.3.8.

Gene	Forward primer	Reverse primer
Cartpt (DLS1)	ATCGGGAAGCTGTGTGACTG	GGGAAAGAGCCCATCCACTC
Tbr1 (DLS2)	CTCGGGGATTTACGAGCAGG	GTAGCCGCCTATGTCCTTGG
Stx1a (DLS3)	GAGGAAGGTCTGAACCGCTC	ATCCCAGAGGCAAAGATGGC
Arc (pDMS1)	TGGGAGTTCAAACAGGGCTC	CTGGTACAGGTCCCGCTTAC
Egr1 (pDMS1)	AAAGCCTTCGCCACTCAGTC	GATCGCAGGACTCAACAGGG
Egr2 (pDMS1)	GAGAGTCAGTGGCAGATAGCC	CATTTGCTCCTCGCACAACC

Table 4.3.8: Candidate genes and associated primer sequences

cDNA was synthesised from 300ng of RNA for each sample, using the iScript™ cDNA Synthesis Kit (BIO-RAD, Hercules, CA). pPCR analysis was then performed using the iQ™ SYBR® Green Supermix (BIO-RAD). PCR amplification was conducted

on the CFX Connect™ Real-Time System (BIO-RAD), according to the following parameters: 1 cycle at 95° for 3min; 39 cycles at 95° for 10s; 58° for 30s; 72° for 30s; with a sample volume of 50ul. Samples were run in technical duplicates and normalised to the housekeeping genes B2M (Beta-2 microglobulin) and HPRT (hypoxanthine guanine phosphoribosyl transferase).

Relative gene expression was calculated using the $\Delta-\Delta$-Ct (ddCt) algorithm[13](Livak and Schmittgen, 2001) against the housekeeping reference genes. Welch's t-test[14] was used to assess differential expression for the target contrast (i.e. the pairwise comparison for which the gene was selected as a candidate) by comparing expression in the longer trained group vs. the shorter trained group (e.g. DLS1: DLS03 vs. DLS00). The expression profiles for each candidate were then compared to Affymetrix intensity values to verify, or refute, the differential expression patterns identified by the microarray analysis.

4.4 Results: Behavioural

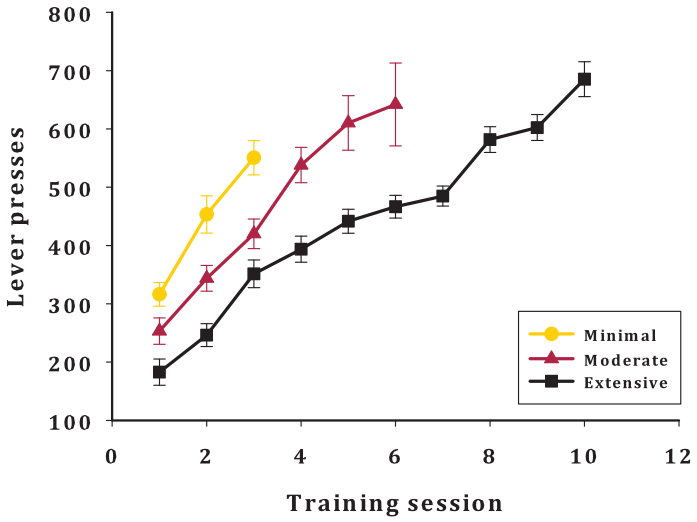
The results of a two-factor ANOVA, with the factors ANALYSIS (*array, qPCR* and *other*) and TRAINING (*minimal, moderate* and *extended*), showed that the session-averaged number of lever press responses (Main effect of ANALYSIS*:* $F[2, 63] = .661$, $p = 0.518$*;* Appendix B, Table B.1.1 a) and magazine entries (Main effect of ANALYSIS: $F[2, 63] = 0.161$, $p = 851$; Appendix B, Table B.1.1 b) did not differ between the three ANALYSIS groups, confirming that the behaviour of those animals from which tissue was taken for molecular analysis was comparable to the other animals in the overall cohort. Furthermore, there was no difference between the total session-averaged number of lever press responses (Main effect of TRAINING:

[13]An approximation method used to estimate *relative* expression of a candidate across different samples.

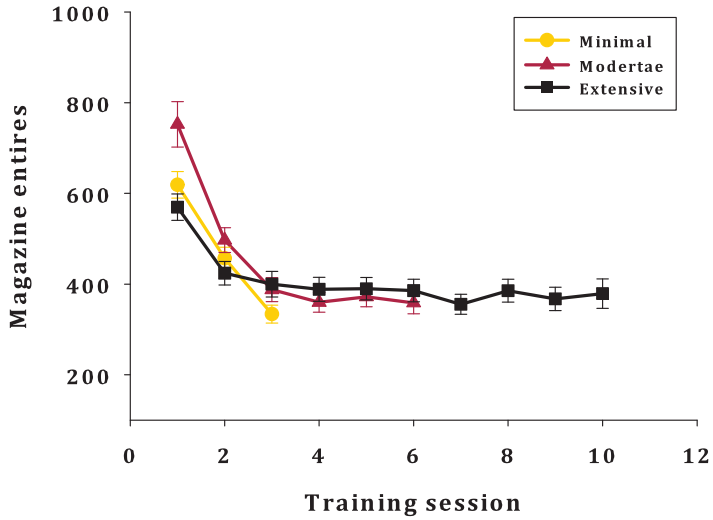
[14]An approximation method used to control for potentially unequal variences

$F[2, 63] = .387$, $p = .679$) between the three training groups. Additionally, although the number of lever presses (Main effect of TRAINING: $F[2, 69] = 11.779$, $p < .001$) and magazine entries (Main effect of TRAINING: $F[2, 69] = 6.922$, $p = .001$) differed across the groups during the first session of training, by the final training session there were no differences in either lever press response rates (Main effect of training group: $F[2, 69] = 1.983$, $p = .146$) or magazine entries (Main effect of TRAINING: $F[2, 69] = .842$, $p = .441$).

Figure 4.4.1 shows the lever press and magazine entry data across the training sessions, for all animals. As expected, lever press response rates increased across the training sessions for all training groups (Main effect of SESSION: *minimal* $F[2, 46] = 47.490$, $p < .001$; *moderate* $F[5, 105] = 26.446$, $p < .001$; *extensive* $F[9, 189] = 50.921$, $p < .001$; Figure 4.4.1 a). Conversely, magazine entries decreased across the training sessions in all training groups (Main effect of SESSION: *minimal* $F[2, 42] = 55.067$, $p < .001$; *moderate* $F[5, 105] = 41.571$, $p < .001$; *extensive* $F[9, 189] = 12.166$, $p < .001$; Figure 4.4.1 b). These data indicate that all groups learnt about the instrumental contingency during training and adjusted their behaviour accordingly.



(a) Lever presses



(b) Magazine entries

Figure 4.4.1: Mean number of lever press responses and magazine entries across training session for the three training groups for all subjects (total N=24; group n = 8 error bars show standard error)

The mean number of lever presses and magazine entries across the training sessions for those animals included in the microarray (N=5) and those intended for biological verification by qPCR analysis (N=6; see Discussion for further details regarding biological verification) are presented alongside the behavioural means for the remaining animals in the experimental cohort (N=13) in Appendix B, Table B.1.1.

4.5 Results: Molecular

Affymetrix Rat 2.0 Gene Arrays (29489 probe sets/gene representations) were used to compare gene expression between samples of tissue from two functionally distinct striatal subregions (DLS and pDMS), taken from rats exposed to different levels of lever press training (see Table 4.3.2 for a summary of the experimental groups). Samples from one rat failed to show sufficient RNA integrity (i.e. RIN < 8) and were replaced by samples with sufficient integrity from a different individual belonging to the same training group. Thus, the final number of arrayed samples per condition were as stated in Table 4.3.2. All arrays passed Affymetrix quality control testing (Table 4.3.1).

4.5.1 Transcriptome properties: Intensity profile plots

As expected for two such anatomically proximal regions, the DLS and pDMS exhibited highly similar overall expression profiles (shown in Figure 4.5.1), providing an initial indication that the data sets were comparable between regions, and that analysis of the tissue was not influenced by any significant technical biases. Plots of various coding and non-coding RNA subclasses also showed similar between-region intensity profiles (see Appendix B, Figure B.1.1) indicating that the relative expression of these subclasses was largely consistent across the two regions. Despite this general similarity, patterns of *training-related* gene expression changes differ considerably between the DLS and pDMS (see below), suggesting that small, but anatomically dissociable, and possibly functionally important, subsets of genes are differentially regulated during instrumental learning.

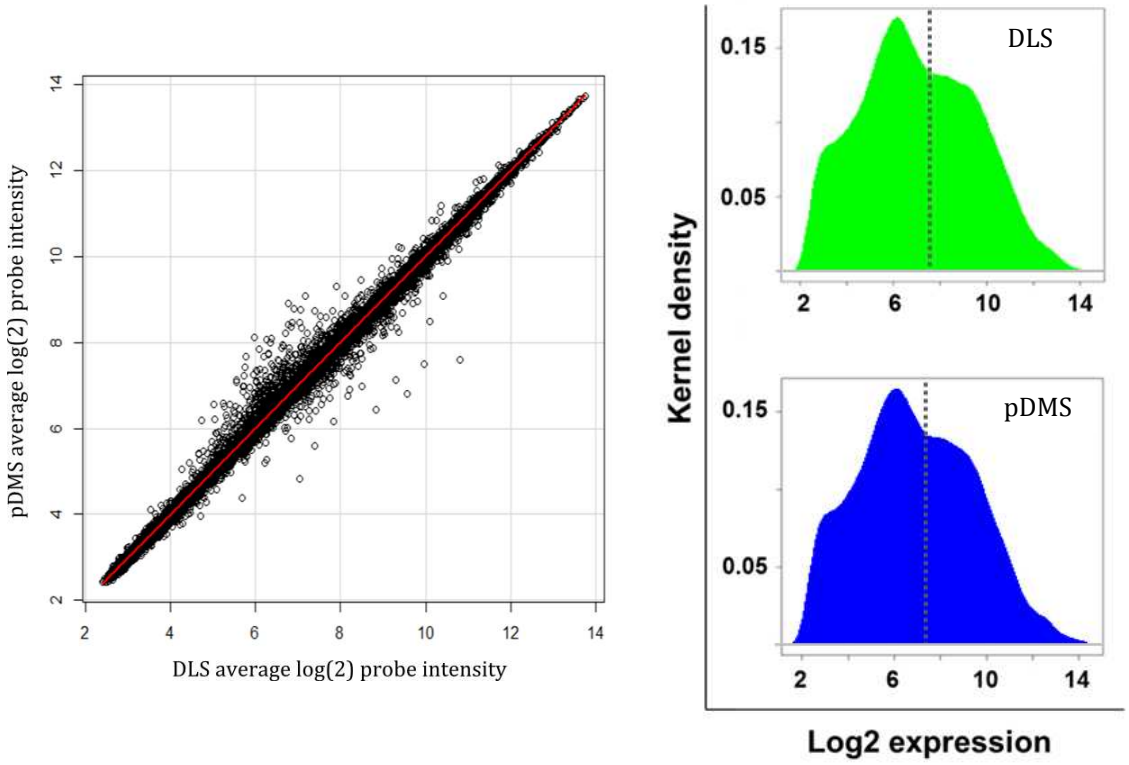


Figure 4.5.1: XY plot comparing average log2 intensity values for all probes between the 2 regions (left) and kernel density plots showing the density distribution of log2 intensities for all probes for the DLS (dorsolateral striatum; top right) and pDMS (posterior dorsomedial striatum; bottom right).

4.5.2 Multivariate analysis: Principle components analysis

Unsupervised, pareto-scaled Principle Components Analysis (PCA) of log2 intensity values for all probes resulted in the extraction of six principle components (PCs) with Eigenvalues > 1, which together explained 47.6% of variation in the model (Table 4.5.1) .

Component	R2X	R2X(cum)	Eigenvalue
0	Cent.		
1	0.142	0.142	5.68
2	0.109	0.251	4.36
3	0.0705	0.322	2.82
4	0.0698	0.391	2.79
5	0.0436	0.435	1.74
6	0.0409	0.476	1.64

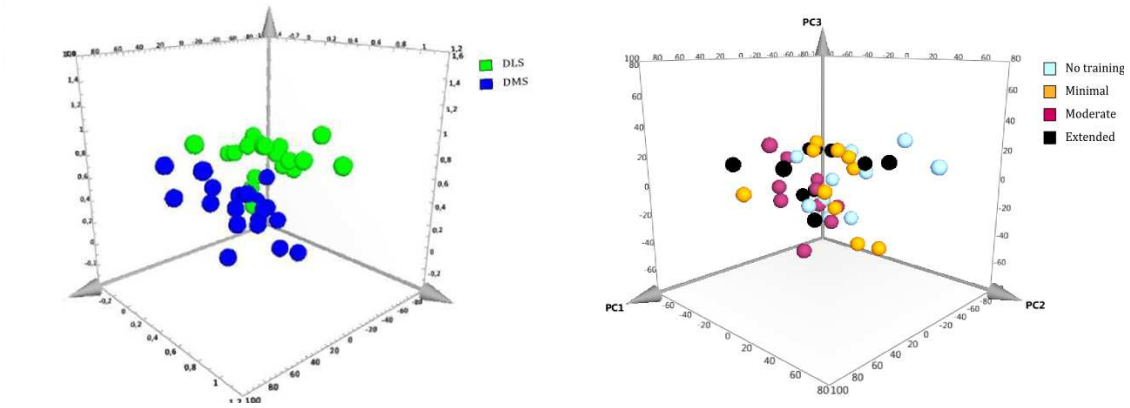
Table 4.5.1: All extracted PCs with Eigenvalues > 1; R2X: Proportion (as a fraction) of variation withing the data set modeled by component; R2X(cum): R2X: Cumulative proportion (as a fraction) of variation withing the data set modeled by successive components.

PCA plots were constructed to visualise between-region and between-training group separation. Only the first three PCs were relevant for between-region separation, with PC1 (first extracted PC) and PC3 (third extracted PC) providing the clearest dissociation between DLS and pDMS samples (R2X[cum] = 0.2125; Figure 4.5.2 a). Given the high degree of similarity between the intensity profiles of the two regions (Figure 4.5.1), the systematic between-region variance evidenced in the PCA model was likely due to subtle, bi-directional differences in gene expression. In order to better understand the nature of the between-brain region separation evident in Figure 4.5.2 a, the PCA model was also applied to data from each of the training groups separately (see Table 4.5.2) and plots coded for region were constructed for each of the four groups (see Appendix B, Figure B.2.2). Model fit was best for the *Extended* training group and between-brain region separation along the first two PCs is only apparent in the trained groups, with no separation evident for the *No training* control group.

Group	R2X PC1	Eigenvalue	R2X PC2	Eigenvalue	Total variance
Control	.262	2.62	.15	1.5	41.2%
Minimal	.231	2.31	.186	1.86	41.7%
Moderate	.220	2.2	.161	1.61	38.01%
Extended	.303	3.03	.179	17.9	47.93%

Table 4.5.2: All extracted PCs with Eigenvalues >2; PC: principle component; R2X: Proportion (as a fraction) of variation withing the data set modeled by component; R2X(cum): R2X: Cumulative proportion (as a fraction) of variation withing the data set modeled by successive components.

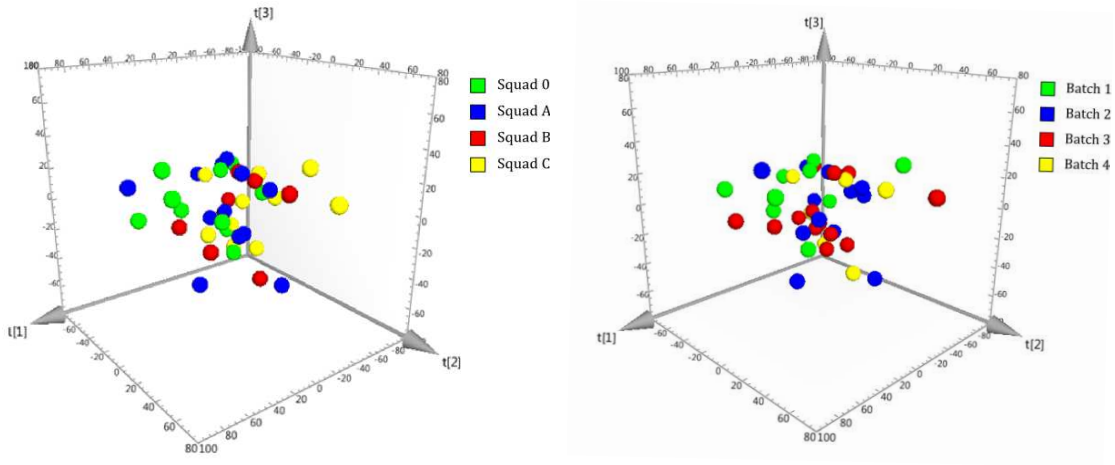
In contrast to the relatively large regional separation, there was no evidence of between-training group separation (see Figure 4.5.2 b; also see Appendix B, Figure B.2.1 for PCA plots showing the lack of between-training group separation for each region separately). Whilst this suggests that a greater proportion of variance in the data set is explained by regional differences than by training effects, it does not mean that training effects are not present; it is possible that there was simply too much noise in the entire probe data set to detect any systematic variance attributable training group. It is important to remember that here we are dealing with whole probe set-level data; given that the samples being analysed were taken from the same species of animal, from the same tissue type (i.e. brain) it is, perhaps, not especially surprising that the effects of the behavioural manipulation were not clearly detectable.



(a) Between-region separation **(b)** Between-training group separation

Figure 4.5.2: 3D PCA plots showing between-region (a) and between-training group (b) separation along PCs 1-3.Total variance explained = 32.2%.

The PCA model was also used to look for evidence of systematic variation in the data attributable to technical artifacts. There was no evidence of separation between the four squads or between the RNA preparation batches, indicating that these were not confounding factors.



(a) Data coded for training squad **(b)** Data coded for RNA preparation batch

Figure 4.5.3: 3D PCA plots coded for training squad (a) and RNA preparation batch (b).

4.5.3 Univariate analysis: pairwise comparisons

Differentially expressed probe sets for each pairwise comparison were defined based on the LIMMA moderated t-statistics. Figure 4.5.4 shows the absolute number of significant differentially expressed probes across the training groups for each region at two different p-value cut off points ($p < .05$ and $p < .001$). From this graph it is evident that more probes exhibited differential expression between the training groups in the DLS than in the pDMS samples, and that the number of differentially expressed probes was highest for the DLS3 comparison (i.e. DLS *moderate* vs. DLS *extended*)

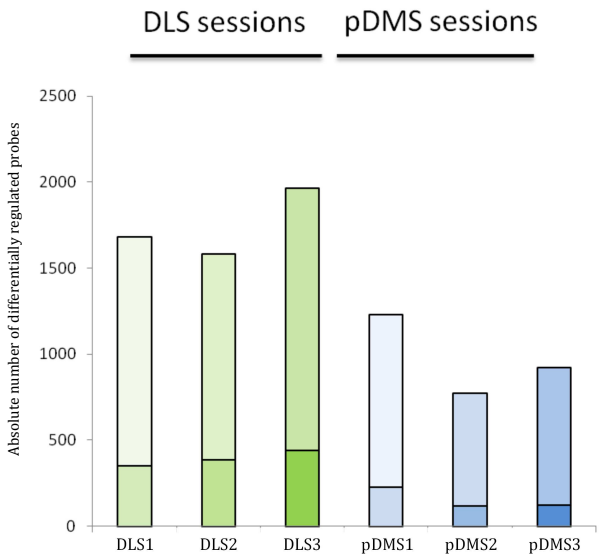


Figure 4.5.4: Number of differentially expressed probes identified by moderated t-tests (top bar [lighter]: p < .05; bottom bar [darker] p < .001)

The Venn diagrams in Figure 4.5.5 show the number of unique (i.e. those probes showing differential expression for one comparison) and shared probes (i.e. those probes showing differential expression for more than one comparison) exhibiting significant differential expression (defined here as moderated $p < .05$) for between-region (left column) and between-training group (right column) contrasts. There is a relatively sparse overlap in differentially expressed probes between the regions for all training groups, indicating that, to a large extent, qualitatively different sets of genes are regulated by instrumental training in the DLS compared to the pDMS; a

pattern which may reflect the functional dissociation between the striatal subregions suggested by lesion studies (e.g.Yin and Knowlton, 2006). This contrasts with the similarity in expression profiles between the regions (Figure 4.5.1); however, whilst the xy plot shows data for all array probes, the Venn diagrams in Figure 4.5.5 specifically compare probes classified as being differentially expressed between the training groups ($p < .05$). Therefore, taken together, these data suggest that, whilst the overall gross pattern of gene expression is very similar between the two regions, there are large regional differences in training-sensitive gene expression changes.

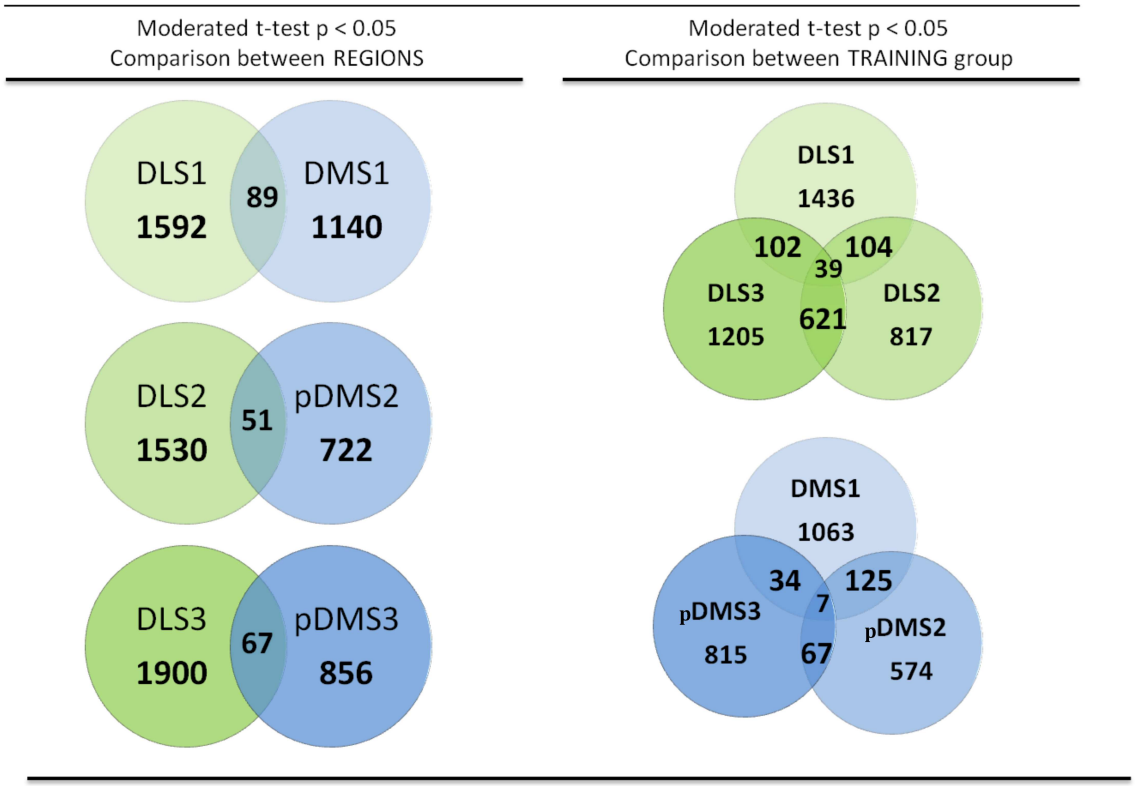


Figure 4.5.5: Numbers of unique and shared differentially expressed probes for between region (left panel) and training group (right panel) comparisons. DLS: dorsolateral striatum; pDMS: posterior dorsomedial striatum; DLS1 / pDMS1: *no training* group vs. *minimal* training group comparison; DLS2 / pDMS2: *minimal* training group vs *moderate* training group comparison; DLS3 / pDMS3: tissues samples from the *moderate* training group; DLS3 / pDMS3: *moderate* training group vs. *extended* training group comparison.

A similar discrepancy between the proportion of unique and shared probe candidates

is apparent for between-training group, within-region, comparisons, although DLS2 (unique probes: 817) and DLS3 (unique probes: 1205) do exhibit a higher degree of overlap, sharing a total of 621 differentially expressed probes (Figure 4.5.5).

4.5.4 Biological interpretation

Ontology enrichment: over-representation analysis. Over-representation analysis (ORA) was conducted on gene ontology (GO) annotated probes from each of the six p-value ranking sets (see Table 4.3.4). Significantly enriched GO class clusters were defined as containing > 3 functionally related significant GO classes[15] within at least one up- or down-regulated p-value ranking set. [16] For each pairwise comparison, a heat map was generated to visualize the normalized difference in gene counts within the identified significant GO classes across the six ranking sets. The results of the hierarchical clustering analysis of significant GO classes, identified by ORA, are shown in Figures 4.5.6 to 4.5.11.

[15]Significant GO classes were defined as sets of genes containing > 5 GO annotated genes, with an EASE score $p<0.01$, kappa test similarity threshold of 0.85 and an enrichment score of > 2

[16]Note the distinction between functional classes and class clusters: functional classes are the first ontological level of description up from individual gene terms (instances), and consist of groups of genes involved in a single specific biological process. GO class clusters form the subsequent level of a gene ontology, and consist of groups of GO classes which share a significant number of genes (the degree of overlap in gene terms required to form a cluster can be user defined: here classes with a Kappa similarity threshold of ≥ 0.85 were clustered and clusters needed to contain a minimum > 3 functionally related significant GO classes to be included in the analysis)

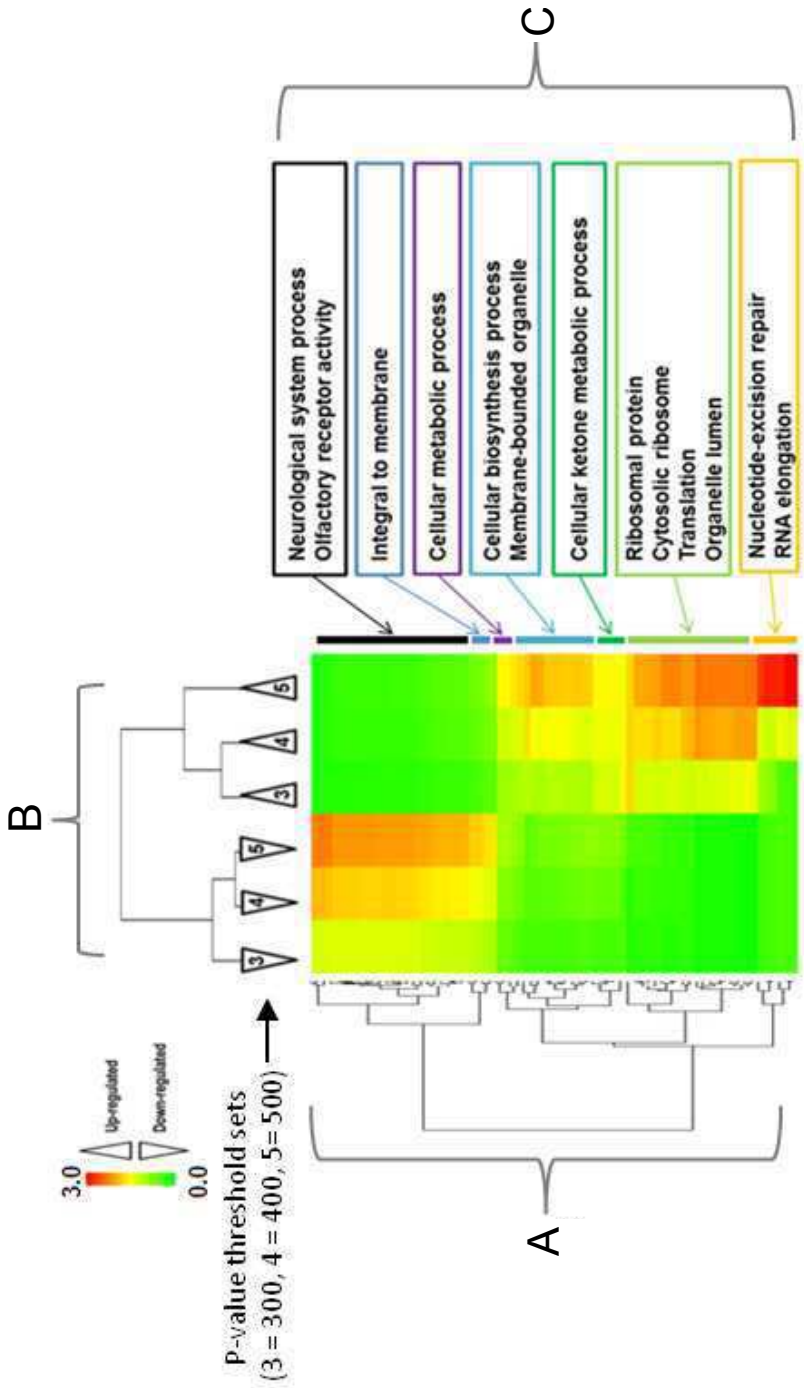


Figure 4.5.6: Heat map showing ontology class enrichment of significantly up- and down-regulated GO annotated probes for the DLS1 pairwise comparison, across all ranking sets. **A**: Between-class hierarchical clustering; **B**: Between-set heirarchical clustering; **C**: cluster annotations. Colour scale reflects the relative normalized difference in gene counts within significantly enriched GO classes for each pairwise comparison (Red » Green = Increase [i.e. normalized difference > 1] » Decrease [i.e. normalized difference < 1]). Dendrograms to the left of the map shows the distance between significant class clusters based on the results of the complete linkage hierarchical clustering analysis, using the Euclidean distance measure. Between ranking set distances are shown above the heat maps.

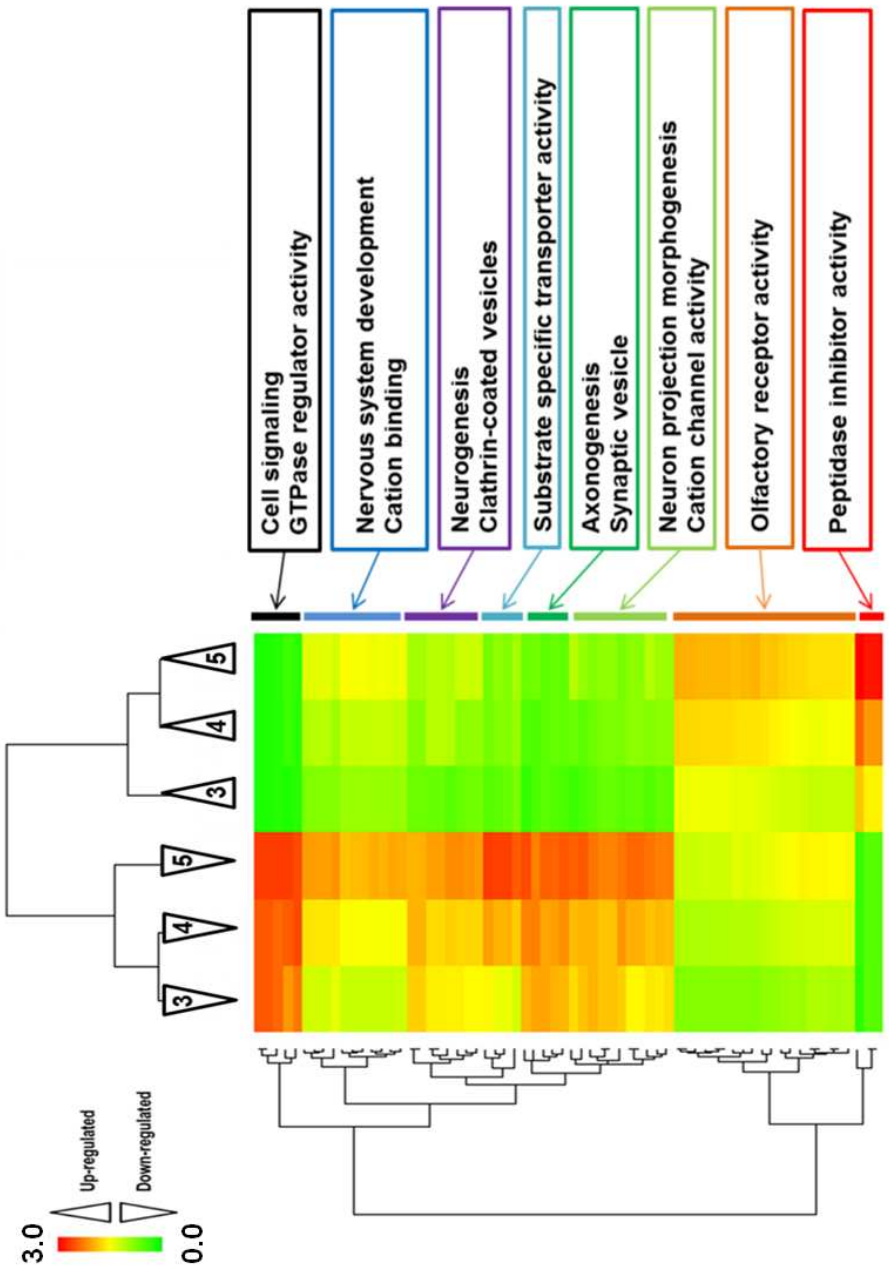


Figure 4.5.7: Heat map showing ontology class enrichment of significantly up- and down-regulated GO annotated probes for the DLS2 pairwise comparison, across all ranking sets. Colour scale reflects the relative normalized difference in gene counts within significantly enriched GO classes for each pairwise comparison (Red » Green = Increase [i.e. normalized difference > 1] » Decrease [i.e. normalized difference < 1]). Dendrograms to the left of the map shows the distance between significant class clusters based on the results of the complete linkage hierarchical clustering analysis, using the Euclidean distance measure. Between ranking set distances are shown above the heat maps.

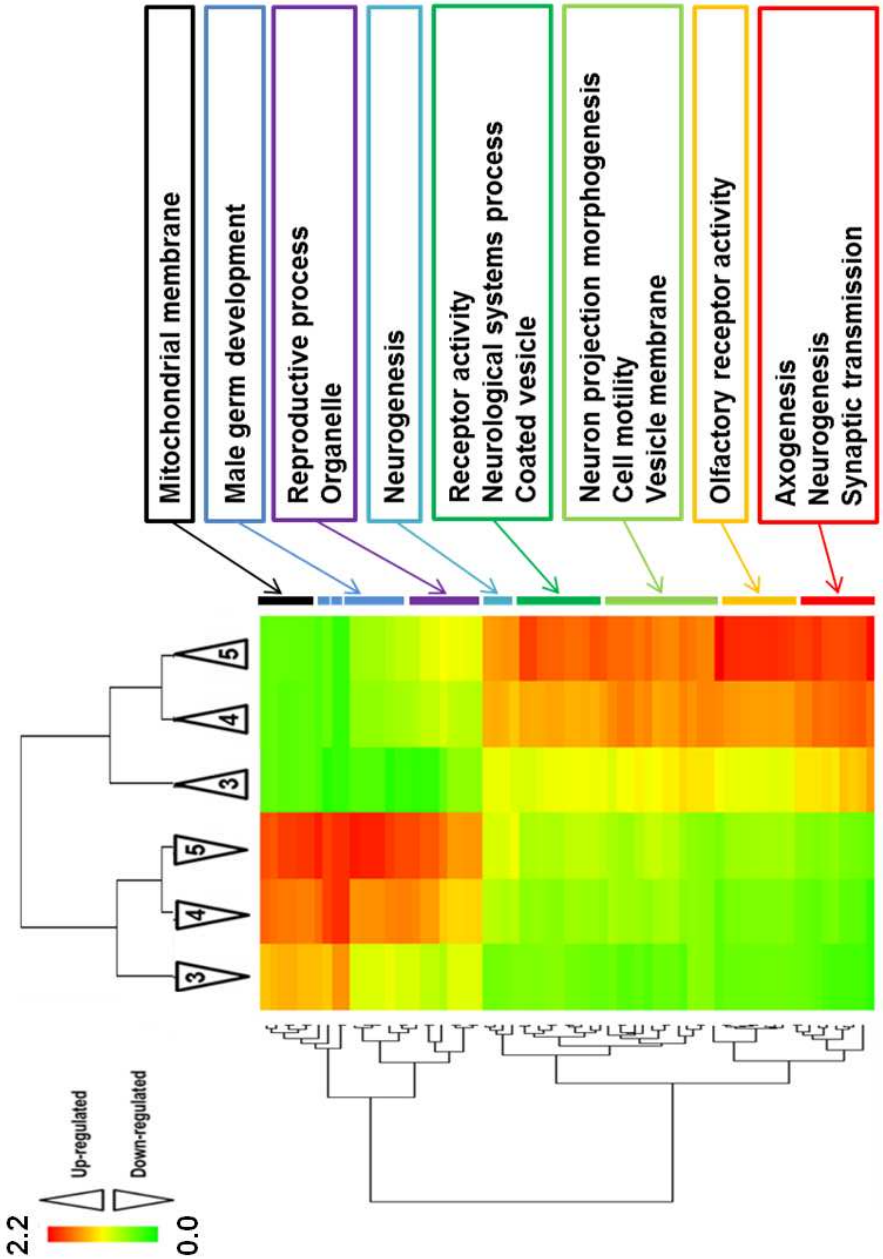


Figure 4.5.8: Heat map showing ontology class enrichment of significantly up- and down-regulated GO annotated probes for the DLS3 pairwise comparison, across all ranking sets. Colour scale reflects the relative normalized difference in gene counts within significantly enriched GO classes for each pairwise comparison (Red » Green = Increase [i.e. normalized difference > 1] » Decrease [i.e. normalized difference < 1]). Dendrograms to the left of the map shows the distance between significant class clusters based on the results of the complete linkage hierarchical clustering analysis, using the Euclidean distance measure. Between ranking set distances are shown above the heat maps.

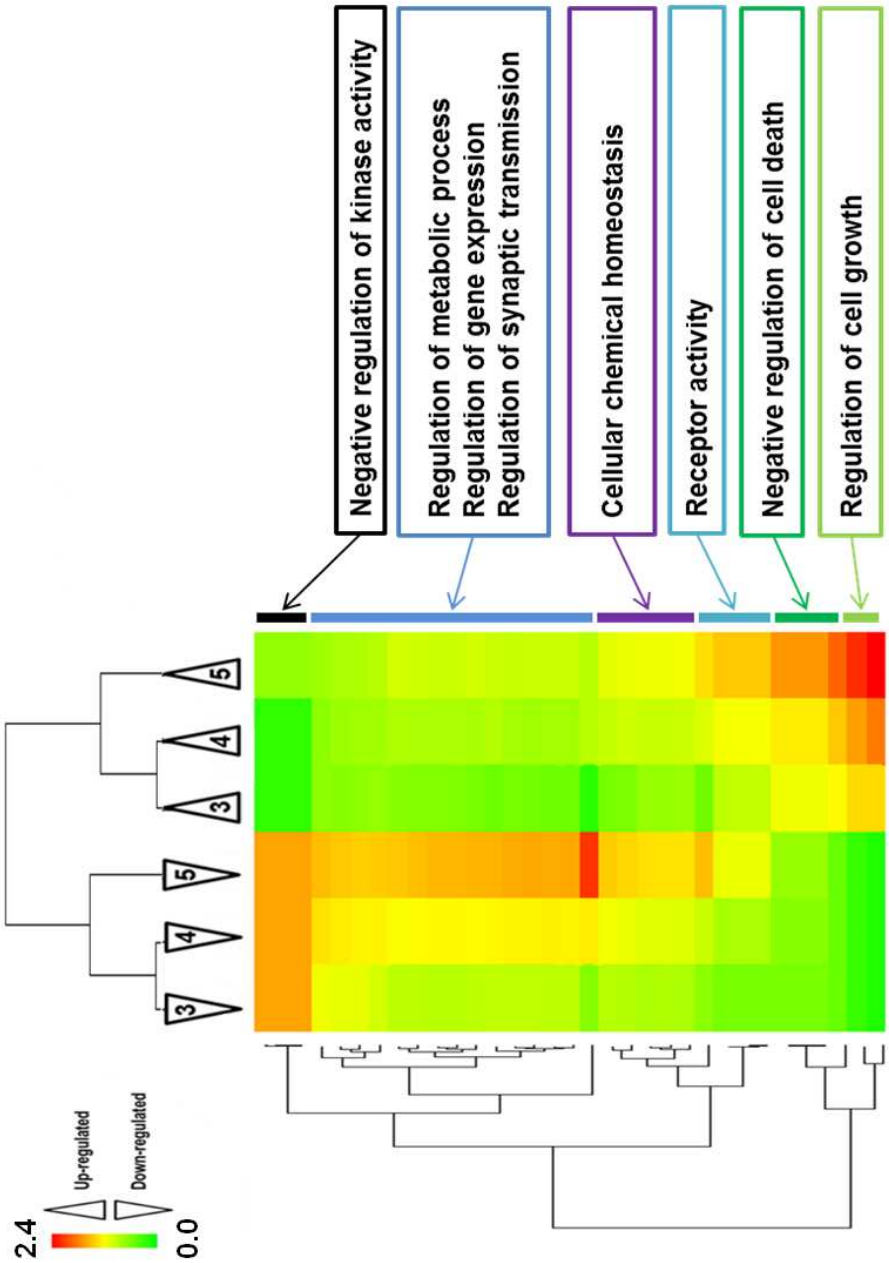


Figure 4.5.9: Heat map showing ontology class enrichment of significantly up- and down-regulated GO annotated probes for the pDMS3 pairwise comparison, across all ranking sets. Colour scale reflects the relative normalized difference in gene counts within significantly enriched GO classes for each pairwise comparison (Red » Green = Increase [i.e. normalized difference > 1] » Decrease [i.e. normalized difference < 1]). Dendrograms to the left of the map shows the distance between significant class clusters based on the results of the complete linkage hierarchical clustering analysis, using the Euclidean distance measure. Between ranking set distances are shown above the heat maps.

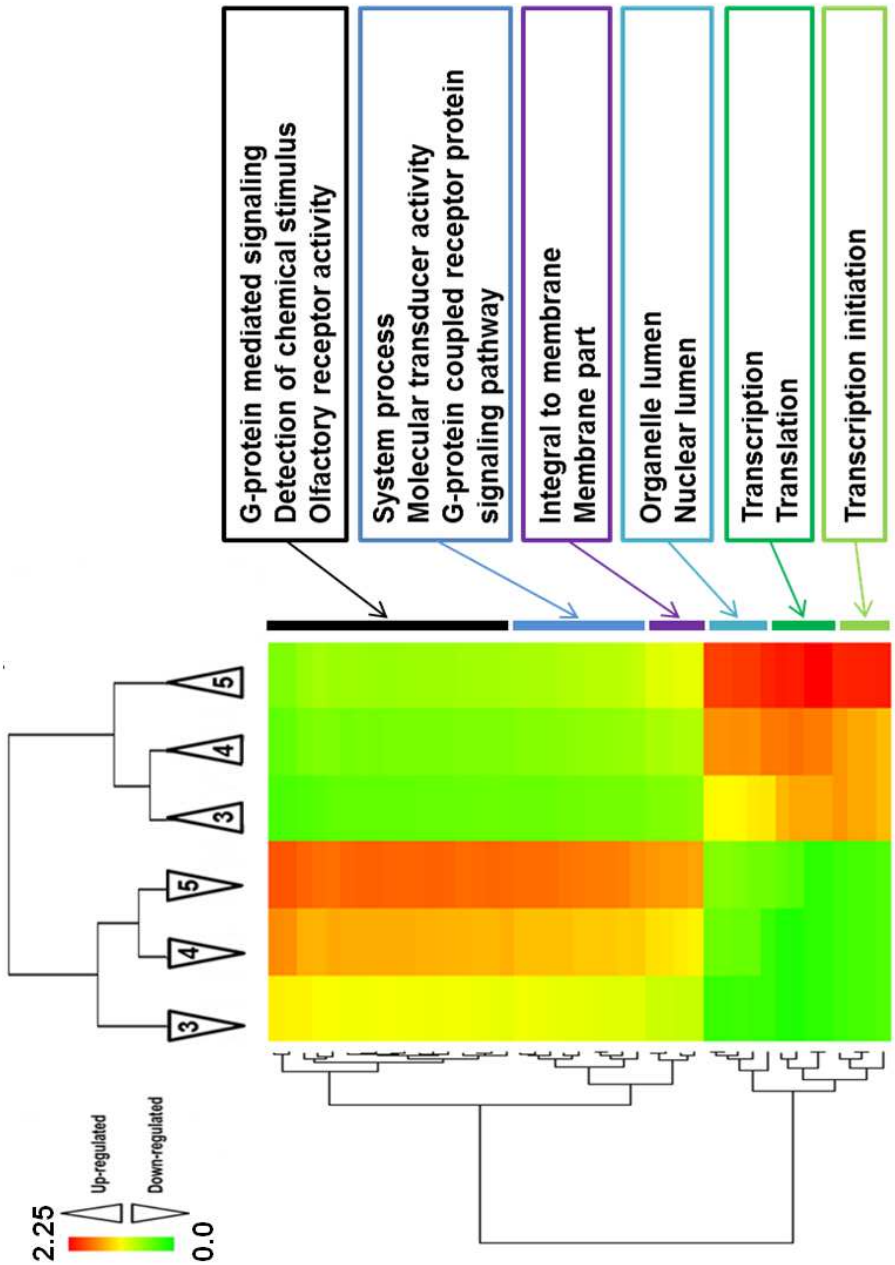


Figure 4.5.10: Heat map showing ontology class enrichment of significantly up- and down-regulated GO annotated probes for the pDMS2 pairwise comparison, across all ranking sets. Colour scale reflects the relative normalized difference in gene counts within significantly enriched GO classes for each pairwise comparison (Red » Green = Increase [i.e. normalized difference > 1] » Decrease [i.e. normalized difference < 1]). Dendrograms to the left of the map shows the distance between significant class clusters based on the results of the complete linkage hierarchical clustering analysis, using the Euclidean distance measure. Between ranking set distances are shown above the heat maps.

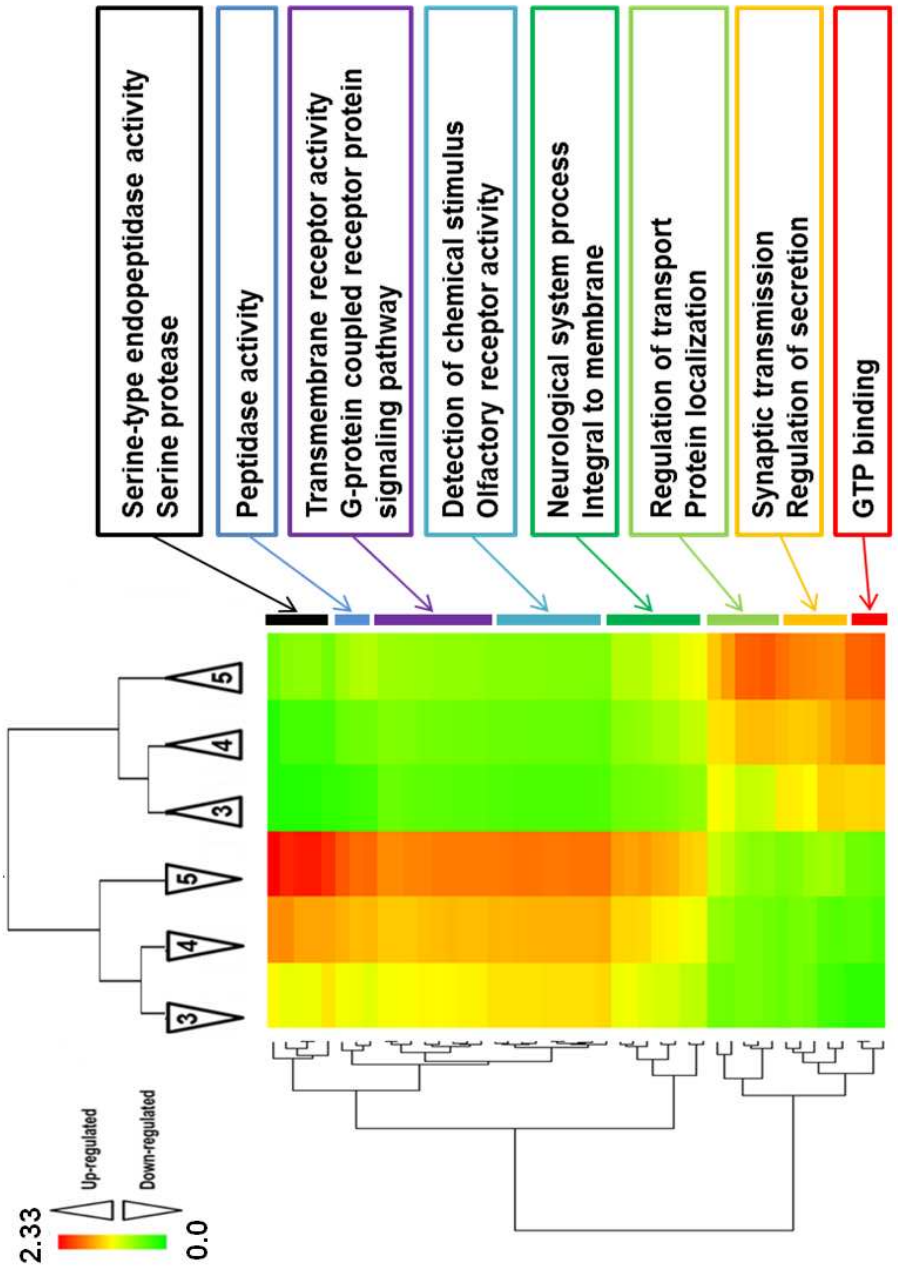
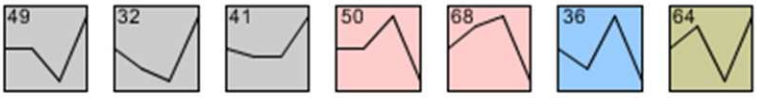


Figure 4.5.11: Heat map showing ontology class enrichment of significantly up- and down-regulated GO annotated probes for the pDMS3 pairwise comparison, across all ranking sets. Colour scale reflects the relative normalized difference in gene counts within significantly enriched GO classes for each pairwise comparison (Red » Green = Increase [i.e. normalized difference > 1] » Decrease [i.e. normalized difference < 1]). Dendrograms to the left of the map shows the distance between significant class clusters based on the results of the complete linkage hierarchical clustering analysis, using the Euclidean distance measure. Between ranking set distances are shown above the heat maps.

Across-group expression profiles: Short Time-series Expression Miner. STEM analysis was used to identify enriched across-session expression profiles from a set of predefined model profiles optimized for modelling microarray expression data across small time series (Ernst and Bar-Joseph, 2006). The significantly enriched (FDR $p < 0.01$) log2 fold change profiles for GO annotated probes are shown for the DLS in Figure 4.5.12 and for the pDMS in Figure 4.5.14 (the full set of DLS and pDMS STEM profiles can be seen in Appendix B for: All probes [Figure B.2.3], GO-annotated probes [Figure B.2.4] and ncRNA probes [Figure B.2.5]).



Observed Genes	137	57	10	145	59	43	32
Expected Genes	26	6	2	25	6	7	6
FDR adjusted p-value	3.4e-57	2.1e-36	1.8e-04	3.0e-65	3.4e-37	2.3e-20	9.5e-14
DLS123 cluster	A			B		C	D
nr-Cluster genes	200			194		39	32
nr-Human orthologues	144			148		27	20
nr-Mouse orthologues	151			143		27	20

Figure 4.5.12: Significantly enriched profiles GO annotated probe sets for DLS (FDR: $p < 0.01$; minimum fold change difference per comparison: > 0.5). Like-coloured profiles are meta-clustered and have correlation factor $r > 0.85$.

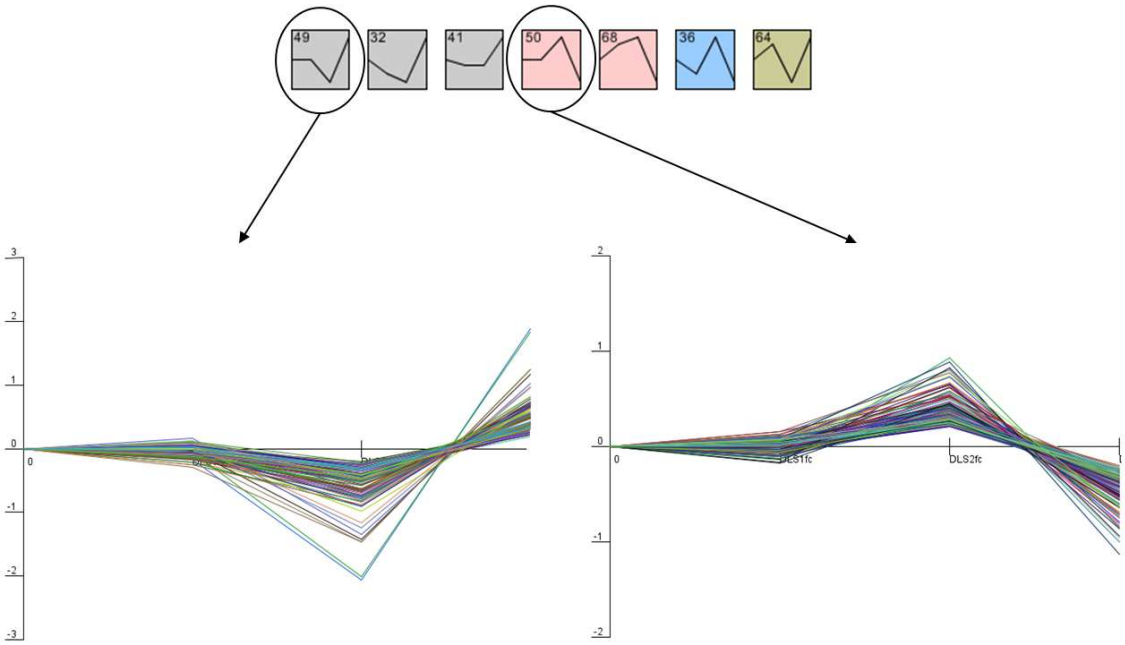
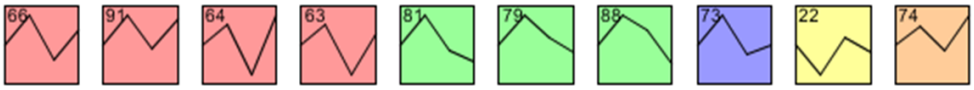


Figure 4.5.13: Log2 fold changes for all probes matched to the 2 most significantly enriched STEM expression profiles for the DLS (#49 [left]: FDR = 3.4e-57; #50 [right]: FDR = 3.0e-65)

The two meta-clusters of significantly enriched profiles in the DLS (DLS Cluster A and DLS cluster B) both show a peak in fold change activity between DLS2 and DLS3 comparisons.[17]. In Cluster A, the profile is indicative of an increase in transcription between the *minimal* and *moderate* training groups, followed by a down-regulation in transcription between the *moderate* and *extended* training groups. Conversely, for Cluster B, the profile cluster indicates a down-regulation of transcription between the *minimal* and *moderate* training groups and a subsequent increase in transcription between the *moderate* and *extended* training groups. Log2 fold change profiles for all GO annotated probes, matched to the most significantly enriched profiles from DLS Cluster A and DLS Cluster B (i.e. profile # 49 and # 50), can be seen in Figure 4.5.13.

[17]Meta-clustered profiles were those profile clusters correlated to $r > 0.85$



Observed Genes	38	25	15	10	33	20	14	23	16	16
Expected Genes	4	2	3	2	5	4	5	7	2	3
FDR adjusted p-value	3.2 e-24	6.5 e-21	1.2 e-06	1.1 e-04	1.1 e-16	4.2 e-09	2.9 e-04	3.7 e-07	2.5 e-09	6.6 e-09
DMS123 cluster	A				B			C	D	E
nr-Cluster genes	85				63			23	16	16
nr-Human orthologues	64				52			20	15	15
nr-Mouse orthologues	65				54			20	16	15

Figure 4.5.14: Significantly enriched profiles GO annotated probe sets for pDMS (FDR: $p < 0.01$; minimum fold change difference per comparison: > 0.5). Like-coloured profiles have correlation factor $r > 0.85$.

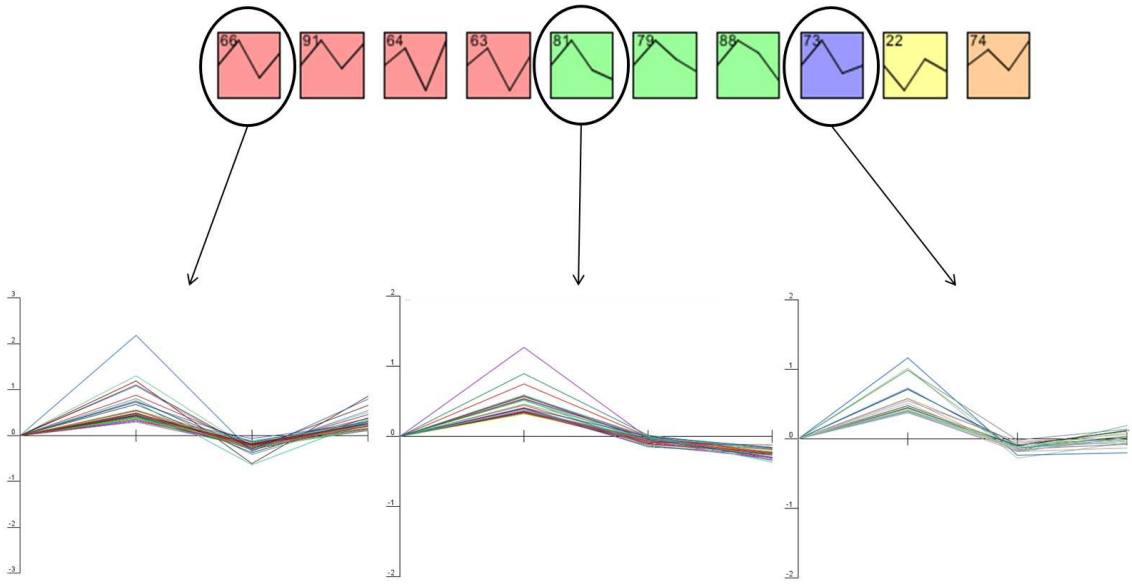


Figure 4.5.15: Log2 fold changes for all probes matched to the most significantly enriched STEM expression profiles for the pDMS

Two enriched profile meta-clusters were also identified for pDMS GO annotated probes (see Figure 4.5.14), although the numbers of probes matched to these profiles was much lower than for the DLS profiles, as shown in Figure 4.5.15.

A comparison of the profiles identified for each region shows that there is a trend for relatively larger fold changes between *no training* controls and the *minimal* training

group in the pDMS (pDMS1 comparison) compared to the DLS (DLS1 comparison). Furthermore, whilst the pDMS enriched profile clusters show a distinct trend for transcriptional up-regulation between these two groups, patterns in the DLS are mixed, with some profile clusters showing an increase in transcription and others showing a down-regulation. Conversely, the enriched DLS profiles show a much greater degree of change between both the *minimal* and *moderate* training groups (DLS2 comparison) and the *moderate* and *extended* sessions (DLS3 comparison) relative to the pDMS (pDMS2 and 3). Frequency histograms showing the distribution of moderated p-values for the most significantly enriched profile cluster, for all pairwise comparisons, are shown for both regions in Appendix B, Figure B.2.6. Consistent with the trends highlighted in the STEM profiles included in these clusters, the plots show a relatively higher frequency of smaller p-values for comparisons DLS2 and 3 than for comparison DLS1. Conversely, comparisons pDMS1 and pDMS2 have a higher frequency of smaller p-values than comparison pDMS3. Furthermore, the bias towards smaller p-values is only ever apparent for the region analysed, highlighting the brain-region specific nature of the regulatory patterns identified.

STEM ORA: GO annotated probes. To identify significantly enriched GO class clusters, GO annotated probes, matched to significantly enriched profile meta-clusters, were submitted for further ORA in DAVID. Figure 4.5.3 shows the significantly enriched class clusters (FDR: $p < 0.01$) within the profile meta-clusters for each region.

The probes matched to DLS Cluster A show an enrichment in functional classes involved in transport of proteins and synaptic vesicles, as well as in receptor activity and binding and morphological neuronal changes. There is a reversal in the regulation of all enriched classes between DLS2 and DLS3, from down- to up-regulation. In contrast, Cluster B has an enrichment of functional classes related to microtubule based intracellular transport and cell cycle phase, and shows the opposite reversal of regulation between DLS2 and DLS3 to that seen for Cluster A (i.e. a switch from up-

to down-regulation). For profile Clusters C and D, the enriched classes are involved in receptor activity and cell-to-cell signal transduction.

Probes matched to the enriched pDMS Clusters A and B also show an enrichment of genes involved various types of receptor and transporter activity, with a change from up- to down- to up-regulation across the comparisons (i.e. pDMS1→pDMS2→pDMS3). Enriched genes in profile Cluster C are involved in cellular hormonal response and switch from up-regulation to down-regulation between pDMS1 to pDMS2.

Profile	#Genes	DLS1	DLS2	DLS3	Property	GO classes	N genes
A	200	→	↓	↑	Substrate-specific transporter activity	16	28
A	200	→	↓	↑	Potassium channel activity	3	10
A	200	→	↓	↑	Voltage-gated cation channel activity	4	9
A	200	→	↓	↑	Synaptic vesicle	3	8
A	200	→	↓	↑	Transporter actvity	3	35
A	200	→	↓	↑	Synaptic transmission	3	11
A	200	→	↓	↑	Receptor activity	3	41
A	200	→	↓	↑	Cation binding	3	42
A	200	→	↓	↑	Carboxylic acid transmembrane transport	6	5
A	200	→	↓	↑	Cell motility	3	10
A	200	→	↓	↑	Neuron projection development	8	9
B	194	→	↑	↓	Dynein complex	3	8
B	194	→	↑	↓	Microtubule motor activity	3	8
B	194	→	↑	↓	Cell phase	3	8
C	39	↓	↑	↓	Receptor activity	7	12
D	32	↑	↓	↑	Signal transducer activity	3	12
D	32	↑	↓	↑	G-protein coupled receptor activity	3	9

(a) DLS enriched GO class clusters

Profile	#Genes	DMS1	DMS2	DMS3	Property	GO classes	N genes
A	85	↑	↓	↑	Ion transmembrane transporter activity	9	11
A	85	↑	↓	↑	Cation channel activity	7	7
A	85	↑	↓	↑	Voltage gated ion channel activity	4	5
B	85	↑	↓	↓	Substrate-specific transmembrane transporter activity	5	6
C	85	↑	↓	→	Response to hormone stimulus	3	6

(b) pDMS enriched GO class clusters

Table 4.5.3: Significantly enriched GO clusters for STEM profile meta-clusters for the DLS (a) and pDMS (b). Each row represents a significantly enriched GO cluster (FDR: $p < 0.01$). The GO classes column shows the total number of classes in each cluster (including genes shared between the clusters, since some of genes, due to their multiple annotation nature can be included in multiple clusters). The number of genes unique to each cluster is shown in the "N genes" column. Arrows illustrate the direction of fold change for each pairwise comparison: ↑ = up-regulated; ↓ = down regulated; → = no change.

Fold change dependent enrichment analysis of ncRNA probes. Cross-condition comparisons of the total number of differentially expressed unique and shared mRNA and ncRNA probes ($p < .05$; categories derived based on Affymetrix annotations) are summarised for the DLS in Figure 4.5.16 and the pDMS in 4.5.17. For each comparison, approximately 10-20% of the all differentially expressed probes were identified as ncRNA transcripts, and there was no evidence of difference in the proportion of differentially regulated ncRNA probes between the two brain regions.

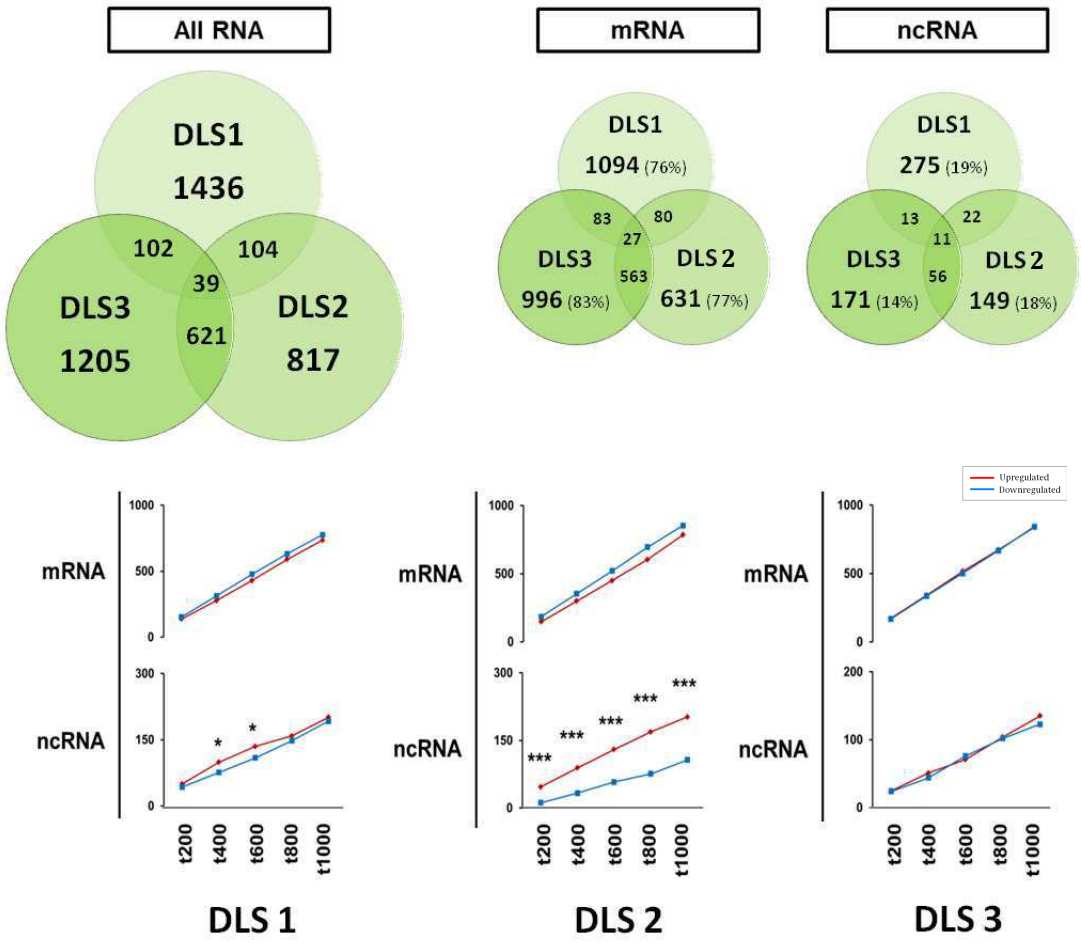


Figure 4.5.16: Overlap of differentially expressed probes from between training-group comparisons for mRNA and ncRNA probes for the DLS. Values in brackets refer to the percentage of all differentially regulated probes belonging to each RNA class

Given the paucity of ncRNA annotations currently available in popular gene ontology databases, it was not possible to conduct ORA on the ncRNA STEM probe sets. Therefore, a Fischer's exact test was used to assess whether there was a significant up- or down-regulation of ncRNA probes (two-tailed p-values * $p < .05$, ** $p < .01$, *** $p < .001$) in the various p-value ranking sets (t200 to t1000) for each comparison in the DLS (Figure 4.5.16) and the pDMS (Figure 4.5.17). For comparison DLS1 there was some evidence of a slight up-regulation of ncRNA transcripts in the t400 and t600 ranking sets. However, this effect disappeared for the larger ranking sets (t800 and t1000), meaning there was no consistent proportional difference between up- and down-regulated probes. Nevertheless, for comparison DLS2 there is a highly significant proportional difference, indicative of a strong trend towards up-regulation of ncRNA transcripts for this comparison, which disappeared completely for comparison DLS3.

There was a weak, non-significant trend for up- and down-regulation of ncRNA probes for comparisons pDMS1 and pDMS2, but, in contrast there was a large, highly significant, proportional down-regulation of ncRNA transcription at pDMS3.

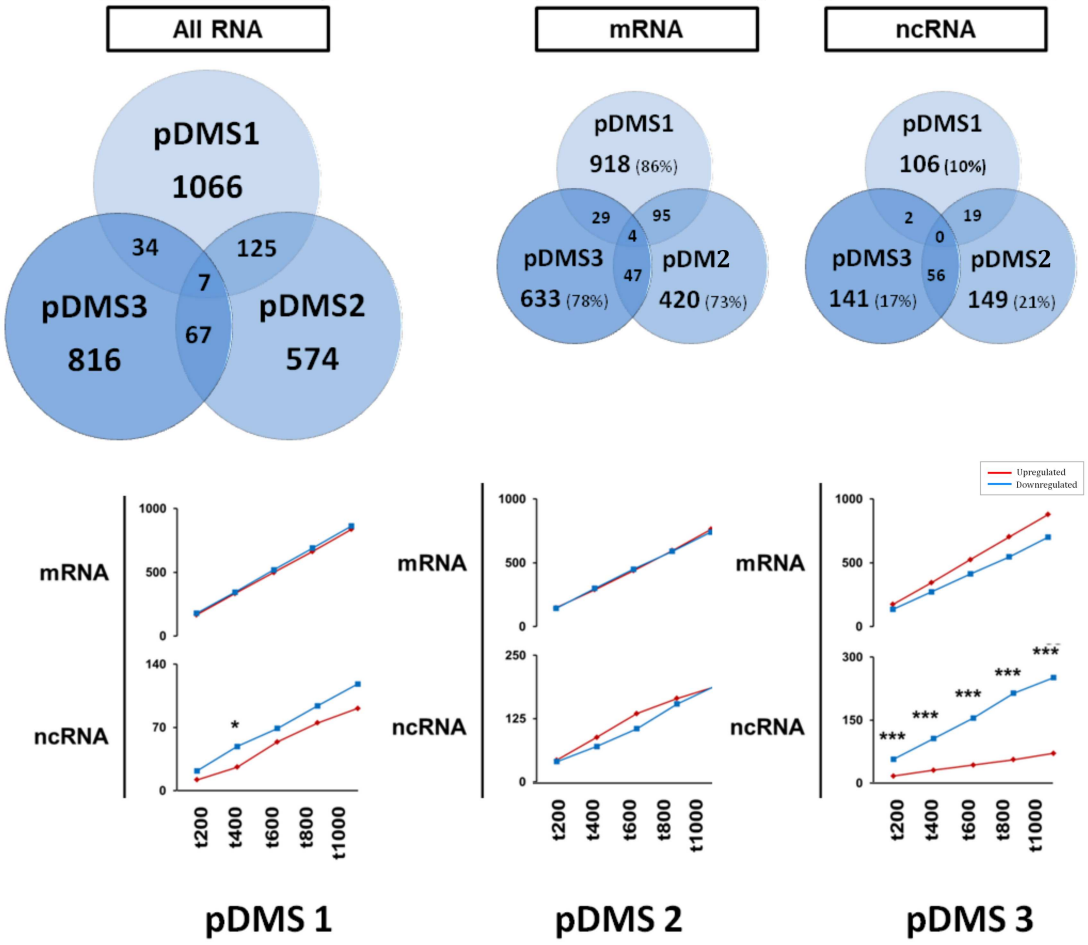


Figure 4.5.17: Overlap of differentially expressed probes from between training-group comparisons for mRNA and ncRNA probes for the pDMS. Values in brackets refer to the percentage of all differentially regulated probes belonging to each RNA class

4.5.5 Technical verification of microarray data: Quantitative real-time polymerase chain reaction (qPCR)

Technical verification of the microarray results was conducted with qPCR, in the same RNA samples used for the arrays. Welch's t-tests were used to analyse average 2ddCT values of selected candidate genes for each pairwise comparison. For all comparisons, qPCR expression profiles were consistent with Affymetrix intensity values. For the DLS comparisons, expression of DLS1 candidate, Cartpt, was significantly lower in the *minimal* training group compared to the *no training* controls; for DLS2 the expression of the selected candidate, Tbr-1, was significantly down-regulated between the *minimal* and *moderate* training groups; and for the DLS3 candidate,

Stx1a, expression was significantly lower in the *moderate* training group compared to the *extended* training group (see Figures 4.5.18 to 4.5.20).

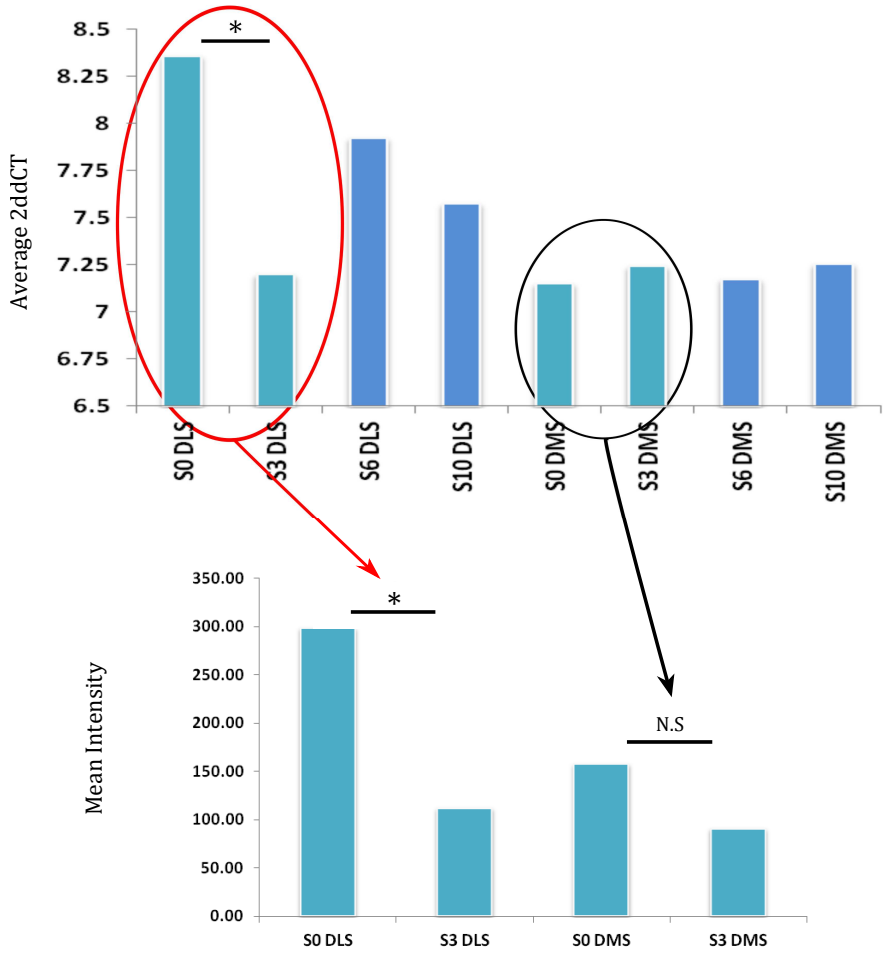


Figure 4.5.18: Comparison of Affy intensity (top figure) and qPCR (bottom figure) expression profiles for **Cartpt** (CART propeptide), selected as DLS1 candidate. Red rings highlight the training-group comparison for which gene was a candidate (i.e. the most significantly regulated gene [based on limma generated p-values] identified from the ORA STEM gene sets [** $p<0.01$, * $p<0.05$, # $p<0.10$]) and black rings indicate the same comparison in the pDMS samples.

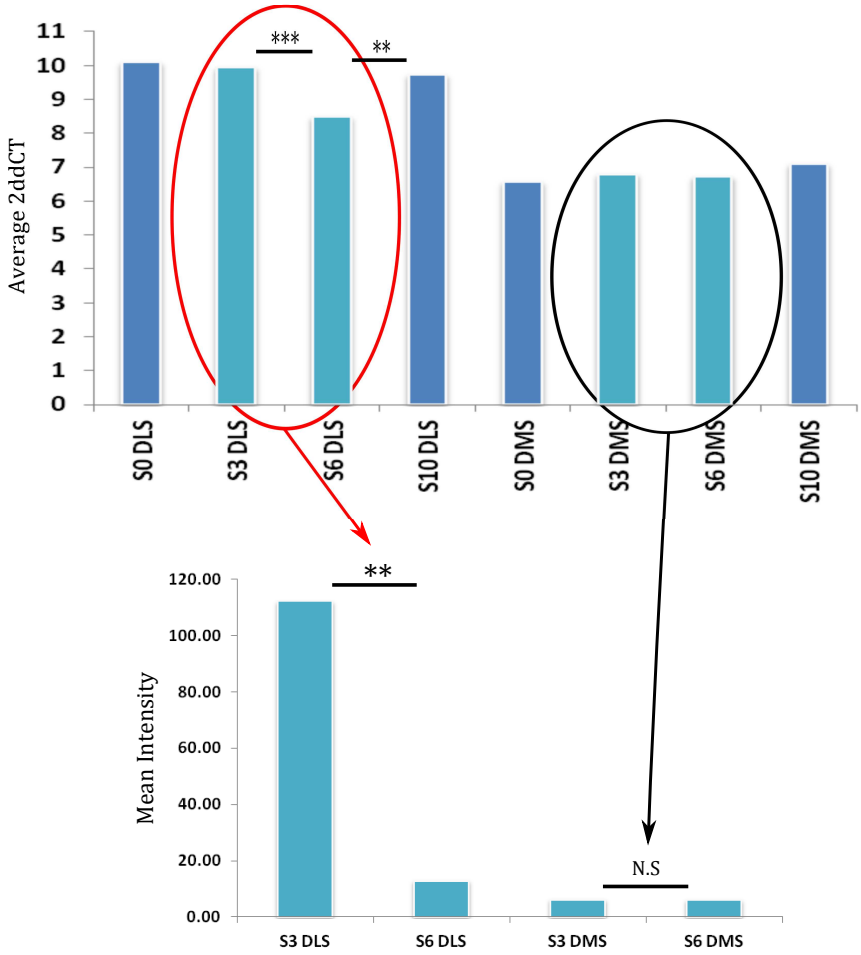


Figure 4.5.19: Comparison of Affy intensity (top figure) and qPCR (bottom figure) expression profiles for **Tbr-1** (T-box, brain, 1), selected as DLS1 candidate. Red rings highlight the training-group comparison for which gene was a candidate (i.e. the most significantly regulated gene [based on limma generated p-values] identified from the ORA STEM gene sets [** $p<0.01$, * $p<0.05$, # $p<0.10$]) and black rings indicate the same comparison in the pDMS samples.

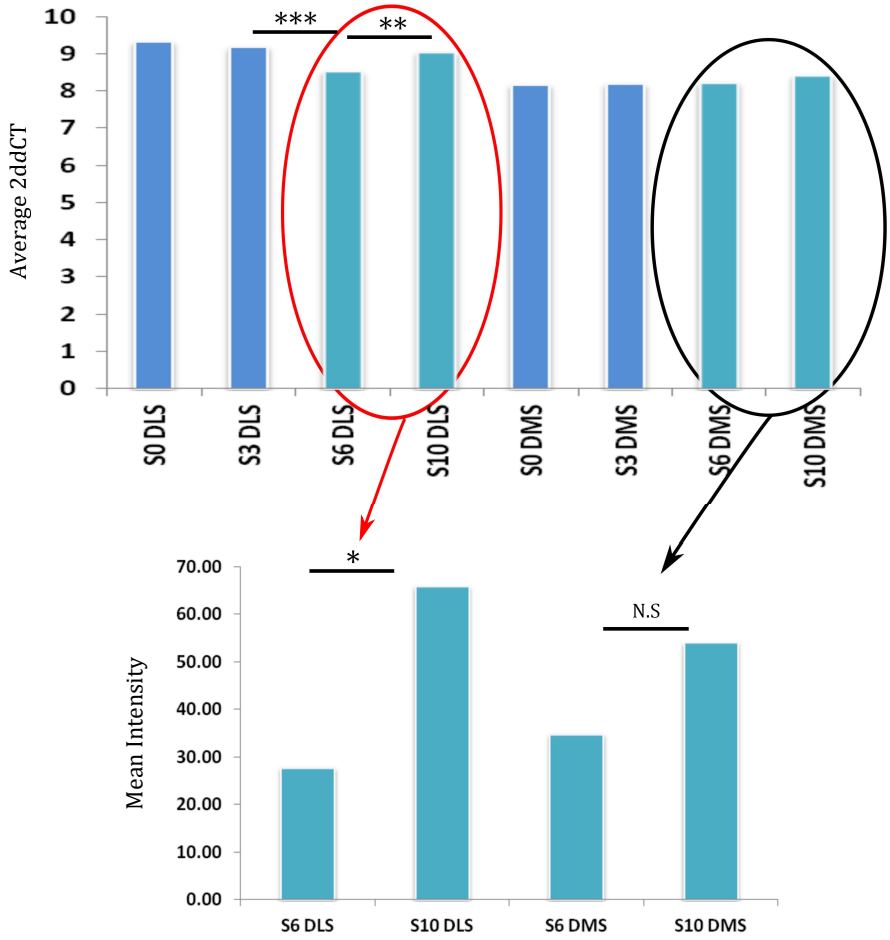


Figure 4.5.20: Comparison of Affy intensity (top figure) and qPCR (bottom figure) expression profiles for **Stx1a** (Syntaxin 1 brain), selected as DLS1 candidate. Red rings highlight the training-group comparison for which gene was a candidate (i.e. the most significantly regulated gene [based on limma generated p-values] identified from the ORA STEM gene sets [** $p<0.01$, * $p<0.05$, # $p<0.10$]) and black rings indicate the same comparison in the pDMS samples.

Because the Affymetrix data for pDMS intensity values pointed to a trend towards the down-regulation of a number of immediate early genes (IEGs) between *no training* controls and the *minimal* training group (i.e. pDMS1 comparison), for this comparison, qPCR verification was conducted on three IEG candidates (Arc, Erg1 and Erg2). As shown in Figures 4.5.21 to 4.5.23, consistent with Affymetrix intensity data, Arc expression was significantly lower in the *minimal* training group compared to the *no training* controls; similarly, expression of the IEG Egr1, was also reduced

in the the *minimal* training group compared to the *no training* controls, although this effect failed to reach significance. For both of these genes this initial training dependent repression was not evident in DLS samples. In contrast to the brain-region specific regulation of these two IEGs, but in line with the Affymetrix data, Egr2 expression was significantly down-regulated in the *minimal* training group compared to the *no training* controls in both pDMS and DLS samples. Fold change, p-value, and FDR values are displayed in Appendix B for the DLS (Table B.2.1) and the pDMS (Table B.2.2).

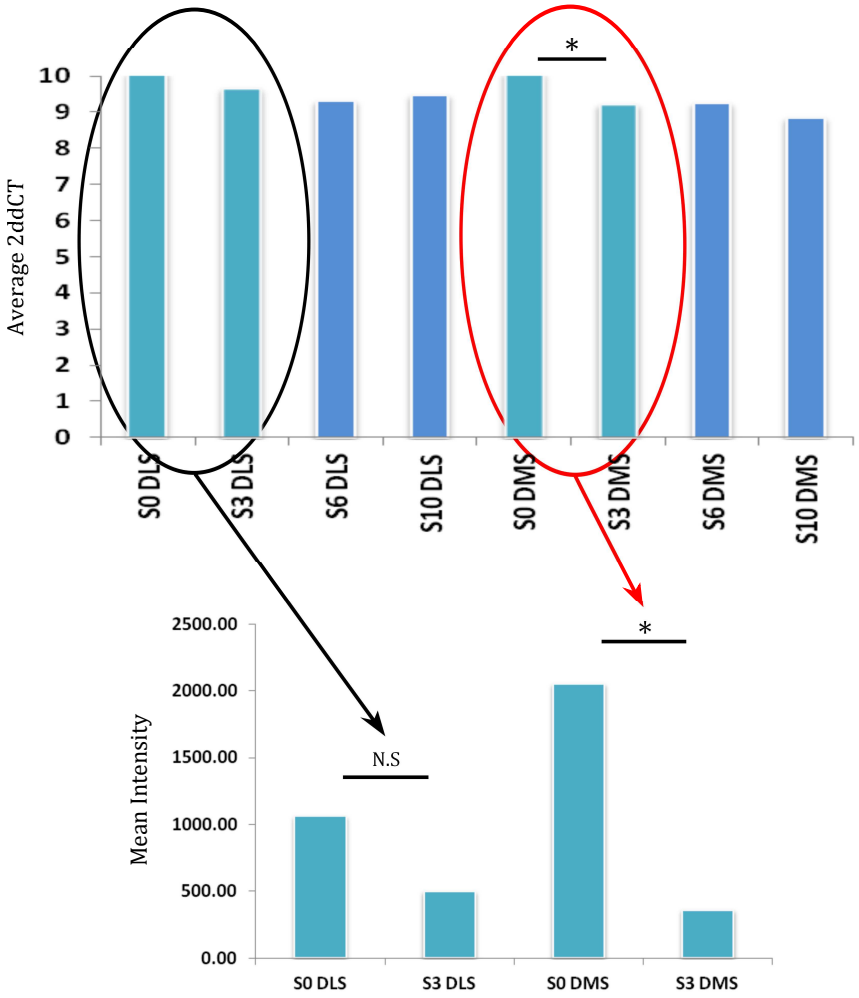


Figure 4.5.21: Comparison of Affy intensity (top figure) and qPCR (bottom figure) expression profiles for **Arc** (Activity-regulated cytoskeleton-associated protein), selected as pDMS1 candidate. Red rings highlight the training-group comparison for which gene was a candidate (i.e. the most significantly regulated gene [based on limma generated p-values] identified from the ORA STEM gene sets [** $p<0.01$, * $p<0.05$, # $p<0.10$]) and black rings indicate the same comparison in the DLS samples.

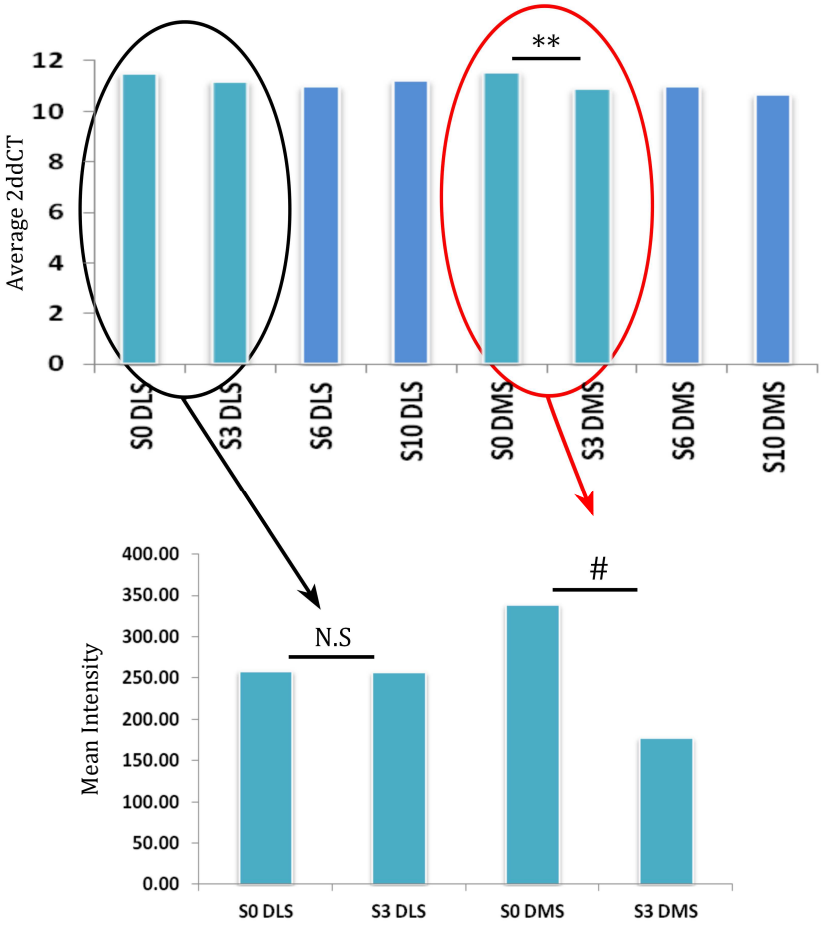


Figure 4.5.22: Comparison of Affy intensity (top figure) and qPCR (bottom figure) expression profiles for **Egr1** (Early growth response 1), selected as pDMS1 candidate. Red rings highlight the training-group comparison for which gene was a candidate (i.e. the most significantly regulated gene [based on limma generated p-values] identified from the ORA STEM gene sets [** $p<0.01$, * $p<0.05$, # $p<0.10$]) and black rings indicate the same comparison in the DLS samples.

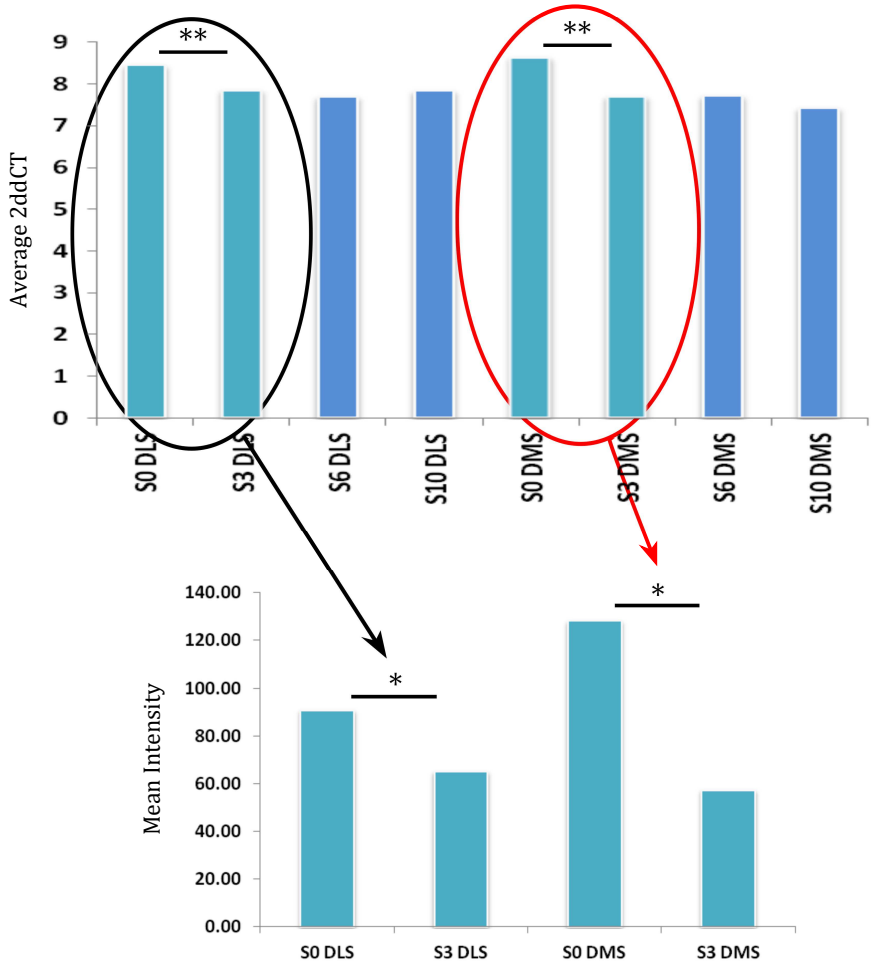


Figure 4.5.23: Comparison of Affy intensity (top figure) and qPCR (bottom figure) expression profiles for **Egr2** (Early growth response 2), selected as pDMS1 candidate. Red rings highlight the training-group comparison for which gene was a candidate (i.e. the most significantly regulated gene [based on limma generated p-values] identified from the ORA STEM gene sets [** $p<0.01$, * $p<0.05$, # $p<0.10$]) and black rings indicate the same comparison in the DLS samples.

4.6 Discussion

4.6.1 Review of Behavioural data

Motivated by the paucity of evidence relating to the molecular correlates of goal-directed and habitual behaviours, the experiments presented in this chapter employed Affymetrix gene arrays to identify training-dependent, brain-region specific,

gene expression profiles between four groups of rats receiving different amounts of instrumental training. Specifically, genes, and clusters of functionally related genes, which were differentially regulated between the training groups, and brain regions, were identified. The current section will provide a brief general summary of the experimental results before commencing on an in-depth interpretive discussion of the data.

Analysis of the behavioural data revealed the expected pattern of responding across the training sessions, with lever presses increasing and magazine entries decreasing, as animals learnt the instrumental contingency. Furthermore, there were no significant differences in either lever press responding or magazine entry behaviour between the three ANALYSIS groups, confirming that the Array and qPCR groups were behaviorally comparable and that the tissue samples included in the molecular analyses were taken from subgroups of animals whose behaviour was representative of the rest of the cohort.

Although session-averaged lever press response levels were comparable across the three training groups, the number of lever presses, and magazine entries, differed across the groups during the first session of training. This was likely due to an increased motivation to work for food caused by differences in the length of food restriction the training groups experienced prior to the start of training. One potential difficulty with designing experiments such as this is that the training manipulation necessitates that the groups start or finish training at different times. Here, it was important to have the groups finishing training at the same point so that they could be dissected on the same day, and, therefore, the necessary staggering occurred at the start of training. However, this required that, either the different groups experienced different periods of food restriction over the course of the whole experiment, or they started training in a different motivational state. Since it would have introduced a potentially important confound into the training manipulation if the groups

had experienced different periods of food restriction at the point of dissection, all the groups were food restricted at the same time. This meant, of course, that the animals in the *minimal* training group had been food restricted for a longer period prior to the start of training which probably explains the trend for lever press response rates to be higher for groups with shorter training periods during the first session of training (see 4.4.1).

Importantly though, statistical analysis did not reveal any main effect of TRAINING group on overall, session-averaged response rates, indicating that within-session exposure to the instrumental contingency did not differ between the groups. Indeed, the variability in lever pressing possible was constrained by the fact that the length of each training session was determined by the number of reinforcers earned, rather than by time. Furthermore, there were no differences in response rates between the groups during their respective final training sessions. Therefore, it seems that any differences in motivation that might have existed between the groups at the start of training did not have a significant effect on learning over the course of the experiment. Furthermore, there is evidence that exposure to the instrumental contingency is key in determining habit formation, rather than the absolute number of lever presses an animal makes (Adams, 1982). Importantly, the within-subjects, brain region-specific dissociations in gene expression profiles, indicated by the array data presented below, are inconsistent with the argument that any molecular effects of training group were the result of differences in the level of food restriction at start of training (or any potential differences in learning resulting from this) between the groups.

4.6.2 Molecular data quality

The microarray data were also used to check for other potential confounds and sources of technical bias. Importantly, the PCA model did not reveal any system-

atic variation attributable to either squad or RNA-preparation batch. Furthermore, since all the arrays were hybridized in parallel, we can discount the possibility of confounding by hybridisation batch. Apart from samples from one animal, which were subsequently replaced, there were no issues with RNA quality and the array data passed all Affymetrix quality control requirements (e.g. RNA degradation and normalisation artifacts). The kernel density plots and intensity profiles indicated, as expected, that there were no large differences in the unfiltered expression profiles between the DLS and pDMS, confirming that sample preparation and analysis were consistent between the regions, and that the dissected tissue types did not respond differently in any way to the RNA extraction and array protocols.

Taken together, these results indicate that both the behavioural and molecular data sets exhibited the expected general patterns and were free from any obvious technical or analytical artifacts. Furthermore, qPCR, in the same samples used for the array, was employed to verify microarray expression data for the top most differentially regulated STEM-ORA genes for each comparison. Patterns of differential expression were consistent between the microarray and qPCR data for all six genes analysed.

4.6.3 Multivariate analysis

Having confirmed data quality, initial multivariate analysis (MV) analysis, using unsupervised PCA, provided a general structural overview of the data. The extracted model, which accounted for approximately half of the total variance in the data set, indicated that brain-region separation was more evident than separation between the training groups. It is possible, of course, that the brain-region separation simply reflects baseline (i.e. non-training related) expression differences between the DLS and pDMS. However, given the high degree of similarity in intensity distributions for the two regions, combined with the fact that the regional separation only becomes apparent for the trained groups (i.e. *moderate* to *extended* training groups), suggests

that instrumental learning has differential effect on gene regulation between the two brain regions studied here. Nevertheless, although PCA is useful for summarizing the data set in a hypothesis-free manner, it is important to recognise that it does not provide any information regarding the nature of this variation (i.e. directionality, functional relevance etc).

4.6.4 Pairwise comparisons and ORA

Following the initial multivariate (MV) analysis, the data were interrogated for statistically significant expression differences between the brain regions and training groups. The results of the pairwise comparisons were used to identify those probes exhibiting differential expression between the training groups based on arbitrarily defined α-values. A simple comparison of the numbers of significant differentially expressed probes (at both $p < .05$ and $p < .001$) indicated that expression changes were greatest in the DLS relative to the pDMS, and that the biggest change occurred in the DLS between the *moderate* and *extended* training conditions (see Figure 4.5.4). For all comparisons, the numbers of differentially expressed probes unique to a particular comparison were generally much greater than for differentially expressed probes common to more than one comparison, indicating a high degree of separation between both the regions and the training groups (see Figure 4.5.5).

Taken alone, the results of the pairwise comparisons do not provide any functionally relevant information. Therefore, in order to move beyond purely numerical comparisons and towards a biological interpretation of the data set, ORA was conducted on variable sized sets of the most differentially expressed probes, as identified by moderated p-value significance ranking. Significantly enriched classes of up- and down-regulated GO annotated probes were identified for each pairwise comparison and visualized using heat-maps coded for the relative normalized difference in gene counts. ORA analysis identified a number of significantly enriched ontology classes,

and hierarchical clustering analysis was used to explore the relationships between these classes. A more detailed analysis of the functional relevance of enriched clusters will focus on the results of the STEM-ORA, discussed below.

4.6.5 STEM

Whilst the significance ranking approach is useful for identifying those probes most likely to be sensitive to the training manipulation in each region, the use of arbitrary significance cut-offs for the p-value based enrichment analysis risks excluding potentially important genes. Furthermore, since statistical power limitations precluded the comparison of non-adjacent training groups (e.g. minimal vs. extensive), ORA only provided information for discrete contrasts between adjacent training groups, preventing the analysis of expression changes across all groups. Nevertheless, each comparison can be viewed as part of a sequence of comparisons across the training groups, providing cumulative information regarding expression changes over the course of behavioural training. STEM analysis exploits the sequential order inherent in the experimental design, and was used to identify common expression profile patterns across all training groups, from lower to higher levels of training. Significant, between-training group expression profiles for the DLS and pDMS were derived from sequentially ordered pairwise log2 fold changes and meta-clustered based on profile similarity.

For the DLS comparisons, the two most significant profile clusters were both indicative of an increased fold change between the *minimal* and *moderate* training groups (i.e. DLS2; see Table 4.5.3). Importantly, the peak in fold change activity occurred in opposite directions for the two clusters, with one cluster showing increased down-regulation between *minimal* and *moderate* training groups (DLS Cluster A) and the other showing an increase in up-regulated probes for the same comparison (DLS Cluster B). In contrast to the the enriched DLS profiles, which indicate relatively

small differences in expression between the *no training* controls and *minimal* training group, the enriched pDMS profiles point to marked transcriptional up-regulation between these groups. Interestingly, pDMS profiles show relatively small fold change levels between the *moderate* and *extended* training groups. Thus, taken together, these patterns are consistent with the literature positing a role for the pDMS in the initial acquisition of goal-directed actions and may reflect an increasing involvement of the DLS in the regulation of instrumental responding as training progresses.

These seemingly paradoxical patterns of up- and down-regulation may in fact be indicative of gross synaptic reorganization. The arrays were conducted on sub-regions of striatal tissue containing heterogeneous populations of neurons (a point which is especially relevant for striatal tissue, since it is well documented that the dorsal striatum contains multiple different cell types with differing roles in mediating plasticity processes; see Kreitzer, 2009) and, therefore, these oppositional patterns of expression may reflect bidirectional gene regulation across different neuronal populations relevant for learning-induced synaptic plasticity.

Interestingly, the two meta-clusters of enriched profiles identified for GO annotated probes in the pDMS samples contained far fewer probes than the DLS meta-clustered profiles. The fact that far more genes appear to be differentially regulated by training in the DLS compared to the pDMS could reflect one of two processes. Either, the DLS may be more involved in regulating training-induced molecular changes, or, alternatively, the pDMS sample may have contained a number of functionally distinct critical regions of tissue, each displaying different training-dependent expression patterns, and thus diluting any clear regulatory patterns, making it more difficult to detect true biological differences.

The progression from purely statistical bioinformatics approaches to biologically relevant analysis is not unidirectional. It is possible, and useful, to modify and refine the results of statistical tests based on the information derived from functional charac-

terisation. Here, a second ORA, conducted on those genes included in the significant model profiles, was used to identify enriched ontology classes showing similar patterns of regulation across the training groups; providing an insight into the biological relevance of these patterns. Perhaps unsurprisingly, given the fact that we are looking at the effects of learning, the STEM-ORA revealed an enrichment of genes involved in receptor activity and transmembrane transport, for example, voltage gated cation transport and receptor activity (see Table 4.5.3). Interestingly, however, the directional regulation of of these functional classes was specific to both brain region and training group comparisons, pointing to brain region-specific patterns of gene expression over the course of instrumental training.

Specifically, the genes included in DLS Cluster B profiles show an enrichment in functional classes related to protein transport and transport of vesicles along axons towards synapses. Conversely, the enriched functional classes included in DLS Cluster A point to a reduction in synaptic transmission and cell-to-cell signalling, as well as a down-regulation of transcripts coding for proteins involved in synaptic vesicle formation and ion-gated channel activity. Significant pDMS Clusters A and B also showed an enrichment of genes involved various types of receptor activity and transmembrane transport, with a change from up- to down- to up-regulation across the comparisons.

4.6.6 Towards specific molecular mechanisms

The few studies that have looked at the molecular correlates of instrumental learning have employed candidate driven approaches. In contrast, here we used Affymetrix microarrays to measure and compare genome-wide expression profiles in tissue samples from animals with different amounts of instrumental learning experience. Whilst the power of this approach lies in the fact that it enabled us to conduct hypothesis-free, data-driven analysis, it is important to recognise that these experiments are

only a first step in understanding the molecular pathways underlying habit formation. As such, it is not possible to formulate any firm mechanistic interpretations of the findings presented here without further investigation (see Chapter 7 for further discussion of the planned future directions for this work).

However, by focusing on a few interesting features of the data set, we are able to form some speculative ideas regarding potential avenues for further exploration. For example, several immediate early genes (IEGs), including Egr1, were down-regulated in the pDMS in minimally trained animals relative to the no training controls. This is contrary to previous findings (Hernandez et al., 2006) and seems intuitively at odds with the notion that IEG expression patterns provide a indirect measure of experience-dependent brain activity. However, it is important to remember that, in the current study, brain dissections were conducted 24 hours post-training, possibly exceeding the time window for learning-induced IEG activation. Indeed, there is some evidence of delayed post-training repression of certain genes. For example, using a passive avoidance paradigm, O'Connell et al. (2000) found that expression of the transcription factor, CREB binding protein (CBP), was significantly down-regulated from basal levels in the dentate gyrus 12 hours post-training. Thus, it is possible to tentatively interpret the down-regulation of IEG transcription at the early stage of instrumental learning as reflecting the involvement of some kind of brain region-specific negative feedback mechanism in the regulation of certain IEGs. Alternatively, given the lesion study evidence suggesting a dissociable role for the pDMS and DLS in goal-directed and habitual behaviour, respectively, this brain region-specific reduction in IEG transcription may be indicative of the early-stage molecular processes underlying a gradual functional repression of the pDMS. Clearly, these speculations need further investigation, for example using gene-specific inhibitors or gene knockdown models.

The issue of temporal specificity in the context of IEG expression is also relevant

when comparing the results of this work with work showing evidence of training-dependent ERK activation discussed in the introduction to this chapter (e.g Shiflett et al. 2010). In contrast to previous studies, the current experiments found no evidence of training-dependent regulation of any of the Map-Kinase signalling pathways. Since the time-course of ERK activation is known to vary significantly across a range of stimuli (Marshall, 1995), these evidential differences are most likely explained by the relatively greater time interval between training experience and dissection employed in the current experiments compared to those of Shiflett et al. (2010).

4.6.7 Non-coding RNA

The majority of the analysis conducted on this data set focused on protein coding transcripts (i.e. mRNA) with identifiable functionality (i.e. GO annotated). Whilst this enabled us to interpret the data in terms of recognised molecular processes and pathways, it meant that potentially important uncharacterised, often non-coding, RNA transcripts may have been ignored. Given the growing interest in the role of ncRNA in epigenetic modifications (Kaikkonen et al., 2011), the regulation of non-coding transcript expression was of particular relevance for the current thesis. Therefore, significant differentially expressed probes, identified by the pairwise comparisons, were categorised, based on their Affymetrix annotations, into coding (i.e. mRNA) or nc-RNA groups. The proportion of differentially regulated ncRNA probes was approximately equivalent between the two regions and there was no evidence of an enrichment of ncRNA probes for a particular training group comparison. However, analysis of the relative levels of up- and down-regulated probes revealed clear bidirectional regulation patterns in ncRNA probes for specific pairwise comparisons. For example, in the DLS, for comparison DLS2, there was a large bias towards up-regulation of ncRNA transcripts between the *minimal* and *moderate* training groups (i.e. comparison DLS2). Conversely, in the pDMS, expression of ncRNA transcripts was significantly down-regulated between the *moderate* and *extended* training groups

(i.e. comparison pDMS3). Whilst, given the lack of annotation information available for non-coding transcripts, it is difficult to make any claims regarding the functional significance of these patterns, as mentioned previously, it has been suggested that ncRNA transcripts may be involved in epigenetic processes (Costa, 2008). Clearly, this hypothesis needs to be explored further but it is possible to speculate about the role of region specific epigenetic changes in regulating the molecular processes involved in the co-ordination of instrumental behaviour. Indeed, in light of the findings discussed in the previous section, it is interesting to note that one current view is that histone modifications act as down-stream mediators of IEG activation by intracellular signalling cascades (Sng et al., 2004).

4.6.8 Potential caveats and conclusions

An important caveat in interpreting the data presented here is the fact that the sensitivity of instrumental responses to outcome devaluation was not explicitly tested in the current experiments. As noted in the introduction to this chapter, it was decided that including a devaluation manipulation and an extinction test would, potentially, introduce too much noise to the data and reduce the probability of detecting specific, training-related changes in gene expression by increasing the temporal distance between training and dissection. Furthermore, the inclusion of a devaluation manipulation would have necessitated the addition of an extra factor to the microarray design, greatly increasing the cost of the analysis, and complexity of the data set. Therefore, in order to maximise the probability of detecting robust, training- and region-specific molecular signals, all brain tissue samples were collected 24 hours after the end of training. Clearly, this precluded the ability to draw direct inferences regarding the degree to which lever press responding in the respective training groups was goal-directed or habitual, limiting the extent to which the data collected here can be used to inform us about the molecular mechanisms underlying habit

formation. However, based on the assumption that the data presented in Chapter 3 provide a general insight into the conditions under which instrumental behaviour is either goal-directed or habitual, indirect inferences can be made which could inform future investigations by highlighting potentially important candidate targets for manipulation studies. Furthermore, molecular level analyses of the effects of repeated training on the brain are of general interest, beyond their applicability to habit formation (Shiflett and Balleine, 2011b).

Another issue, common to all microarray experiments, with interpreting the data presented here is the degree to which RNA transcript levels can be used to infer protein expression levels. An implicit assumption in the functional interpretation of many microarray experiments, at least those that focus on coding RNA, is that mRNA transcription is an indirect index of protein expression. However, there is evidence that mRNA levels are weakly, and unreliably, correlated with protein levels (Maier et al., 2009). This point, added to the fact that the functional activity of proteins within a cell is influenced by many factors other than just expression levels, highlights the need to exercise caution when drawing conclusions about the functional relevance of differential mRNA levels identified by microarray analyses (Chuaqui et al., 2002). Nevertheless, these points do not negate the importance of the results presented in this chapter, they simply highlight the need to refrain from over-interpreting the functional relevance of these findings before more targeted investigations have been conducted.

In light of these last two points, and in order to move towards a more detailed understanding of the molecular processes underlying the regulation of goal-directed and habitual behaviour, it will be necessary to conduct a number of follow up experiments. Following biological verification of relevant candidate genes (for example, with qPCR analysis conducted on tissue samples from a different subgroup of animals behaviourally trained alongside those used for array analysis), it may be possible

to identify particular regulatory pathways of interest and disrupt their functioning using pharmacological reagents (e.g. receptor specific antagonists). Genetic manipulation techniques offer another potentially informative avenue to pursue, the most obvious of these being targeted gene knock out models (Picciotto and Wickman, 1998), although the burgeoning field of optogenetics may also offer a way to disrupt the function of specific molecular candidates at discrete time points during training (Bernstein and Boyden, 2011; Bernstein et al., 2012). Further discussion of future directions for this work can be found in Chapter 7.

At this stage the relevance of these data to the co-ordination of goal-directed and habitual behaviour remains unclear, but this experiment represents an important first step in exploring the molecular substrates of these two psychologically and neurophysiologically distinct behaviours. Indeed, showing, as they do, brain-region specific patterns of gene expression over the course of instrumental training, these data raise the prospect that individual regulatory pathways, discrete to functionally dissociable subregions of the dorsal striatum, may be involved in the co-ordination of instrumental behaviour.

4.7 Chapter summary

- This chapter presents data from Affymetrix microarray experiments investigating the effects of different amounts of instrumental training on gene expression in striatal subregions of interest, the DLS and pDMS; areas thought to have dissociable roles in the regulation of goal-directed and habitual behaviour.

- Animals were assigned to one of three training groups: *minimal*, *moderate* and *extended* and trained to lever press across three, six or ten sessions respectively.

- Bilateral dissections of the brain-regions of interest were taken from a proportion of animals from each training group and transcriptome changes were

measured and compared across both regions, and across the different training groups, using Rat Gene 2.0 ST Affymetrix arrays.

- Bidirectional, complex non-linear, shifts in gene regulation were found between the training groups in both the DLS and pDMS and distinct populations of genes were regulated in the two brain region respectively, suggesting that discrete, region-specific pathways may underlie these training-related expression changes.

Chapter 5

Systemic administration of an epigenetic modifier: effects of HDAC inhibition on the *acquisition*, *consolidation* and *retrieval* of an instrumental response

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5.1 Introduction

The experiments presented in Chapter 4 examined the molecular processes underlying instrumental learning by comparing patterns of gene expression, in key brain regions, between groups of animals which had received different amounts of lever press training. The data from these experiments point to bidirectional, region-specific differences between training groups in the expression of a number of genes, and provide an important initial step in understanding the transcriptional correlates of early- and late-stage instrumental learning. However, a number of factors limit the extent to which it is possible to infer a role for these molecular changes in habit formation *per se*. Firstly, as described in section 4.2.2, the design of the study was such that it was not possible to explicitly test whether lever pressing was goal-directed or habitual at

the point of dissection. Secondly, the fact that the observed transcriptional changes were correlational and did not involve any assessment of the effects of interfering with gene expression precludes any attribution of causality in the context of linking molecular and behavioural effects. Finally, changes in gene expression are relatively distal to the environmental inputs that drive learning and only represent one stage in the complex biological pathways that link environmental experience to changes in brain function and behaviour.

As detailed in the General Introduction of this thesis (section 1.3.3), interest is growing in the involvement of chromatin modifications (notably DNA methylation and post-translational modifications to chromatin associated histone proteins) in the brain in regulating learning-related molecular and cellular processes and many researchers anticipate that studying these dynamic epigenetic processes will help to advance our understanding of the neurophysiological mechanisms underlying learning and memory (Levenson and Sweatt, 2005; Day and Sweatt, 2011). Motivated by these factors, and also in the light of the ncRNA results presented in the previous chapter (see section 4.6.7), which potentially point to changes in epigenetic regulation occurring over the course of instrumental training (Kaikkonen et al., 2011), the experiments presented in this chapter aimed to investigate the molecular mechanisms involved in the transition from goal-directed to habitual responding more directly, by using a systemically delivered drug compound to manipulate endogenous chromatin remodelling processes.

When designing studies that involve the use of pharmacological agents to investigate learning processes, a number of key questions need to be considered. Firstly, it is necessary to specify *what* neurobiological process (or processes) is to be targeted and to then select the most appropriate agent available to achieve this. Drug suitability will be determined by a number of factors including, but not limited to: desired specificity (e.g. for particular protein complexes), toxicity and *in vivo* phar-

macokinetics and pharmacodynamics. The current experiments focused on the role of histone acetylation (HA) in habit formation. As discussed in section 1.3.3, one of the reasons HA was selected as the principle mechanism for investigation in this thesis is that there exist a number of well utilised drugs which can be employed to indirectly alter HA levels by inhibiting the families of enzymes which regulate acetylation (i.e HATs and HDACs). A detailed review of the literature regarding the studied effects of HDAC inhibitors (HDACi) and HAT inhibitors, in the context of learning and memory, can be found in section 1.3.3. The experiments presented here used the HDACi sodium butyrate (NaB) to alter the regulation of HA. As shown in Table 5.1.1, this general HDACi has been well used in the field of neuroscience and previous work has shown that systemic administration of NaB enhances memory performance in a number of different tasks, including: fear conditioning (Levenson et al., 2004), fear extinction (Lattal et al., 2007), spatial learning (Fischer et al., 2007) and novel object recognition (Stefanko et al., 2009).

The second factor that needs to be considered when designing psychopharmacological studies is *where* the drug is administered. In neuropharmacological research, drugs are typically administered via one of two routes: systemic administration (e.g. via intraperitoneal [IP] injection) or direct microinfusion into the central nervous system, and there is evidence that administering HDACis via both of these routes can produce behavioural effects (Lattal et al., 2007). Whilst the spatial specificity of microinfusions provides a powerful tool by which to target discrete brain regions, the breadth afforded by systemic administration, combined with its (potentially) greater clinical relevance, made it a more appropriate method for the completely novel work presented here. Therefore, in the current set of studies, NaB was administered systemically, by IP injection.

Finally, it is important to specify *when* the drug will be administered. There is an increasing awareness, in the field of neuroscience, that the specific molecular and

behavioural effects of a number of drugs are significantly influenced by both the learning-state of the brain at the point of administration and by post-administration experience (Ikegami et al., 2007; Dash et al., 2009; Raybuck et al., 2013). These experience-dependent effects highlight the interaction between neuroactive drugs and the neurophysiological processes involved in normal learning and memory functions.

Learning is typically described as consisting of a series of biologically and psychologically distinct phases and such a multi-process view provides a useful framework within which to study and explain the temporally specific effects of certain drugs on learning, cognition and behaviour (Abel and Lattal, 2001). In behavioural neuroscience these stages are typically described as follows: *acquisition*, the stage at which a behaviour or piece of knowledge is initially learnt; *consolidation*, a variable period of time during which a memory trace is strengthened and becomes more stable; *retrieval*, the point at which a memory trace is reactivated, frequently by re-exposure to specific context; *reconsolidation*, a process during which a reactivated memory trace becomes labile and sensitive to modification. Each of these various stages has been shown to involve specific brain networks and molecular pathways that can be discretely targeted by various behavioural and neurobiological manipulations (Abel and Lattal, 2001). Figure 5.1.1 provides a highly simplified summary of the multiple stages of memory and describes how how they relate to the current experimental design.

This conceptual framework was used to inform the design of the the series of experiments presented in the current chapter in which the hypothesised involvement of HA in habit formation was investigated by systemically administering NaB at specific time points during training as summarised in Figure 5.1.2.

The emphasis on when drugs are administered is particularly relevant to the effects of HDACis on behaviour, where emerging evidence suggests such effects can vary

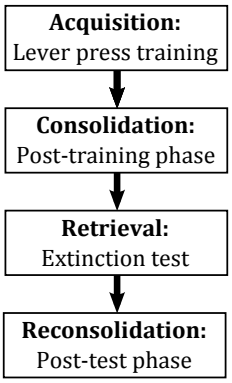


Figure 5.1.1: Summary of the multiple stages of memory illustrating how they relate to the current experimental design.

substantially according to stage of learning. For example, Raybuck et al. (2013) used a cocaine conditioned place preference (CCCP) procedure to examine the effects of systemic NaB administration on three distinct stages of learning: the initial acquisition of a context-drug memory, the extinction of context-dependent drug seeking behaviour and the renewal of previously extinguished drug-context associations. Whilst NaB administration prior to CCPP training enhanced the acquisition of drug-context memory, post-extinction NaB administration had no effect on the strength of reconditioned memory. Interestingly, the the effects of post-training administration on extinction were dose dependent; whilst lower doses enhanced extinction learning (as indexed by an increased resistance to reconditioning), higher doses led to weaker extinction. As well as illustrating the learning-stage dependency of HDACi effects, this study highlights the importance of selecting an appropriate drug dosage, indicating, as it does, that HDACis (or, at least, NaB) may exhibit an inverted u-shaped dose-effect curve. The authors suggest that at lower doses the interaction between exogenous epigenetic effects of NaB and endogenous learning induced molecular changes facilitated learning, but at higher doses these learning-induced effects are obscured by more global changes in HA.

Interactions between HDACis and post-administration experience have been shown elsewhere. For example, Dash et al. (2009) found that NaB helped to reduce brain

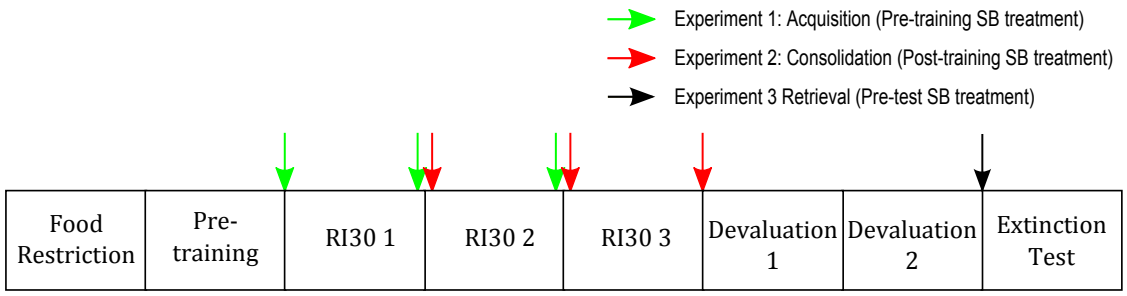


Figure 5.1.2: Summary of the various time points of systemic NaB administration presented in this section

injury-induced cognitive impairments, but only when administered in combination with environmental enrichment. These, and similar studies (e.g. Intlekofer et al. 2013), studies illustrate how the behavioural effects of HDACis are sensitive to both pre- and post-administration experience and emphasize the importance of considering how such experiential factors may interact with with the specific parameters of a given epigenetic manipulation, including dosage.

Task	Dosage	Point of administration (*Learning stage*)	Behavioural effects	Reference
CFC	1.2 g/kg	Prior to extinction session *(Retrieval)*	Enhanced contextual fear extinction 24 hrs post-IP	Lattal et al. (2007)
NOR	1.2 g/kg	After NOR training *(Consolidation)*	Increased retention of NOR memory in WT and CBP mice [1]	Stefanko et al. (2009)
CCPP	0.3, 0.6 & 1.2g/kg	Immediately after each trial *(CCP consolidation)*	0.3 and 1.2g/kg enhanced CCP	Raybuck et al. (2013)
CCPP	0.3, 0.6 & 1.2g/kg	After extinction (x 4 trials) *(Extinction consolidation)*	0.3 g/kg: facilitated extinction (impaired reconditioning) 1.2 g/kg: weakened extinction of NIH	Raybuck et al. (2013)
CCPP	0.3, 0.6 & 1.2g/kg	After reconditioning trial *(Reconsolidation of CCP)*	No effects of NaB at any dose	Raybuck et al. (2013)
CFC	1.2g/kg	1 hr prior to CFC trials *(Acquisition)*	Increased retention of CFC memory 24 hrs after training	Levenson et al. (2004)
CCLM CCPP	100 or 200mg / kg	30 min prior to CCCP trials *(Acquisition)*	Enhanced CCPP (24 hrs post-final conditioning session)	Hui et al. (2010)
NOR	1.2g/kg	After NOR training *(Consolidation)*	Increased retention of NOR memory 24 hrs after training	Reolon et al. (2011)
Extinction of CCPP	1.2g/kg	After CCPP extinction session *(Consolidation)*	Enhanced extinction of CCPP	Malvaez et al. (2010)
EBC Mice	250mg/kg	30 min prior to EBC training *(Acquisition)*	Increased rate of conditioning	Fontán-Lozano et al. (2008)
CFC & CuFC Rat	1.2g/kg	1 hr prior to CFC trials	Long term & acute acceleration of extinction learning.	Itzhak et al. (2012)
CTA	1.2 g/kg 0.4 g/kg	10 minutes post-CTA	Increased resistance to CTA extinction	Kwon and Houpt (2010)
CCLM CCPP	100 mg/kg	CCLM: After 75 min of habituation to chamber CCPP: within 1 hr of control sessions (x2)	Short-term dose-dependent enhancement of CCLM & CCPP.	Schroeder et al. (2008)
FST NIH	100 mg/kg	3 injections over 24 hrs	FST: increased immobility NIH: Increase in latency to consume in a novel environment.	(Gundersen and Blendy, 2009)

Table 5.1.1: A summary of the NaB dosage and administration details published to date. For all experiments the route of administration was via intraperitoneal (IP) injection. CCCP: Cocaine-induced conditioned place preference; CCLM: Cocaine-induced locomotor activity; CFC: Contextual fear conditioning; CTA: conditioned taste aversion; CuFC: Cued fear conditioning; EBC: Eyeblink conditioning; FST: Forced swim test; NIH: novelty-induced hypophagia; NOR: Novel object recognition

Again, because of the novelty of this work it was logical, at the outset, to use a dose range of NaB that has been shown to give rise to behavioural and (in some cases) molecular effects in previous work. As illustrated in Table 5.1.1, the majority of published studies have used a dose of 1.2g/kg (IP). This dose has been shown to give rise to both behavioural (Levenson et al., 2004; Lattal et al., 2007; Stefanko et al., 2009) and neurophysiological effects (Fischer et al., 2007) and thus was an obvious choice for these initial experiments. However, extensive pilot studies conducted by us, revealed that, at the 1.2mg/kg (IP) dose, NaB produced an adverse reaction in treated rats, the main feature of which was an extended period of profound hypo-activity following administration (see Figure 5.1.3). This effect was not due to pH, since they were still evident when the drug solution was buffered to physiological pH with 10% citric acid solution, but was related to the particular dose of NaB. On communicating with others in the field we found that these effects were a frequent occurrence but were rarely (if ever) mentioned in publications (personal communication from Sara Orsi, in the laboratory of Professor P. Dash, Texas Medical School). Clearly however, such gross side-effects, which persisted into the time period we would have been testing the animals, precluded the use of the 1.2mg/kg dose in the current set of experiments on both animal welfare and scientific grounds. When an anticipated experimental effect depends on the comparison of differential response rates (i.e. Devalued vs. Non-devalued groups) between drug treated animals and controls (i.e. NaB vs. Saline), as was the case here, any baseline differences in general levels of motor activity could give rise to confounding effects (e.g. floor effects). Therefore, for this set of experiments we used a lower dose of NaB (0.6g/kg, IP) which was within the range shown to have behavioural and molecular effects in other published studies (Fontán-Lozano et al., 2008; Hui et al., 2010; Kwon and Houpt, 2010; Raybuck et al., 2013) and, which did not have significant effects on levels of general locomotor activity (see Figures 5.3.3, 5.4.3 and 5.5.3).

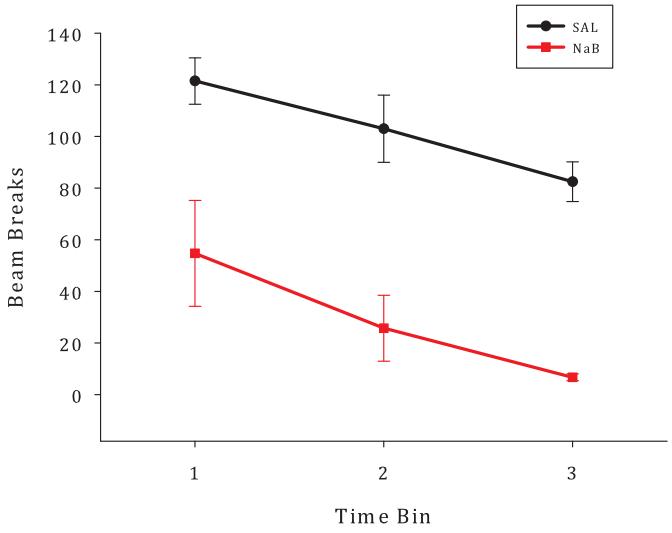


Figure 5.1.3: Mean number of beam breaks made by NaB (1.2g/kg; IP) and vehicle treated animals over a 30 minute locomotor activity test session (N=4; further details regarding this pilot experiment can be found in Appendix C, section C.1).

5.2 Common Methods

This Chapter presents three separate experiments, all of which followed essentially the same experimental design; differing only in the point in training at which NaB was administered. Methodological details specific to each experiment are described in the individual experimental sections. All common methods are described in this section.

5.2.1 Subjects

Each experiment used a separate group of 32 naive male Lister Hooded rats, supplied by Charles River (Margate, Kent, UK). All subjects were acclimatised to the holding room for at least one week before being food restricted to above 80% of their *ad libitum* feeding weight prior to the start of instrumental training. Subjects were then maintained on a restricted diet for the remainder of the experiment. Specific details of the husbandry for each experimental cohort (e.g. weight information) are reported in the individual experimental sections. General husbandry details were as

described in section 2.1.1.

5.2.2 Apparatus

All apparatus details (e.g operant boxes and locomotor activity chambers) were as described in Chapter 2.

5.2.3 Drug administration

A review of the literature, which is summarised in Table 5.1.1, was used to inform decisions regarding the dosage of NaB used in this series of experiments. As described above, due to the adverse side-effects produced by IP administration of NaB at a dose of 1.2g/kg (the most commonly reported dosage in behavioural studies), a lower dose of 0.6g/kg (IP) was used in these experiments. NaB (Sigma, Aldrich) was dissolved in physiological saline (0.9%) before being microfiltered though a syringe filter and buffered to approximately pH 7.4 using a 10% citric acid solution. Both NaB and vehicle (0.9% saline) were administered at a volume of 10ml/kg (IP). Based on previous work showing effects of NaB in the brain 30 minutes post-administration (Schroeder et al., 2007; Gundersen and Blendy, 2009; Kwon and Houpt, 2010), NaB was administered 30 minutes prior to the relevant stage of behavioural training (or immediately after training for Experiment 2).

5.2.4 Training, devaluation and extinction test

The basic training procedure and extinction test were as described in Chapter 3.5, Experiment 1. Outcome devaluation and the consumption test were conducted as described in Chapter 3.5, Experiment 2.

5.2.5 Locomotor activity

Details of the locomotor activity apparatus can be found in section 2.2.2. Animals were placed in the chambers after they had completed the targeted stage of be-

havioural training (e.g. after each RI30 session) and left in the dark for the duration of the session. Each session lasted 1 hour and the total numbers of beam breaks were summated across six 10 minute bins for each session.

5.2.6 Measures and data analysis

Training. Lever press responses and magazine entries were analysed using a 3-factor mixed ANOVA, with the between-subjects factors: HDACi group (*Sal* vs. *NaB*) and DEVALUATION group (*Devalued vs. Non-devalued)* and the within-subjects factor SESSION. In Experiments 1 and 2 it was important to assess the effects of repeated injections of NaB on instrumental learning and, for all experiments it was necessary to ensure that there were no pre-exsisting differences between the devaluation groups.

Test. To assess behavioural sensitivity to outcome devaluation, lever press responses and magazine entries for the whole extinction test were analysed using a 2-factor between-subjects ANOVA with the factors: HDACi group (*Sal* vs. *NaB*) and DEVALUATION group (*Devalued vs. Non-devalued).*

Locomotor activity. To ascertain whether NaB administration had any effects on gross locomotor activity levels, overall beam break data across the three 10 minute time bins for each LMA session were compared using a mixed ANOVA, with the within-subjects factors: BIN (*1 vs. 2 vs. 3*) and SESSION (*1 vs. 2 vs. 3*, where multiple LMA session were completed) and the between-subjects factor HDACi group.

5.3 Experiment 1: The effects of NaB administration on the *acquisition* of a minimally trained instrumental response

5.3.1 Methods

Experiment 1 was designed to target the acquisition phase of learning. Systemic injections of NaB (0.6 g/kg; IP) were given 30 minutes prior to each RI30 training session. After each training session (and $\sim$ 70 minutes post-injection) all animals completed a 60 minute locomotor activity session. Figure 5.3.1 summaries the basic experimental design for this experiment. All other experimental details were as described in section 5.2. Weight details for the cohort are displayed in Table 5.3.1

		Ad lib	Restricted
SAL	Mean	288.3125	240.375
	Range	(266-315)	(219-274)
NaB	Mean	291.1875	243.6875
	Range	286-309	219-258

Table 5.3.1: The ranges and mean *ad libitum* and restricted feeding weights for the two experimental groups in grams. *Ad libitum* weights were taken immediately prior to food deprivation. Restricted weights were recorded at the end of the experiment.

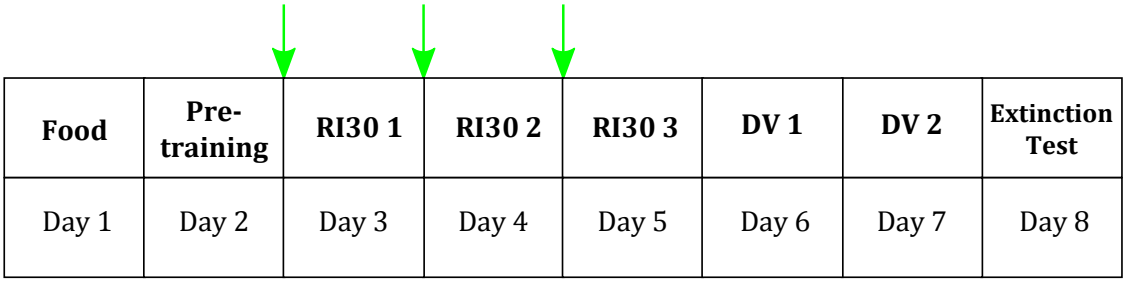


Figure 5.3.1: Basic design for Experiment 1

5.3.2 Experiment 1: Results

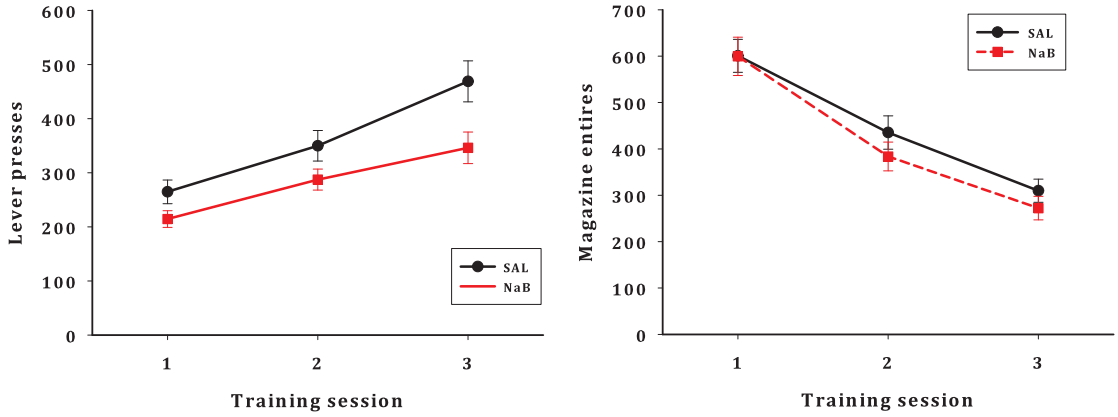
One animal was excluded from analysis because of poor training performance (i.e. earned < 4SD from the mean number of rewards across all RI30 training sessions; as described in section 2.5.3).The final group sizes of the experimental groups, after exclusions, are shown in Table 5.3.2

	Devalued	*Non-devalued*
Saline	8	8 *(7)*
NaB	8	8

Table 5.3.2: Initial groups sizes of the 4 experimental groups. Figures in brackets show the final groups sizes used in statistical analysis after exclusions.

Training Figure 5.3.2 a. shows the lever press responding across the three RI30 training sessions for the two HDACi groups. Lever presses increased across the sessions in both groups (Main effect of SESSION: $F[2, 54] = 39.601$, $p < .001$), with NaB-treated animals pressing the lever, on average, less than the saline-treated animals for all three sessions (Main effect of HDACi: $F[1, 27] = 6.486$, $p = .017$). Conversely, as shown in Figure 5.3.2 b, magazine entries decreased across the training sessions (Main effect of SESSION: $F[2, 54] = 102.004$, $p < .001$), but there was no difference between the two HDACi groups (Main effect of HDACi: $p = >.05$). Taken together, these data indicated that all animals acquired the basic lever press response and learnt about the instrumental contingency as expected, reducing their magazine approach behaviour in favor of lever pressing; and also that NaB pre-treatment specifically reduced lever press responding relative to saline-treated controls. Mean data for all conditions can be found in Appendix C, Table C.3.1.

Activity Beam break data across the locomotor activity sessions are presented in Figure 5.3.3. Data from two additional animals were excluded from this analysis due to equipment failure during one of sessions (NaB n = 15; Saline n = 14). Both groups habituated to the locomotor activity chambers over the course of training,



(a) Lever presses **(b)** Magazine entries

Figure 5.3.2: Mean number of lever press responses and magazine entries across training session. Total N= 31; error bars show standard error.

as indexed by a decline in beam breaks across the three 10 minute time bins (Main effect of BIN: $F[5, 135] = 129.310$, $p < .001$). Between session habituation was also evidenced by decrease in beam breaks from the first to the last activity session (Main effect of SESSION: $F[2, 54] = 3.302$, $p = .045$). Importantly, there was no effect of HDACi group on activity levels (Main effect of HDACi group: $p > .05$) indicating that, at this dose, NaB had no non-specific effects on levels of general behavioural arousal and providing further evidence of the behavioural specificity of the effect of NaB on lever press response rates (see Figure 5.3.2 a).

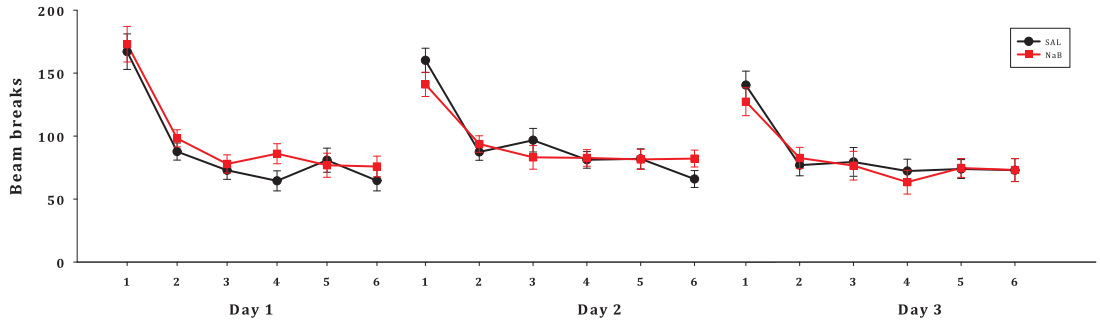
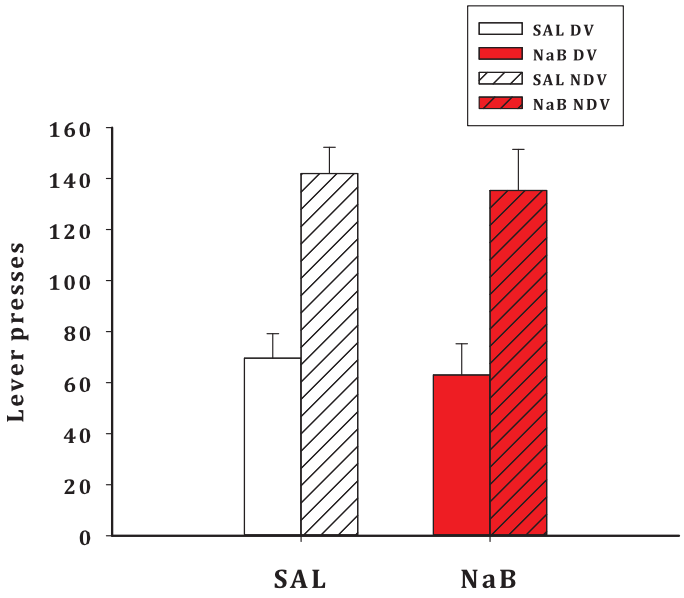


Figure 5.3.3: Mean number of beam breaks across 10 minute bins for each training day. Total N= 31; error bars show standard error

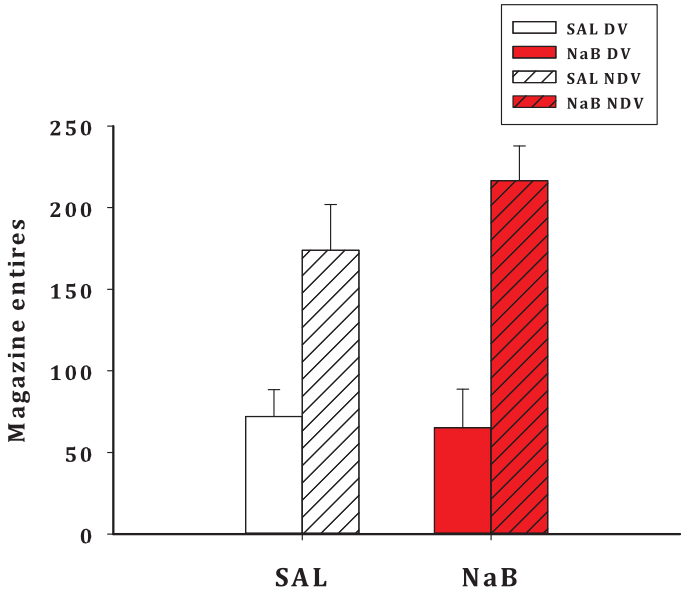
Extinction Test As shown in Figure 5.3.3 NaB had no effect on lever pressing or magazine entries in extinction (Main effect of HDACi group: $p > .05$) and both

groups showed the same degree of behavioural sensitivity to outcome devaluation, with *Devalued* animals reducing both lever press responses (Main effect of DEVALUATION: $F[1, 28] = 29.110$, $p < .001$) and magazine entries (Main effect of DEVALUATION: $F[1, 28] = 31.877$, $p < .001$) for a devalued reinforcer.



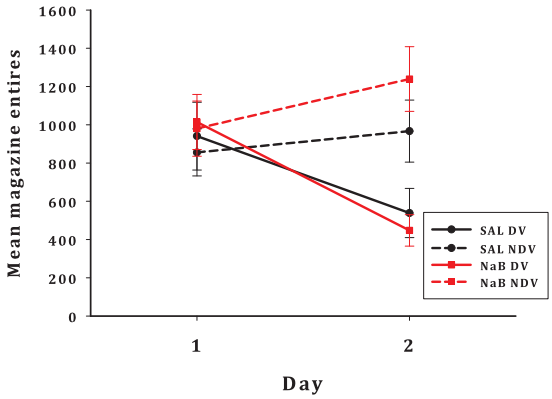
(a) Lever presses in extinction

Devaluation and consumption As shown in Figure 5.3.4 a, *Devalued* animals reduced magazine entry behaviour following the first devaluation session (SESSION x DEVALUATION group interaction: $F[1, 28] = 36.323$, $p < .001$) and the magnitude of this effect was the same for both NaB- and Saline-treated groups (SESSION x HDACi x DEVALUATION group interaction: $p > .05$). More importantly, at test, the *Devalued* groups consumed significantly less than their equivalent *Non-devalued* group (Main effect of DEVALUATION group: $F[1, 28] = 112.362$, $p < .001$) and there was no difference in the size of this effect between the two HDACi groups (DEVALUATION x HDACi group interaction: $p > .05$).

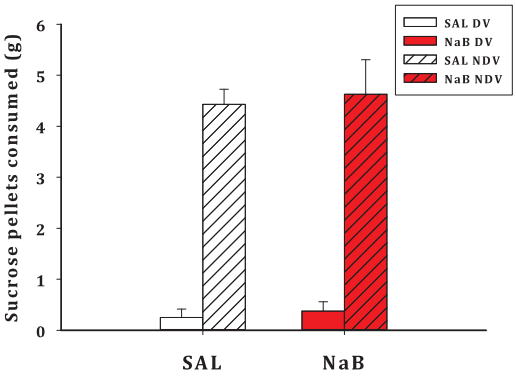


(b) Magazine entries in extinction

Figure 5.3.3: Mean number of lever presses (a) and magazine entries (b) during extinction test. Total N= 31; error bars show standard error



(a) Magazine entries across devaluation sessions. Total N= 31; error bars show standard error



(b) Sucrose pellets eaten at consumption test (in grams). Total N= 31; error bars show standard error

Figure 5.3.4: Conditioned taste aversion: Mean magazine entries across devaluation sessions (a) and mean amounts of sucrose pellets eaten across consumption test (b).

5.4 Experiment 2: The effects of NaB administration on the *consolidation* of a minimally trained instrumental response

5.4.1 Methods

Experiment 2 was designed to target the consolidation phase of learning. Systemic injections of NaB (0.6 g/kg; IP) were given immediately after each RI30 training session. Locomotor activity sessions were conducted $\sim$ 30 minutes post-injection on each training day. Figure 5.4.1 summarises the basic experimental design for this experiment. Group size and weight details for the cohort are displayed in Tables 5.4.1 and 5.4.2 respectively.

	Devalued	*Non-devalued*
Saline	8	8
NaB	8	8

Table 5.4.1: Groups size of the 4 experimental groups

		Ad lib	Restricted
SAL	Mean	367.0625	327.9375
	Range	326-407	284-371
NaB	Mean	361.75	324.875
	Range	330-390	295-350

Table 5.4.2: The ranges and mean *ad libitum* and restricted feeding weights for the two experimental groups in grams. *Ad libitum* weights were taken immediately prior to food deprivation. Restricted weights were recorded at the end of the experiment.

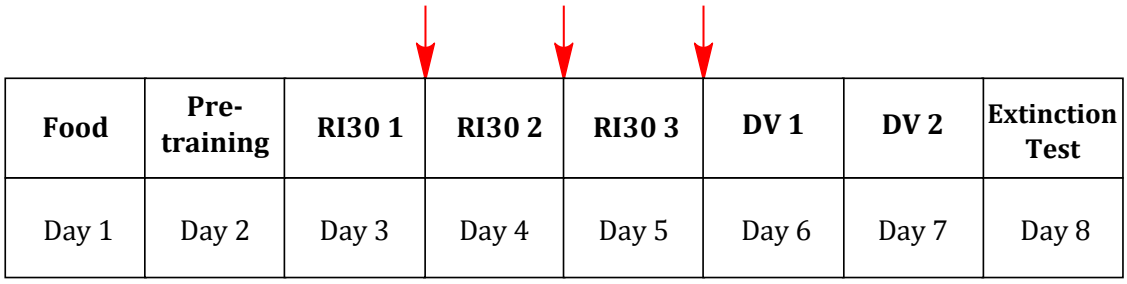
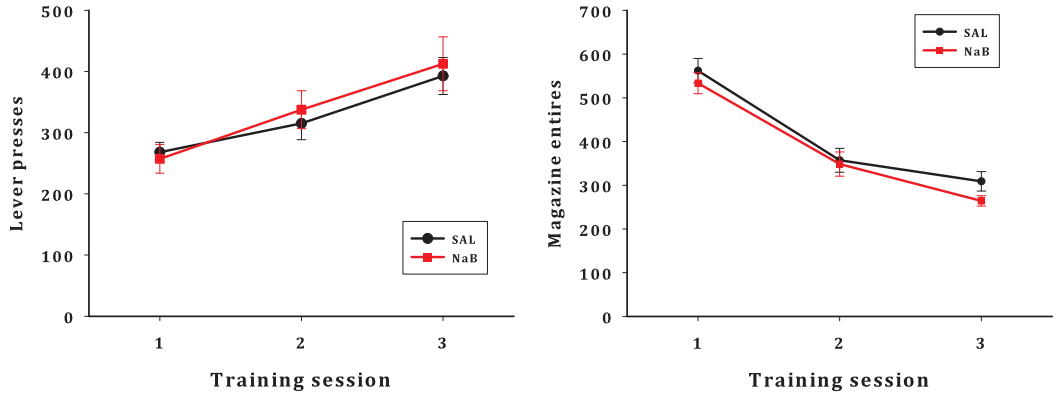


Figure 5.4.1: Basic design for Experiment 2

5.4.2 Experiment 2: Results

All animals completed training and no exclusions were made for statistical analysis (n [per group] = 8; N [total] = 32 ; see Table 5.4.1).

Training Figure 5.4.2 a shows the lever press responding across the three RI30 training sessions for the two HDACi groups. Lever presses increased across the sessions in both groups (Main effect of SESSION: $F[2,\ 56] = 38.803$, $p < .001$). Conversely, as shown in Figure 5.4.2 b, magazine entries decreased across the training sessions (Main effect of SESSION: $F[2,\ 54] = 95.162$, $p < .001$). There was no difference in either lever press responding or magazine entry behaviour between the two HDACi groups (Main effect of HDACi $p > .05$). Taken together, these data indicate that all animals acquired the basic lever press response and learnt about the instrumental contingency as expected, reducing their magazine approach behaviour in favor of lever pressing; and that post-training administration of NaB had no enduring effects on behaviour during subsequent training sessions. Mean data for all conditions can be found in Appendix C, Table C.3.2.



(a) Lever presses **(b)** Magazine entries

Figure 5.4.2: Mean number of lever press responses and magazine entries across training sessions. Total N= 32 (group n = 8); error bars show standard error

Activity Beam break data across the the locomotor activity sessions are presented in Figure 5.4.3. Both groups habituated to the locomotor activity chambers over the course of training, as indexed by a decline in beam breaks across the six 10 minute time bins (Main effect of BIN: $F[5, 140] = 148.179$, $p < .001$). Again, there was no evidence that NaB had any effect on locomotor activity over the course of training (Main effect of GROUP $p > .05$). Although, in contrast to Experiment 1, here there was no evidence of between-session habituation (Main effect of SESSION: $p > .05$) with the total number of beam breaks made in a single session remaining relatively stable across the three sessions. However, NaB administration had no effect on either within- or between-session habituation (BIN x SESSION and GROUP x SESSION interaction: $p > .05$).

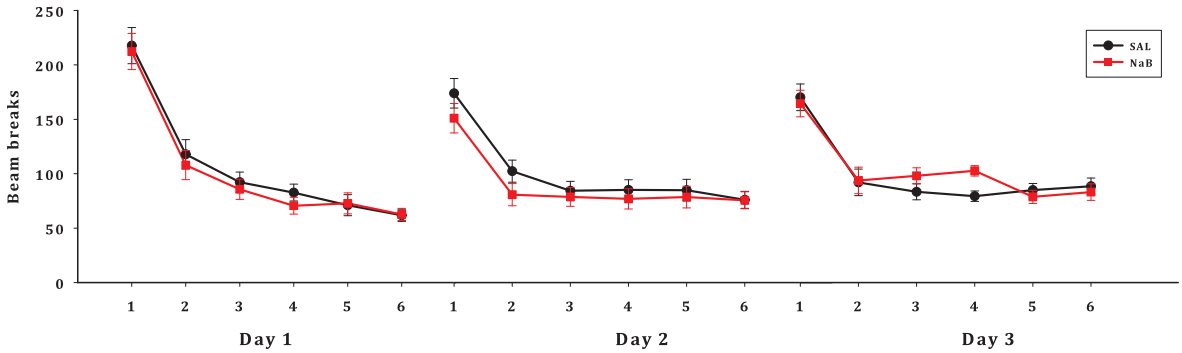
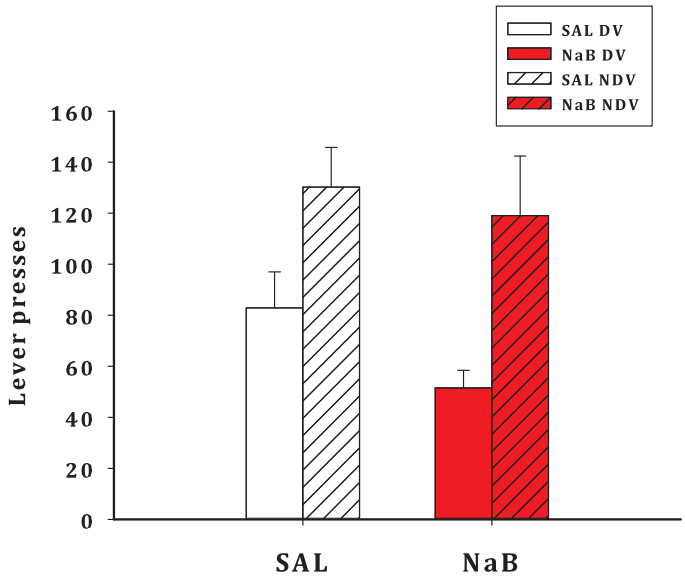
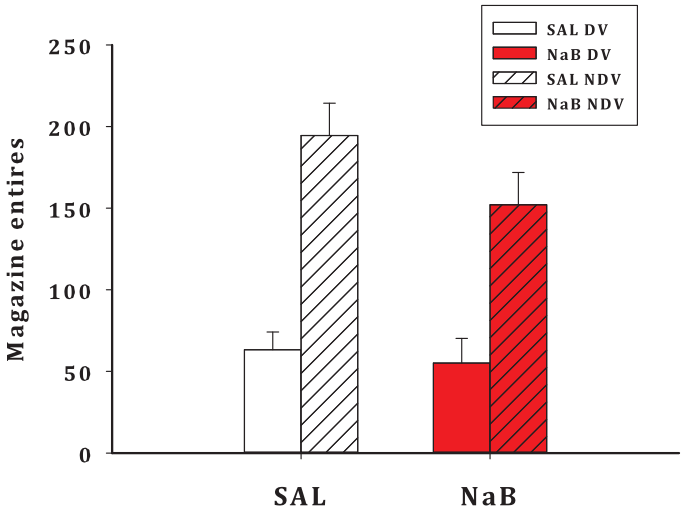


Figure 5.4.3: Mean number of beam breaks across 10 minute bins for each training day. Total N= 32 (group n = 8); error bars show standard error.

Extinction Test As shown in Figure 5.4.3, NaB had no effect on overall levels of lever pressing or magazine entries in extinction (Main effect of HDACi group: $p > .05$) and both HDACi groups showed the same degree of behavioural sensitivity to outcome devaluation, with *Devalued* animals reducing both lever press responses ($F[1, 28] = 12.821$, $p = .001$) and magazine entries ($F[1, 28] = 45.998$, $p < .001$) for a devalued reinforcer.



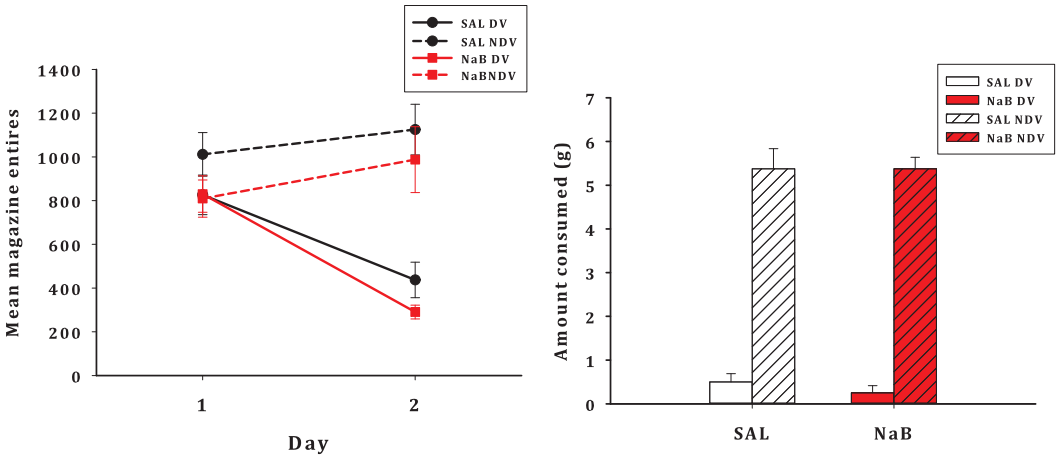
(a) Lever presses in extinction



(b) Magazine entries in extinction

Figure 5.4.3: Mean number of lever presses (a) and magazine entries (b) during extinction test. Total N= 32 (group n = 8); error bars show standard error.

Devaluation and consumption As shown in Figure 5.4.4 a, all *Devalued* animals reduced magazine entry behaviour following the first devaluation session (SESSION x DEVALUATION group interaction: $F[1, 28] = 39.105$, $p < .001$) and the magnitude of this effect was the same for both NaB- and Saline-treated groups (SESSION x HDACi x DEVALUATION group interaction: $p > .05$). More importantly, at test, the *Devalued* groups consumed significantly less than their equivalent *Non-devalued* groups (Main effect of DEVALUATION group: $F[1, 28] = 290.909$, $p < .001$) and there was no difference in the size of this effect between the two HDACi groups (DEVALUATION x HDACi group interaction: $p > .05$).



(a) Magazine entries across devaluation sessions

(b) Sucrose pellets eaten at consumption test (in grams)

Figure 5.4.4: Conditioned taste aversion: Mean magazine entries across devaluation sessions (a) and mean amounts of sucrose pellets eaten across consumption test (b).Total N= 32 (group n = 8); error bars show standard error.

5.5 Experiment 3: The effects of NaB administration on the *retrieval* of a minimally trained instrumental response

5.5.1 Methods

Experiment 3 was designed to target the retrieval phase of learning. A single systemic injection of NaB (0.6 g/kg; IP) or saline (0.9%; IP) was given 30 minutes prior to extinction test. A 60 minute locomotor activity session was conducted ~50 minutes post-injection. Figure 5.5.1 summarises the basic experimental design for this experiment. All other experimental details were as described in section 5.2. Group size and weight details for the cohort are displayed in Tables 5.5.1 and 5.5.2 respectively.

	Devalued	*Non-devalued*
Saline	8	8
NaB	8	8

Table 5.5.1: Groups size of the 4 experimental groups

		Ad lib	Restricted
SAL	Mean	268.87	233
	Range	256-285	219-258
NaB	Mean	269.13	230.87
	Range	248-285	216-246

Table 5.5.2: The ranges and mean *ad libitum* and restricted feeding weights for the two experimental groups in grams. *Ad libitum* weights were taken immediately prior to food deprivation. Restricted weights were recorded at the end of the experiment.

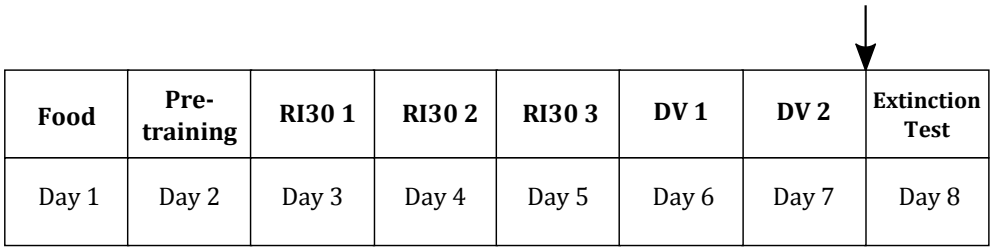


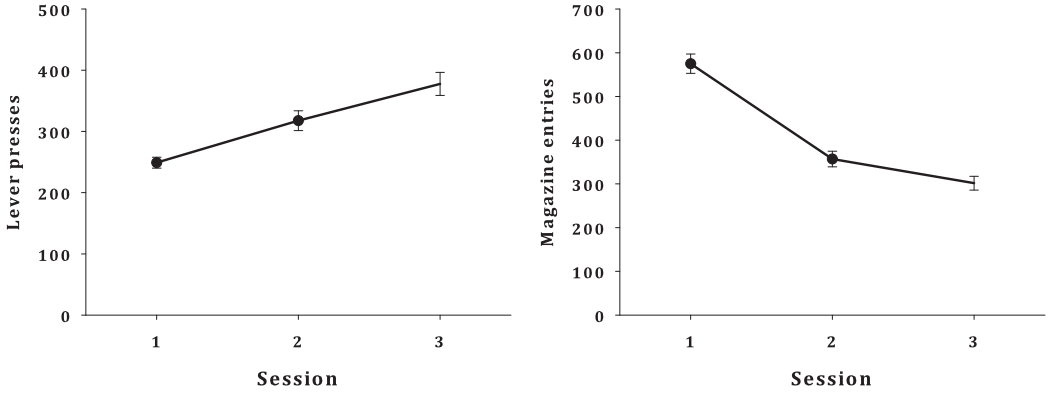
Figure 5.5.1: Basic design for Experiment 3

5.5.2 Experiment 3: Results

All animals completed training and no exclusions were made for the statistical analysis (n = 8; see Table 5.5.1).

Training Figure 5.5.2 shows the lever press responding and magazine entry behaviour across the three RI30 training sessions for all animals. Lever presses increased across the sessions (Main effect of SESSION: $F[2, 56] = 30.025$, $p < .001$) whilst magazine entries decreased over the course of training (Main effect of SESSION: $F[2, 56] = 82.372$, $p < .001$). These data indicate that all animals acquired

the basic lever press response and learnt about the instrumental contingency as expected, reducing their magazine approach behaviour in favor of lever pressing. Importantly, there were no pre-exsisting differences between the HDACi or DEVALUATION groups prior to drug treatment ($p > .05$; mean data for all conditions can be found in Appendix C, Table C.3.3).



(a) Lever presses **(b)** Magazine entries

Figure 5.5.2: Mean number of lever press responses and magazine entries across the training sessions. Data shown is for all animals since no experimental manipulations had taken place at the point of training. Total N= 32 (group n = 8); error bars show standard error.

Activity Beam break data across the the locomotor activity sessions is presented in Figure 5.5.3. Data from one animal were excluded from this particular analysis due to equipment failure during the session (NaB n = 15; Saline n = 16). Both groups habituated to the locomotor activity chambers over the course of training, as indexed by a decline in beam breaks across the six 10 minute time bins (Main effect of BIN: $F[5, 145] = 76.701$, $p < .001$). Importantly, NaB treatment did not effect activity levels (Main effect of HDACi group: $p > .05$) or habituation to the chamber (HDACi x BIN interaction: $p > .05$), indicating that, at this dose, NaB had no non-specific effects on levels of general behavioural arousal or habituation.

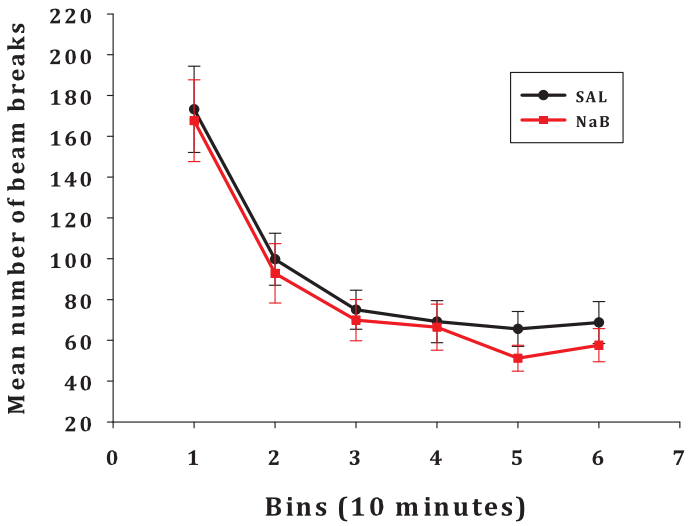
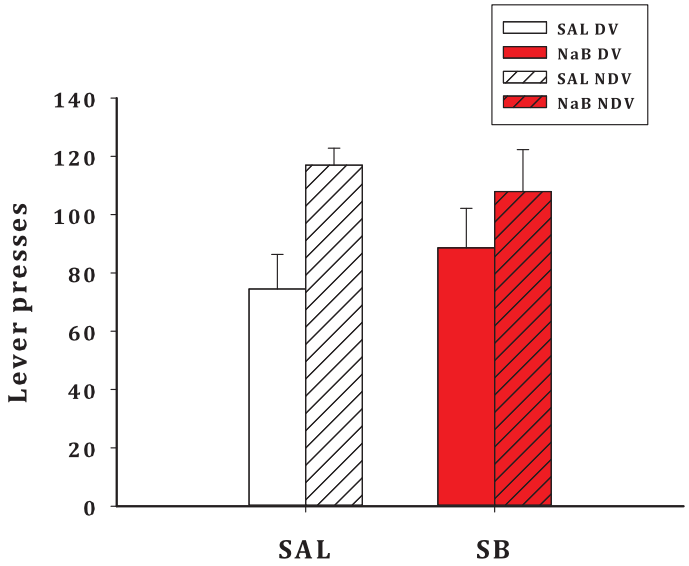
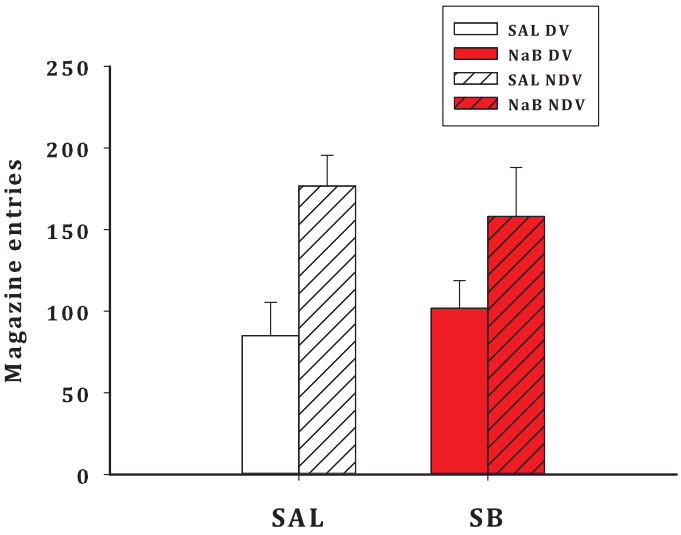


Figure 5.5.3: Mean number of beam breaks across 10 minute bins post-test activity session. Total N= 32 (group n = 8); error bars show standard error.

Test Lever press and magazine entry data for the whole 10 minute extinction test are shown in Figure 5.5.3. Whilst, overall, *Devalued* groups pressed the lever less than *Non-devalued* groups (Main effect of DEVALUATION: $F[1, 28] = 6.748$, $p = .015$), as shown in Figure 5.5.3 a, the devaluation effect was smaller in the *NaB*-treated animals (Simple effect of DEVALUATION: $F[1, 28] = 1.312$, $p = .263$) relative to *Saline*-treated animals (Simple effect of DEVALUATION: $F[1, 28] = 6.393$, $p = .017$). Similarly, although, overall, magazine entries were sensitive to outcome devaluation (Main effect of DEVALUATION: $F[1, 28] = 11.146$, $p = .002$), this effect was, again, much bigger in the *Saline*-treated animals (Simple effect of DEVALUATION: $F[1, 28] = 8.558$, $p = .007$) than in *NaB*-treated animals (Simple effect of DEVALUATION: $F[1, 28] = 3.225$, $p = .084$). Importantly, there was no difference in overall levels of responding between the HDACi groups for either lever presses (Main effect of HDACi: $F[1, 28] = .044$, $p = .834$) or magazine entries (Main effect of HDACi: $F[1, 28] = .002$, $p = .966$).



(a) Lever presses

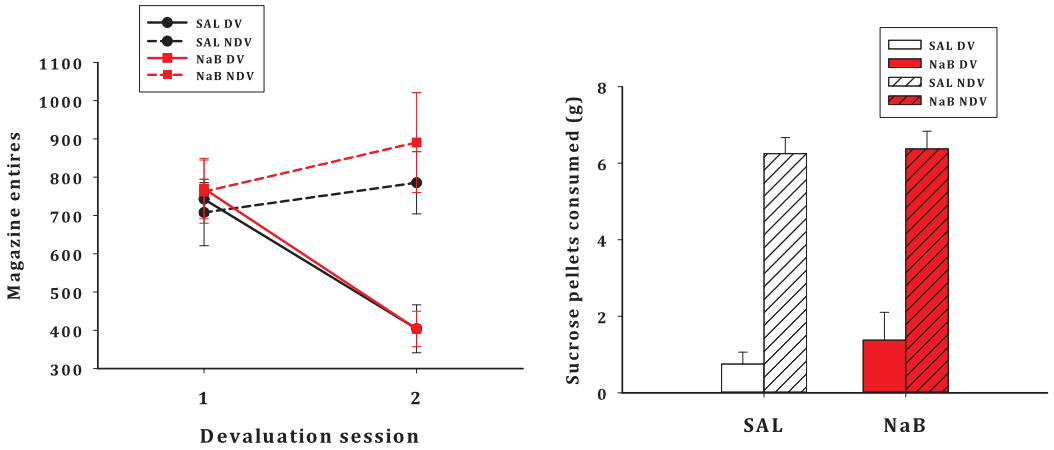


(b) Magazine entries

Figure 5.5.3: Mean number of lever presses (a) and magazine entries (b) during extinction test. Total N= 32 (group n = 8); error bars show standard error.

Devaluation and consumption A shown in Figure 5.5.4 a, *Devalued* animals reduced magazine entry behaviour following the first devaluation session (SESSION x DEVALUATION group interaction: $F[1,\ 28] = 26.225$, $p < .001$) and the magnitude of this effect was the same for both NaB- and Saline-treated groups (SESSION x

HDACi x DEVALUATION group interaction: $p > .05$). More importantly, at test, the *Devalued* groups consumed significantly less than their equivalent *Non-devalued* group (Main effect of DEVALUATION group: $F[1, 28] = 108.793$, $p < .001$) and there was no difference in the size of this effect between the two HDACi groups (DEVALUATION x HDACi group interaction: $p > .05$), indicating that the devaluation manipulation was successful in reducing the motivational value of the sucrose pellets and that the strength of the conditioned taste aversion was not effected by the HDACi manipulation.

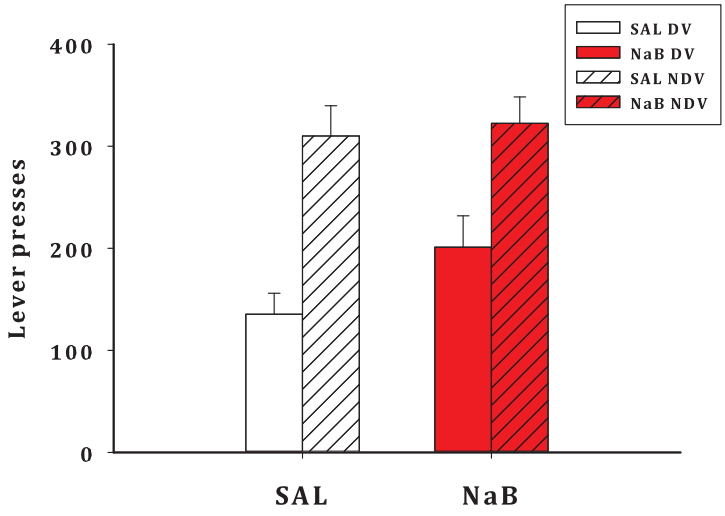


(a) Magazine entries across devaluation sessions

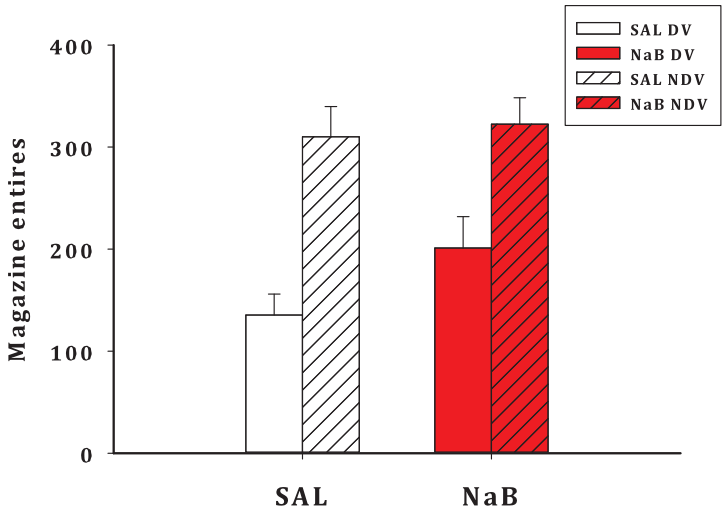
(b) Sucrose pellets eaten at consumption test (in grams)

Figure 5.5.4: Conditioned taste aversion: Mean magazine entries across devaluation sessions (a) and mean amounts of sucrose pellets eaten across consumption test (b). Total N= 32 (group n = 8); error bars show standard error.

Reacquisition test In order to further investigate the effects of NaB-treatment on outcome devaluation sensitivity, a reinforced reacquisition test was conducted 24 days after the initial extinction test. The lever press and magazine entry data for the whole session are presented in Figure 5.5.5.



(a) Lever presses



(b) Magazine entries

Figure 5.5.5: Mean number of lever presses (a) and magazine entries (b) during reacquisition test. Total N= 32 (group n = 8); error bars show standard error.

In contrast to the extinction test data, lever pressing during the reacquisition test was sensitive to outcome devaluation in both Saline-treated (Simple effect of DEVALUATION: $F[1, 28] = 20.951$, $p < .001$) and NaB-treated animals (Simple effect of DEVALUATION: $F[1, 28] = 10.142$, $p = .004$). Saline-treated animals exhibited devaluation sensitivity in magazine entry behaviour (Simple effect of DEVALUATION:

$F[1, 28] = 8.113$, $p = .008$); however, whilst magazine entries in NaB-treated animal showed a trend towards a devaluation sensitivity, this difference was not statistically significant (Simple effect of DEVALUATION: $F[1, 28] = 3.430$, $p = .075$).

5.6 Discussion

The experiments presented in this chapter were designed to systematically investigate the effects of pharmacological manipulation of histone acetylation (HA) levels on goal-directed (i.e. minimally trained) instrumental learning. The general HDACi inhibitor, NaB, was administered systemically at discrete points during training, with the objective of targeting specific stages of learning.

Experiment 1 investigated the effects of NaB administration prior to each of the instrumental training sessions, with the aim of investigating the role of HA in the initial acquisition of an instrumental response. During training, NaB-treated animals pressed the lever significantly less than Saline-treated controls, however NaB had no effect on overall response rate in the extinction test. Furthermore, both Saline and NaB treated animals showed the expected devaluation effect, reducing responding for a devalued reinforcer, and the degree of devaluation sensitivity did not differ between the groups. Taken together these data suggest that the suppressing effects of NaB on lever press responding during training were acute and did not influence the acquisition and encoding of the action-outcome association. It is not possible to determine the reason for this acute suppressing effect from the data presented here, but magazine entries during training did not differ between the groups and NaB had no effect on general locomotor activity, suggesting that the reduced lever press responding was not a reflection of a non-specific effect of NaB on behavioural arousal. Furthermore, it is unlikely that this effect was simply the result of chance variation in the propensity to lever lever press between the groups, since they did not

differ their levels of responding during the CRF pre-training session (see Appendix C, section C.2). Moreover, the effect appeared to be cumulative, with the difference in response rates between the two groups increasing over the course of the sessions. It may be, therefore, that these effects were caused by transient changes in brain function, perhaps mediated by short-term alterations in neurotransmitter system function. Indeed, NaB treatment, and HA in general, have been linked to altered dopaminergic (Lee and Workman, 2007; Harrison and Dexter, 2013) and serotonergic function (Schroeder et al., 2007) and we can speculate, tentatively, that the reduced lever press responding in NaB-treated was a reflection of either an reduced motivation to work for food (i.e. 'wanting'), perhaps due to altered dopamine function (Wyvell and Berridge, 2001) or, alternatively, a reduced 'liking' of the reinforcer, via some effect on opioid receptors (Richardson et al., 2005; Berridge et al., 2009).

Experiment 2 investigated the effects of NaB administration on the post-training consolidation of instrumental behaviour. Here, systemic administration of NaB at the end of each training session had no effect on response rates during the subsequent days' training, again highlighting the apparently acute nature of the training effects observed in Experiment 1. As in Experiment 1, neither overall rates of responding nor devaluation sensitivity were affected by NaB treatment.

The lack of effects of NaB on goal-directed responding, under conditions designed to favour effects on acquisition (Experiment 1) and consolidation (Experiment 2) of learning, may be attributable to a number of different reasons. Firstly, and most fundamentally, it may have been that the 0.6g/kg dose of NaB used here was simply not physiologically or behaviourally effective. As discussed in the introduction to this chapter, for welfare and scientific reasons, we used a lower dose than that used in the majority of published studies, and so it could be argued that the null effects found in Experiments 1 and 2 were attributable to the fact that this dose had no effect on HA levels in the brain. Nevertheless, other groups have reported both neurophysiological

and behavioural effects using this dose (see Table 5.1.1). For example Fontán-Lozano et al. (2008) found that systemic NaB, at a dose of 250mg/kg, significantly enhanced recognition memory in mice, as well as inducing an increase in histone H3 acetylation and IEG expression in the brain. Furthermore, our own data showing a specific, acute effect of NaB on lever press response rates indicates that this dose was behaviourally effective.

An alternative, but related, possibility is that this dose did not induce sufficient molecular changes in the brain to alter the nature of learning in this particular behavioural paradigm. A review of current literature reveals a large degree of variability in NaB dosage and experimental design across studies, suggesting that the effects of HDACis may be both dose- and task-specific (see Table 5.1.1, and especially Raybuck et al. 2013). It is intuitively attractive to speculate that the threshold to produce behavioural effects may be positively correlated with task complexity. In contrast to much of the literature in this field, which has relied on simple one trial learning paradigms (e.g. fear conditioning), the lever pressing task used here was relatively complex, requiring the encoding of both Pavlovian (i.e. magazine approach) and instrumental contingencies and the execution of a specific instrumental action (i.e. lever pressing), and it may be that a higher dose is needed to produce sufficient molecular changes to have manifest behavioural effects.

However, such arguments are challenged by the results of Experiment 3, where NaB-treatment, prior to test, reduced behavioural sensitivity to outcome devaluation. In this experiment, whilst Saline-treated control animals exhibited a significant decrease in responding for a devalued reinforcer, NaB-treated animals did not show such a devaluation effect in either their lever press or magazine entry behaviour.[2]

[2]Of course, one caveat in interpreting the nature of this effect is the fact that the simple effects comparisons upon which this discussion is based were neither planned nor were they conducted in order to further interrogate a significant statistical interaction and as such are technically inappropriate. However, it was felt that, given the consistency of the data collected in the two earlier

Importantly, consumption test data confirmed that the devaluation procedure was successful in reducing the motivational value of the sucrose pellets reinforcers in NaB-treated animals, and that the strength of the conditioned taste aversion in this group did not differ from that of the control group. Furthermore, there were no differences in overall response rates (on the lever or in magazine approach) between the NaB- and Saline-treated groups, and their post-test locomotor activity levels were comparable, indicating that any statistical differences in outcome devaluation sensitivity could not be attributed to general behavioural hypo- or hyper-activation effects.

As described in section 1.1, according to the current consensus view in the field of animal learning, insensitivity to outcome devaluation is a feature of habitual responding, reflecting, as it does, the cognitive and behavioural independence of instrumental actions from their consequences (Dickinson et al., 1995). Thus, one interpretation of the effects of NaB on the sensitivity of instrumental behaviour to outcome devaluation is that the HDACi produced molecular changes in the brain which facilitated the *expression* of habitual behaviour. Evidence from lesion studies indicates that, rather than being learnt in serial, the associative structures underlying goal-directed and habitual behaviour are acquired, essentially, in parallel, with executive mechanisms regulating the ultimate expression of one response style over the other (Killcross and Coutureau, 2003). Since here, NaB was given post-training, we can assume that learning was comparable between the groups and thus, the effects of drug-treatment can be interpreted as reflecting a change in the processes mediating the co-ordination of instrumental behaviour in extinction.

studies, the discrepancy between the large numerical difference in lever press responding between devalued and non-devalued saline-treated animals and the much smaller absolute devaluation effect seen in NaB-treated animals warranted further attention. Clearly this, putative effect needs to be verified through replication in ongoing work and, as such, this discussion of this effect should be treated with a degree of caution.

It is not possible to make any confident mechanistic interpretation of these data, but we can speculate about possible explanations for the results. Others have shown a similar acceleration of habit formation following treatment with psychostimulant drugs such as cocaine and amphetamine (Nelson and Killcross, 2006; Zapata et al., 2010; LeBlanc et al., 2013) and this effect has been attributed to alterations in forebrain dopamine (DA) transmission (Faure et al., 2005; Costa, 2007; Belin et al., 2009; Nelson and Killcross, 2013). Furthermore, there is evidence that HA is involved in regulating DA signalling and the downstream neurophysiological and behavioural effects of DA transmission (Mattson, 2003; Gozen et al., 2013; Jordi et al., 2013) and a number of HDACis (including NaB) have been shown to alter dopamine system function when administered systemically (Schroeder et al., 2008; Kidd and Schneider, 2010). Therefore, it is possible that the effects of NaB found here were caused by an epigenetically mediated change in the DA-dependent neuronal mechanisms underlying the co-ordination of actions and habits.

Statistical issues aside (see footnote), clearly, the tentative interpretation of the data presented here requires further investigation. In particular, it will be necessary to confirm that these results are indeed attributable to changes in histone acetylation and are not, in fact, the result of non-specific pharmacological effects of NaB on behaviour (Zovkic and Sweatt, 2013). One obvious fist step will be to assess the nature, degree, and brain-regional localisation of any drug-induced molecular changes using a combination of biochemical and genomic techniques to analyse samples of brain tissue taken from animals treated with the same drug administration protocol as that used in the current experiments (see Chapter 7 for a more in depth discussion of planned molecular work). Furthermore, whilst evidence of drug-induced changes in HA will be important, arguments for the causal relevance of these changes in the observed behavioural effects could be further strengthened by converging evidence from different approaches to manipulating acetylation levels in the brain, for ex-

ample genetic knock out models, or by using different HDACi compounds. Given that different HDACis target different classes of HDACs (Delcuve et al., 2012), this last point has wider pertinence in a mechanistic context. Indeed, by using different drug compounds to manipulate HA in the brain, it may be possible to gain a deeper understanding of the precise nature of the acetylation changes underlying these behavioural effects (again, see Chapter 7 for further discussion).

Interestingly, in Experiment 3, devaluation sensitivity was reinstated, for lever press responding at least, during the reinforced reacquisition test in NaB-treated animals. Such reinforced tests are often used as a way of confirming conditioned taste aversion in drug manipulation studies. The facilitation of habit formation by pharmacological manipulations is often reversed following re-exposure to the devalued outcome (Nelson and Killcross, 2006). However, Nelson and Killcross (2013) have also shown that the lever press performance of amphetamine-sensitized animals treated with the D2 receptor antagonist, eticlopride, remained habitual during a reinforced reacquisition test. The authors interpret this finding as evidence of elevated habit strength characteristic of compulsive behaviour and attributable to a fundamental dysfunction of normal instrumental learning systems. Thus, the relative susceptibility of drug-induced behavioural effects to outcome re-exposure can provide an insight into the nature of the changes induced by a given manipulation. The reacquisition of goal-directed instrumental behaviour seen in the current experiment is consistent with the idea that, rather than inducing permanent changes in brain function, the effects of NaB-treatment on instrumental behaviour are plastic, perhaps mediated by altered neurotransmitter signalling or through mutable changes in synaptic activity.

Taken together, the results of this set of experiments point to learning stage-dependent effects of NaB, which are independent of any general changes in levels of behavioural arousal or motivation. Specifically, whilst the initial learning of a goal-directed instrumental lever press response (i.e. acquisition and consolidation) was not affected

by systemic-NaB administration, NaB given at the point of memory retrieval reduced the sensitivity of both lever press and magazine entry behaviour to outcome devaluation, suggesting that HA may be involved in the the executive co-ordination of instrumental behaviours.

5.7 Chapter summary

- This chapter presents data from three separate studies which examined the role of post-translational histone modifications in the transition from goal-directed to habitual responding using a systemically delivered HDACi, sodium butyrate (NaB), to manipulate endogenous histone acetylation at different stages during instrumental learning.

- In Experiment 1, NaB was administered 30 minutes prior to each of the three RI30 lever press training sessions with the aim of targeting the *acquisition* phase of learning .

- Experiment 2, the consolidation phase of learning was targeted with NaB being administered at the end of each RI30 training session.

- In both Experiments 1 and 2 NaB had no effect on the sensitivity of lever press behaviour to outcome devaluation, suggesting that histone acetylation, as influenced by systemic NaB, is not involved the regulation of goal-directed and habitual responding at either the acquisition or the consolidation phase of instrumental learning.

- Experiment 3 explored the role of HA in the retrieval and expression of a previously acquired instrumental response by administering NaB 30 minutes prior to extinction testing.

- NaB reduced the sensitivity of lever press responding to outcome devaluation. One possible explanation for this finding is that histone acetylation plays a necessary role in the retrieval and / or execution of previously learnt action-outcome contingencies.

Chapter 6

Effects of microinfusions of Sodium Butyrate into the medial prefrontal cortex: Exploratory investigations

6.1 Introduction

The results of the last experiment described in Chapter 5 showed discrete effects of systemic administration of the general histone deacetylase inhibitor (HDACi), sodium butyrate (NaB), on lever pressing behaviour of minimally trained rats; specifically, when given prior to extinction testing (but not when given pre-training or immediately post-training), NaB reduced the sensitivity of instrumental lever press responding to changes in outcome value. In the context of contemporary learning theory, which posits multiple dissociable systems driving instrumental behaviour (Dickinson, 1985), this finding might be interpreted as evidence that NaB, in some way, biases the executive control of instrumental lever press responding in favour of stimulus-response associations, causing an otherwise goal-directed action to become habitual. As described in section 5.6, this interpretation should be treated with a degree of caution and additional behavioural and molecular investigations are required before the mechanistic processes underlying this effect can be fully understood. One

particular difficulty with interpreting the results of systemic pharmacological studies is the lack of spatial specificity afforded by this approach. Direct administration of drug compounds into the brain, via microinfusion, enables the targeted pharmacological manipulation of discrete brain regions and can, in principle, offer information about key brain areas underlying systemic effects (Rauch et al., 2008).

As discussed in section 1.2, lesion studies have provided a large amount of information regarding the brain regions and circuitries involved in the control of instrumental actions and have implicated a number of cortical and subcortical regions (see Table 6.1.1 for a brief summary of this work). Notably, subregions of the dorsal striatum and the medial prefrontal cortex (mPFC) appear to play dissociable roles in the acquisition and co-ordination of goal-directed and habitual behaviours, with the posterior dorsomedial striatum (pDMS) and prelimbic (PL) cortex being required for goal-directed responding and the dorsolateral striatum (DLS) and infralimbic cortex (IL) being required for habitual behaviour (Killcross and Coutureau, 2003; Yin and Knowlton, 2006). Interestingly, *post-training* inactivation of the IL reinstates goal-directed performance in extensively trained animals, implying that the transition from goal-directed to habitual responding is not a one-way, absolute process (Coutureau and Killcross, 2003), and that instead, the associative structures underlying the two types of behaviour may be acquired in parallel and it is their relative influence over behavioural output that changes across the course of training. This notion is intuitively attractive given the need for behavioural flexibility in order to modify well practiced actions in response to changing motivational and environmental demands.

An obvious question then, is what are the mechanisms which mediate the experience-dependent control of instrumental behaviour? In the context of the findings presented in Chapter 5, it is possible to hypothesise that the behaviourally specific effects of post-training, systemic administration of NaB on the expression of goal-directed

responding were caused by some neurophysiological change which altered the executive processes co-ordinating the relative influence of acquired action-outcome and stimulus-reposnse associations over manifest behavioural output. Therefore, in order to investigate this hypothesis further, in the current experiment, NaB was infused directly into the IL cortex prior to extinction testing.

	Under-training (i.e. Goal directed)		Over-training (i.e Habitual)		
Region	Pre-training	Post-training	Pre-training	Post-training	Reference
DLS	Goal directed [n/a]	Goal directed [n/a]	**Goal directed [1]**	Goal directed [?]	1. Yin et al. (2004)
pDMS	**Habit [2]**	**Habit [2]**	Habit [1]	Habit [n/a]	1. Yin et al. (2004) 2. Yin et al. (2005)
mPFC	**Habit [1]**	Goal directed [1]	Habit [?]	Habit [?]	1. Ostlund and Balleine (2005)
PL	**Habit [2]**	Goal directed [2]	Habit [1]	Habit [?]	1. Killcross and Coutureau (2003) 2. Tran-Tu-Yen et al. (2009)
IL	Goal directed [1]	Goal directed [?]	**Goal directed [1]**	**Goal directed [2]**	1. Killcross and Coutureau (2003) 2. Coutureau and Killcross (2003)

Table 6.1.1: A summary of the reported effects of pre- and post-training lesions and temporary inactivation of the DLS and DMS and PL and IL highlighting the dissociable roles these regions play in the regulation of instrumental behaviour. Experimental effects (i.e where the null hypothesis was rejected) are in bold text and [?] refer to untested assumptions. DLS: dorsolateral striatum; IL: infralimbic cortex; mPFC: medial prefrontal cortex; pDMS: posterior dorsomedial striatum; PL: prelimbic cortex.

Whilst this experiment was of interest in its own right, in examining specific hypotheses raised by the data in Chapter 5, it also provided an opportunity to address some of the practical limitations of systemic pharmacological manipulations. Hence, as well as beginning to investigate the neural substrates underlying the observed effects of systemic NaB on lever pressing behaviour using direct drug delivery into the brain, the present study also tested the efficacy of NaB in changing histone acetylation (HA) and, additionally, the extent to which NaB may cause off-target (possibly damaging) effects alongside any on-target chromatin modifications.

Thus, the experiment presented in this chapter was designed to be an exploratory

investigation into the factors discussed above and represents the initial stage of what is intended to be a series of studies examining the effects of target epigenetic manipulation of discrete brain regions of interest on instrumental behaviour.

6.2 Materials and methods

6.2.1 Initial molecular analyses

Preliminary analyses were conducted on NaB-treated tissue to investigate some of the molecular effects of the planned infusion protocol. The procedural details and data from these analyses can be found in Appendix D. To summarise, Western blot analysis of IL tissue samples revealed that, following microinjection, NaB had effects on H4K5/8/12/16ac, with NaB-treated tissue showing an increase level of acetylated H4 than artificial cerebrospinal fluid (aCSF) treated tissue. However, there was also evidence of elevated H4K5/8/12/16ac in the more dorsal PL region, suggesting an upward spread of NaB from the infusion site. However, the Western blot analysis was not sufficiently sensitive to detect any potential qualitative or quantitative differences between these two regions and it will be important to establish whether or not the PL shows levels of H4ac that are comparable to IL samples, using more sensitive molecular measures such as enzyme-linked immunosorbent assays (ELISAs) or immunohistochemistry. It is notoriously difficult to prevent the upward spread of drugs from the infusion site, especially with regions as small and as close together as these mPFC subregions. Therefore, the best possible solution to this issue may be to test whether or not the IL shows significantly higher NaB-induced effects on histone acetylation than the PL. It will also be necessary to probe different acetylation marks (e.g. H3ac) to see if the drug manipulation effects all HA modifications equally. Nevertheless, despite these limitations these preliminary findings were important as they showed a demonstrable effect of NaB infusions on histone acetylation in the brain,

data that is often lacking in behavioural studies using histone deacetylase inhibitors (HDACis).

Western blot analysis also showed that activation of the apoptosis marker protein, Caspase-3 (Casp-3; Porter and Jänicke, 1999), was relatively lower in NaB-treated tissue relative to aCSF-treated samples, suggesting that NaB infusions may have helped to ameliorate some of the damaging effects of the microinfusion procedure. However, levels of phosphorylated histone H2AX (a variant of histone H2A), a sensitive marker of DNA damage (Sharma et al., 2012), were elevated in NaB-treated tissue samples. Taken together these findings give a mixed picture of the adverse effects of NaB infusions into the IL. It is possible that the relatively lower levels of pre-apoptotic cells in NaB-treated samples indicated by the Casp-3 results were merely a reflection of the fact that these samples contained a higher number of necrotic cells, and therefore fewer apoptotic cells. Alternatively, it could be that, whilst NaB may cause more damage to cells near the infusion site (perhaps due to the pH of the solution or because of the animals response to the infusion[1]), NaB may have had neuroprotective effects at regions more distal to the infusion site. Clearly it is not possible to distinguish between these hypotheses on the basis of the data presented in this early pilot work but the implications of these findings will be discussed below in section 6.4.

[1]Indeed, the experimenter did observe that NaB-treated animals tended to move around more during the infusion which may have led to an increase in the physical damage caused by the infusion cannula.

6.2.2 Subjects

32 naive, male Lister Hooded rats, supplied by Charles River (Margate, Kent, UK), with a mean weight of 284.9g (range: 257-322g), were used in the behavioural experiment. Before the start of instrumental training all animals were food restricted to above 80% of their *ad libitum* feeding weight and maintained on a restricted diet for the remainder of the experiment. At the end of testing the cohort had a mean weight of 330.3g (range: 296-374g). All other animal husbandry details were as described in section 2.1.1.

6.2.3 Surgical procedures

Animals were anaesthetised using gaseous isoflurane and placed in a Kopf stereotaxic frame (Kopf Instruments, Tujunga, CA) in a flat skull position. Bilateral, 26-gauge stainless steel guide cannulae (Plastics One Inc Roanoke, VA), extending 2mm below the base of the pedestal, were implanted, through a single pre-drilled hole, at the following co-ordinates (in mm from Bregma): A-P (anterior-posterior) +2.6 and ML (medial-lateral) ±0.6. Three stainless steel anchoring screws were inserted into the skull surface and dental cement was used to hold cannulae in place. All animals were monitored daily for signs of pain and distress (using a scoring system developed by Lloyd and Wolfensohn, 1998) and given *ad libitum* food access for at least 7 days post-surgery.

6.2.4 Microinfusions

Approximately 24 hours after the final devaluation session, *NaB*-treated animals received two bilateral infusions of 0.5μl of sodium butyrate solution (1mg NaB: 1ml aCSF) targeting the infralimbic cortex, with 24 hours between each infusion. The guide cannula was inserted at a depth of 1.6mm from brain surface (dorsal-ventral: -1.6mm). A 33 gauge infusion cannula, which projected 3mm beyond the guide cannula[2], was inserted into each guide cannula and infusions were administered at a

[2]Therefore final depth of infusion site was 4.6mm

rate of 0.25μl/min for two minutes. The cannulae were left in place for one minute after infusion to ensure the liquid bolus was fully absorbed. The remaining 16 animals received bilateral infusions of 1 aCSF. Extinction testing took place 30 minutes after the final infusion.

6.2.5 Behavioural procedures

All animals completed the two pre-training sessions (i.e. magazine training and continuous reinforcement lever press training; see section 2.3.2) prior to surgery. The basic training procedure and extinction test was as described in Chapter 3, Experiment 1. Outcome devaluation and the consumption test were conducted as described in Chapter 3, Experiment 2.

6.2.6 Histology

At the end of behavioural testing animals were killed by exposure to rising concentration of CO_2. Their brains were rapidly removed and one hemisphere was dissected for future molecular analysis. The remaining hemisphere was post-fixed in 10% formal saline before being transferred to 25% sucrose solution for at least 24 hours. 40μm thick coronal sections of frozen tissue were then cut from each of the fixed brain hemispheres using a cryostat and mounted on gelatine coated slides. The slices were stained using cresyl violet and inspected for cannula placement and any obvious cell damage resulting from infusions.

6.3 Results

Three animals were excluded from statistical analysis, two due to poor cannula placement and one due to poor training performance (i.e. earned $<$ 4SD from the mean number of rewards across all RI30 training sessions; as described in section 2.5.3). Final group sizes are shown in Table 6.3.1.

	Devalued	*Non-devalued*
Saline	8	8 *(6)*
NaB	8 *(7)*	8

Table 6.3.1: Groups size of the 4 experimental groups (values in brackets show group sizes after exclusions).

6.3.1 Histology

The two animals whose cannula placement was outside of target area were excluded from statistical analysis. Cannula placements for all other animals were as shown in Figure 6.3.1.[3]

[3]Note that although only one hemisphere was used to check cannula placement, because the bilateral cannulae used here were mounted on a single pedestal, it was possible to accurately estimate the placement of the canuulae in the other hemisphere.

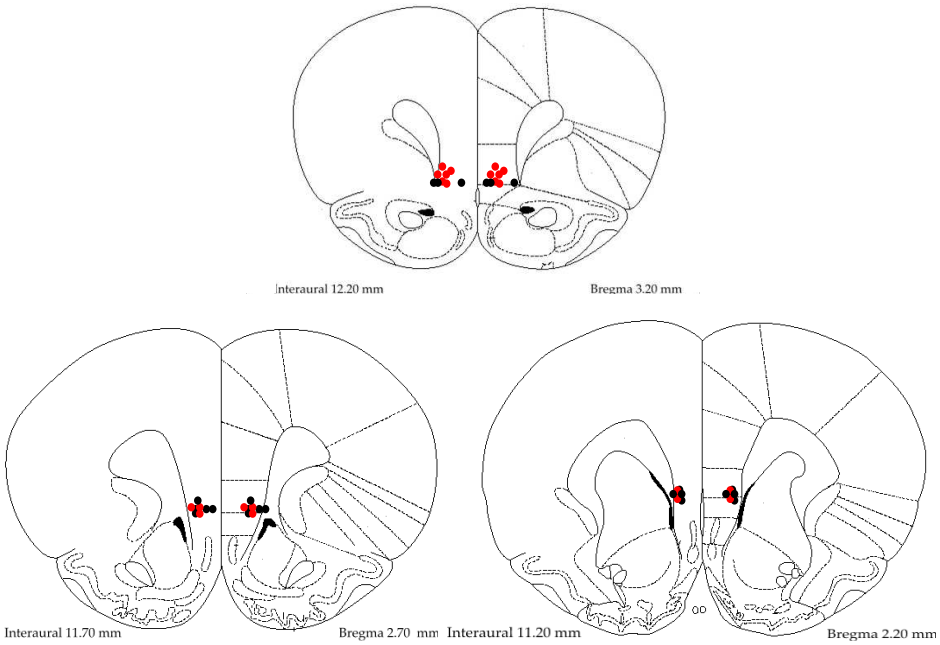
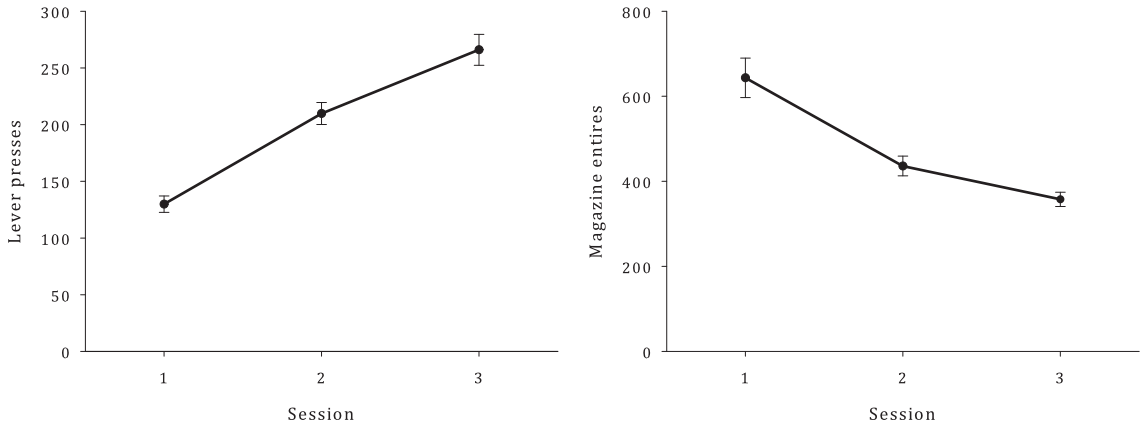


Figure 6.3.1: Summary of histological analysis. Schematic diagram showing area of medial prefrontal cortex (mPFC) within which the injection sites fell (images taken from Paxinos and Watson, 1998). Red spots represent NaB groups and black spots controls.

6.3.2 Training

Figure 6.3.2 shows the lever press responding and magazine entry behaviour across the three RI30 training sessions for all animals. Lever presses increased across the sessions (Main effect of SESSION: $F[2, 50] = 91.510$, $p < .001$; Figure 6.3.2 a), whilst magazine entries decreased across the training sessions (Main effect of SESSION: $F[2, 50] = 39.165$, $p < .001$; Figure 6.3.2 b). Taken together, these data indicated that all animals acquired the basic lever press response and learnt about the instrumental contingency as expected, reducing their magazine approach behaviour in favor of lever pressing. Furthermore, and importantly, there were no pre-existing differences in baseline response rates between the experimental groups ($p > .05$; see Appendix D, Table D.3.1 for mean data for all conditions).

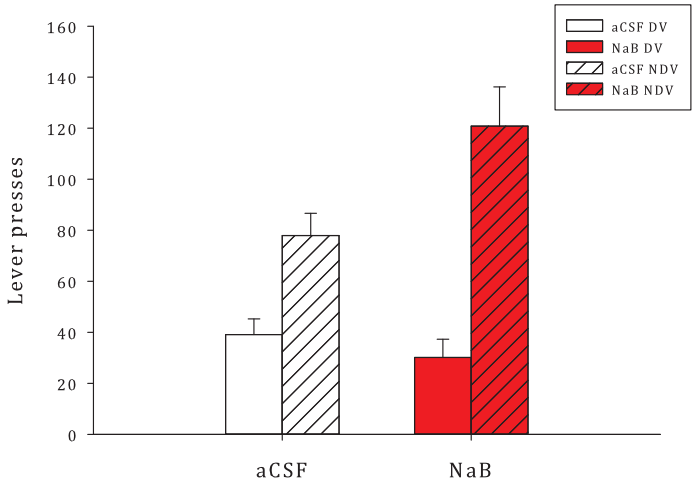


(a) Lever presses **(b)** Magazine entries

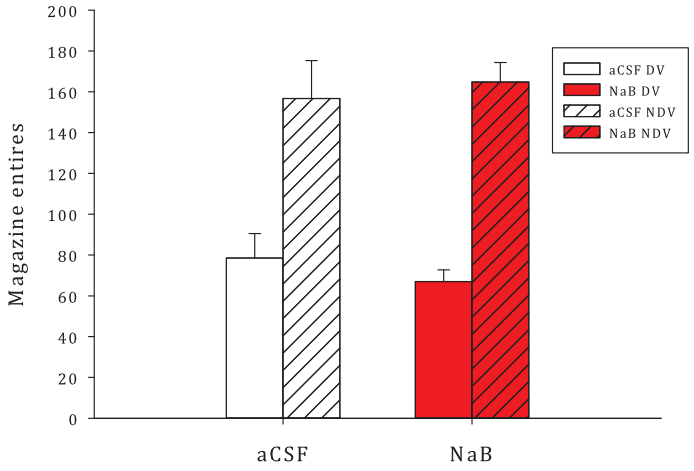
Figure 6.3.2: Mean number of lever press responses and magazine entries across training session. Total N= 29; error bars show standard error.

6.3.3 Test

Extinction. Figure 6.3.2 shows lever press response rates and magazine entry behaviour in extinction for the four experimental groups. There was a significant overall main effect of DEVALUATION for both lever presses ($F[1, 25] = 5.727$, $p = .025$) and magazine entries ($F[1, 25] = 55.197$, $p < .001$), with *Devalued* animals pressing the lever significantly less than *Non-devalued* animals, but there was no main effect of HDACi group ($p > .05$). Despite the fact that there was no evidence of a statistically significant interaction between the two factors (DEVALUATION x HDACi*:* $p > .05$), simple effects analysis revealed that, whilst there was a large devaluation effect in lever press responding in *NaB*-treated animals (Simple effect of DEVALUATION [NaB]: $F[1, 25] = 8.402$, $p = .008$), instrumental lever pressing in *aCSF*-treated animals was insensitive to outcome devaluation (Simple effect of DEVALUATION [aCSF]: $p > .05$). In contrast, both HDACi groups exhibited devaluation sensitivity in their magazine entry behaviour (Simple effect of DEVALUATION [NaB]: $F[1, 25] = 21.796$, $p < .001$; Simple effect of DEVALUATION [aCSF]: $F[1, 25] = 34.085$, $p < .001$).



(a) Lever presses

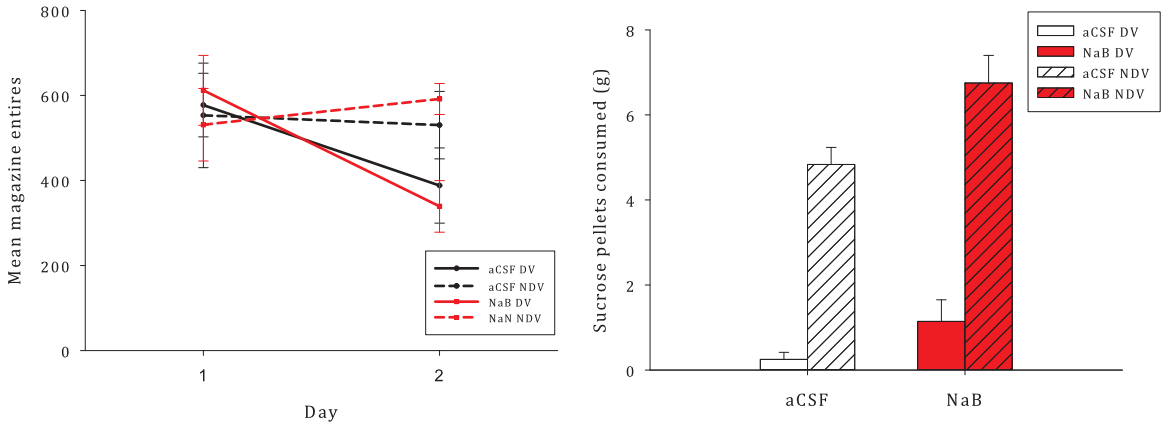


(b) Magazine entries

Figure 6.3.2: Mean number of lever presses (a) and magazine entries (b) during extinction test. Total N= 29; error bars show standard error.

Devaluation and consumption. A shown in Figure 6.3.3a, both *NaB* and *aCSF*-treated *Devalued* groups their reduced magazine entry behaviour following the first LiCl injection (SESSION x DEVALUATION group interaction: $F[1, 25] = 4.689$, $p = .04$) and the magnitude of this effect was the same for both NaB- and Saline-treated groups (SESSION x HDACi x DEVALUATION group interaction: $p > .05$).

At test, the *Devalued* groups consumed significantly less than *Non-devalued* groups (Main effect of DEVALUATION: $F[1, 25] = 114.030$, $p < .001$) and there was no difference in the size of this effect between the two HDACi groups (DEVALUATION x HDACi group interaction: $p > .05$; see Figure 6.3.3 b).



(a) Magazine entries across devaluation sessions

(b) Sucrose pellets eaten at consumption test (in grams)

Figure 6.3.3: Conditioned taste aversion: Mean magazine entries across devaluation sessions (a) and mean amounts of sucrose pellets eaten across consumption test (b). Total N= 29; error bars show standard error.

6.4 Discussion

The exploratory investigations presented in this chapter were designed to act as proof-of principle for the development of a procedure for infusing the HDACi, sodium butyrate (NaB), directly in to key brain regions of interest, at different points during instrumental learning. As discussed in the introduction to this chapter, such an approach compliments the systemic work presented in Chapter 5, not only by addressing some of the limitations of systemic pharmacological manipulations, but also by allowing us to test a number of different hypotheses. Here, guided by the results of Experiment 3 of Chapter 5,which showed an effect of systemic NaB on the expression of goal-directed behaviour when administered pre-extinction test, we

successfully infused NaB in to the infralimbic cortex (IL), a region known to be required for both the acquisition and expression of habitual behaviours (Coutureau and Killcross, 2003; Killcross and Coutureau, 2003), of minimally trained animals prior to the end of training.

The data suggest that NaB infusions enhanced the sensitivity of instrumental responding to outcome devaluation relative to vehicle treated animals, with NaB-treated groups significantly reducing responding for the devalued reinforcer. Importantly, in contrast to the systemic work presented in Chapter 5, in the present study, control animals failed to show the anticipated devaluation effect in extinction after three sessions of instrumental training. Since the data do point to a trend towards reduced responding in the devalued aCSF-treated animals it is possible that the lack of a statistically significant effect of devaluation in these groups was due to the increased variability inevitable in experiments involving invasive procedures such as these, although it is difficult to think of a reason why this would affect control groups more than NaB-treated animals. One possibility is that the implantation of microcannula into the IL caused physical damage to the more dorsal prelimbic regions, and in so doing disrupted the functional role this region plays in the acquisition of the action-outcome associations underlying goal-directed behaviour (Tran-Tu-Yen et al., 2009). It is a further possibility that NaB may have acted in some way to ameliorate the effects of this damage, perhaps by its anti-inflammatory properties, which have been evidenced elsewhere (Andoh et al., 1999; Kim et al., 2007). Indeed, Huang et al. (2011) have even shown evidence that NaB can reduce caspase-3 activation and enhance cell survival in TH-postive DA neurones pointing to a neuroprotective effect of NaB which is consistent with out own Western blot analysis (see Figure D.2.1).

Alternatively, it is possible that NaB infusions caused more significant damage than cannula insertion, and, since these effects are likely to have been greatest at the site of infusion, it may be that the degree of damage to this region influenced it's relative

control over behavioural input. Thus, the apparent enhancement of goal-directed behaviour in NaB-treated animals may actually reflect the fact that the IL, which is required for the expression of habitual behaviour (Coutureau and Killcross, 2003), had less influence over behaviour than the PL, which is required for the expression of goal-directed behaviour (Killcross and Coutureau, 2003), in these animals, compared to controls. These possibilities will need to be explored further using more in depth molecular analyses, such as those described above. It will also, obviously, be necessary to demonstrate devaluation sensitivity in control groups before progressing further with this work and in addition, address the possible interpretational issues that arise from the upward spread of drug beyond the the IL cortex and into the PL cortex. These issues are considered further in the General Discussion section, which follows immediately.

6.5 Chapter summary

- This chapter presents data from initial, exploratory investigations into the development of a protocol for infusing HDACis directly into functionally important brain regions.
- After the surgical implantation of microcannula targeting the infralimbic cortex animals were trained to lever press across three sessions of training, before receiving two microinfusions, spaced by 24 hours; 30 minutes after the final infusion animals were tested in extinction.
- Molecular analysis of mPFC tissue samples showed that NaB increased histone H4 acetylation and behavioural data revealed an enhanced sensitivity to outcome devaluation in NaB-treated animals relative to controls.

- The anticipated devaluation effect was not present in the aCSF-treated animals. This may have been due to physical damage to mPFC caused by the microinfusion procedure.

- These preliminary data suggest that NaB may have acted to reduce the damaging effects of the microinfusions and represent the first step in the development of a microinfusion protocol for studying the role of histone acetylation in instrumental learning.

Chapter 7

General Discussion

This thesis explored the molecular substrates of goal-directed and habitual behaviour using a combination of correlational and pharmacological intervention approaches. The following discussion provides a brief overview of the experimental findings before going on to examine some potential caveats and limitations of the experiments, as well as exploring some of the more interesting features of the data and describing how planned future experiments will aim to inform and extend the way in which these findings can be interpreted.

7.1 Summary of experimental chapters

At the outset it was important to develop and refine task parameters for modelling goal-directed and habitual behaviour in rats, in our hands. Therefore, in Chapter 3, groups of Lister hooded rats were trained to lever press across a variable number of sessions. The relative sensitivity and resistance of animals' instrumental lever press responding to lithium chloride (LiCl) induced outcome devaluation was used as an index of goal-directed and habitual responding respectively (Adams and Dickinson, 1981). In Experiment 1, two cohorts of rats, each from a different supplier, were given three sessions of instrumental training, after which they were assigned to

one of two "devaluation type" conditions. Analysis of response levels in extinction showed that only animals sourced from Charles River, in which outcome devaluation was conducted in the training context, exhibited sensitivity to the change in outcome value, reducing responding for a devalued reinforcer. Experiment 2 aimed to model habitual behaviour by extending the findings of Experiment 1. Here, after 10 sessions of instrumental training the responding, in extinction, of rats sourced from Charles River, was insensitive to outcome devaluation conducted in the training context. Taken together, the results of these two studies demonstrated a clear training-dependent shift in the nature of the associative structures underlying instrumental responding indicative of a transition from purposive, goal-directed actions to automatic, stimulus-driven habits (Adams, 1982), and provided the basis for the training protocol employed in the subsequent experimental chapters.

In Chapter 4, Affymetrix microarrays were used to investigate the molecular correlates of minimal and extended instrumental training. Based on the findings reported in Chapter 3, three groups of rats were trained to lever press across three, six and 10 sessions respectively and brain tissue from sub-sets of these training groups was submitted for comprehensive microarray analysis. Comparison of the gene expression profiles of tissue taken from dorsomedial and dorsolateral striatium (brain areas showing clearly dissociable function in goal-directed and habitual behaviours, respectively; Yin and Knowlton, 2006) with respect to training group revealed bidirectional, non-linear expression profiles indicative of qualitative brain region- and training group-dependent changes in gene regulation.

The series of experiments presented in Chapter 5 aimed to directly manipulate endogenous chromatin remodelling processes involved in the control of gene expression, using a systemically delivered drug compound, the general histone deacetylase inhibitor (HDACi) sodium butyrate (NaB). The three experiments included in this chapter were each designed to target a different component of instrumental learn-

ing by manipulating the point at which NaB was administered before and after lever press training and prior to extinction testing. In Experiments 1 and 2, which aimed to target the acquisition and consolidation phases of instrumental learning respectively, NaB had no effect on the lever press responding or magazine entry behaviour, with both NaB-treated animals and saline-treated controls significantly, and comparably, reducing responding for devalued reinforcer. However, in Experiment 3, which aimed to target the retrieval phase of learning by administering NaB 30 minutes prior to extinction test, the lever press responding of NaB-treated animals was insensitive to outcome devaluation, in contrast to control animals which exhibited the expected devaluation effect. The extent to which these results implicate histone acetylation (HA) in habit formation remains unclear in the absence of further experiments (see below for further discussion of planned future work) but, taken together, these findings point to experience-dependent, learning-stage specific effects of systemically administered NaB on the expression of a previously learnt, minimally-trained, instrumental response.

The study presented in Chapter 6 developed a protocol for infusing HDACis directly into the rat medial prefrontal cortex (mPFC). To address some of the limitations of the systemic drug studies presented in Chapter 5, most notably in terms of improving the spatial specificity of drug administration, microcannula were implanted to target the infralimbic cortex (IL) and subjects received two microinfusions of NaB (or aCSF) after lever press training and devaluation had been completed. Then, 30 minutes after the final infusion animals were tested in extinction and lever press and magazine entry behaviour was recorded. Western blot analysis showed an increase in histone H4 acetylation in mPFC samples from animals treated with NaB relative to aCSF-treated samples. In a complex set of data, microinjection into the IL cortex area did not recapitulate the behavioural effects of systemic NaB, administered at this stage of training, reported in Chapter 5. That is, instead of causing insensitivity

to outcome devaluation, goal-directed behaviour was if anything enhanced relative to aCSF-treated controls. However, the effects of NaB were accompanied by the failure of the control rats to show the anticipated devaluation effect in extinction after three sessions of instrumental training. Various explanations for this pattern of results were considered, including the possibility that NaB was in some way reducing the damaging effects of the microinfusions. This conclusion is backed up, to an extent, by data indicating less damage in NaB treated tissue samples, as indexed by a molecular marker of apoptosis. A further complication to the interpretation of the data from Chapter 6 was evidence of the spread of NaB beyond the confines of the IL cortex into the overlying prelimbic (PL) cortex. Clearly, a number of practical issues highlighted by this work (discussed further below) will need to be addressed before it will be possible to fully exploit the advantages offered by targeted drug manipulations of specific brain regions using microinjection methods.

7.2 Differential gene expression patterns in striatal subregions associated with minimal and extended instrumental training

Biological verification of the microarray calls. An important issue in the interpretation of the dissociable patterns of gene expression seen in striatal subregions following minimal and extended instrumental training is the extent to which the microarray calls can be verified. For those genes tested in the current work, qPCR analysis of the same RNA samples as were used for the microarrays was broadly consistent with the Affymetrix data. However, this so called 'technical verification' only provides information about the reliability of the data obtained from a specific array experiment (i.e a specific set of tissue samples). In order to fully test the *validity* of array data, 'biological verification', using samples of tissue taken from different animals,

exposed to the same conditions, is required. For this reason, as noted in section 4.2.4 tissue samples were taken from a total of 12 animals, leaving us with additional samples that were not submitted for microarray. We plan to use these samples for qPCR analysis in order to provide biologically independent information about the expression profiles of key candidate genes highlighted by the microarray data. In addition, if candidates are chosen carefully, this qPCR analysis may also help to build up a picture of the functional mechanisms reflected in the observed transcriptional changes. For example, it may be possible to further explore the immediate early gene (IEG) effects, discussed in Chapter 4, to see if the apparent down-regulation of a number of IEGs in the posterior dorsomedial striatum (pDMS) at the start of training is evident in biologically independent samples. Furthermore, because these samples would only be used for qPCR analysis, there would be more RNA available, allowing us to look at the expression patterns of a greater number of genes than was possible for the technical verification. Of course, the strongest form of verification would come from experimental replication. This wouldn't necessarily need to involve another set of microarrays; instead we could run the same behavioural paradigm but analyse the tissue samples using only qPCR, or, indeed, a combination of qPCR and other molecular analyses (see below for further discussion of what potential analyses could be used and what they may tell us).

Relevance of gene expression changes to goal-directed and habitual behaviour. As already explained at length in Chapter 4, with the design employed in this study it was not possible to explicitly test responding in extinction for sensitivity to outcome devaluation using the LiCl-induced taste aversion protocol developed in Chapter 3, meaning any interpretation of the data must be couched strictly in terms of length-of-training-effects; although it is important to remember that the training protocols developed in the thesis gave rise to robust behavioural dissociations (presented in Chapter 3). One possible way of matching more exactly the molecular and be-

havioural effects would be to run an experiment in which animals are trained as in Chapter 4, but then go on to complete the devaluation and extinction test stages, described in Chapter 3, before being culled. This would provide us with a behavioural index of the nature of instrumental responding at the point of dissection that would help to inform our interpretation of any molecular findings. Furthermore, by comparing any interesting effects with the results of the microarray experiments, it may be possible to parse any effects specific to the training manipulation from those related to outcome devaluation and/or extinction testing. However, if devaluation of the reinforcer was achieved using LiCl, which is neuroactive in itself (Kwon and Houpt, 2010), appropriate controls would be needed to separate out any molecular effects due to training from LiCl-related effects. Looking further ahead, it will be interesting to run additional experiments in which a reacquisition test is also conducted prior to dissection. Since re-exposure to the instrumental contingency has been shown to cause habitual responses to become goal-directed (Nelson and Killcross, 2006), such an experiment may help to further inform our interpretation of the microarray data by allowing us to compare the gene expression profiles of tissue taken from two groups of extensively trained animals, one expressing habitual behaviour at the time of dissection and the other expressing goal-directed behaviour having been re-exposed to the instrumental contingency.

Epigenetic control of gene expression. The training-dependent changes in gene expression, detailed in Chapter 4, clearly represent just one stage in a complex chain of neurophysiological processes underlying the switch from goal-directed behaviour to habits that occurs following extended training. The epigenetic control of gene expression in the brain is being increasingly viewed as an important mechanism linking the effects of exogenous experience to endogenous neuromolecular changes, and the eventual downstream consequences of these changes on brain function and behaviour. Therefore, in future studies it may be possible to build upon the microarray data

by looking at the epigenetic changes which parallel the experience-dependent, and brain-region specific changes in gene expression highlighted by this work.

Given the increasing evidence of behavioural effects resulting from manipulations targeting HA processes (Gräff and Tsai, 2013), an obvious starting point for epigenetic analysis would be to investigate the effects of minimal and extended training on HA in the same striatal subregions used in the microarray study. This extension of the correlational approach, adopted in this chapter, could use a number of methods. Initially, it would be important to assess the extent to which HA is sensitive to the training regimen by comparing acetylation levels between the training groups using Western blotting or enzyme-linked immunosorbent assays (ELISA), with antibodies specific for a particular HA mark (e.g. H3ac). It would then be possible to use chromatin immunoprecipication (ChIP) techniques to look at the genomic regions associated with any training-dependent acetylation changes. Such an approach would allow us to directly compare the histone modification data with the microarray findings, potentially allowing us to begin to attribute training induced gene expression changes to specific epigenetic mechanisms.

7.3 Manipulating instrumental behaviour with the HDAC inhibitor Sodium Butyrate: systemic effects

The results presented in Chapter 5 point to highly specific behavioural effects of systemic sodium butyrate (NaB), administered prior to extinction testing, on the sensitivity of instrumental lever press behaviour to outcome devaluation. The reduced sensitivity to devaluation seen in NaB-treated animals can be interpreted, cautiously, as evidence that general inhibition of histone deacetylase (HDAC) activity disrupted

the mechanisms involved in the retrieval of the action-outcome associations underlying goal-directed responding. As yet, very little is known about the molecular processes underlying habit formation and even less about how these process interact at the different stages of instrumental learning to determine behavioural output and, therefore, it would be premature to speculate about the possible mechanisms mediating this effect at this stage. Nevertheless, these findings provide some valuable insights and there are a number of avenues that can be pursued in future work in order to gain a better understanding of potential mechanisms.

Temporal specificity: Learning stage dependent effects. One of the most interesting facets of the data presented in Chapter 5 is the temporal specificity of the behavioural effects observed; that is, the learning-stage dependency of the effect of NaB on the sensitivity of lever press responding to outcome devaluation. Systemic NaB had no effect on outcome devaluation sensitivity when administered during training (both pre- and post-training session), suggesting the drug had no effect on the acquisition or consolidation of instrumental learning. As discussed above, the fact that a change in outcome devaluation sensitivity was only apparent when the drug was given 30 minutes prior to extinction testing, may be evidence that the drug affected the retrieval of the associative structures driving goal-directed behaviour.

Alternatively, it could be the case that these effects reflected some non-specific effect of NaB on off-target behavioural or cognitive processes, indirectly, or even entirely unrelated, to habit formation. For example, acute NaB administration has been shown to enhance the expression of behavioural indices of anxiety and depression (Gundersen and Blendy, 2009) and increased stress has been linked to impairments in goal-directed behaviour (Schwabe and Wolf, 2011). Thus, if NaB had an acute anxiolytic effect, this may have affected the rats' ability to retrieve the action-outcome associations underlying goal-directed responding. Alternatively, and perhaps more pertinent to the current work, is evidence that NaB alters the strength

LiCl-conditioned taste aversion (CTA) learning (Kwon and Houpt, 2010). However, the strength of the CTA did not differ between the two treatment groups, as shown by the consumption test data. Furthermore, the sensitivity of lever press behaviour to outcome devaluation was reactivated during the reacquisition test indicating that NaB animals learnt the CTA to the same extent as controls. Finally, given that the levels of lever press responding and magazine entry behaviour of non-devalued animals were comparable between NaB-treated animals and controls, as well as the fact that NaB administration had no effect on levels of general locomotor activity, it is difficult to imagine the nature of a general physiological effect that could give rise to such specific behavioural effects.

It is important to note here that, as well as providing evidence that NaB did not alter the strength of the CTA, the reactivation of outcome devaluation sensitivity during the reacquisition test suggests that the circuitry controlling goal-directed behaviour was reversibly influenced by the drug treatment. A similar, transient enhancement of habitual behaviour has been shown following amphetamine sensitization (Nelson and Killcross, 2006) and suggests that, rather than degrading the action-outcome associations underlying goal-directed responding, NaB treatment may have biased the executive processes regulating instrumental responding in favor of the retrieval of stimulus-response associations rather than action-outcome associations, a change which was overwritten by re-exposure to the instrumental contingency during the reacquisition test.

Molecular specificity: Histone acetylation and beyond. Leaving the questions regarding the nature of the psychological effects of NaB treatment aside, the issue of on- and off target effects is highly pertinent when considering the nature of the molecular effects of NaB, and again, here, several questions will need to be addressed in future work.

First and foremost, it is important to consider any potential off-target effects (i.e. non-histone acetylation effects) that could account for the pattern of behavioural effects seen here. One possibility is that NaB had damaging effects on the brain which may have resulted in the gross dysfunction of the circuitries controlling goal-directed behaviour. However, as noted previously, the highly specific behavioural effects found here are inconsistent with general damaging effects. Furthermore, as discussed above, the reinstatement of goal-directed behaviour following the reacquisition session presents a significant challenge to such arguments. Added to this is the molecular data from the microinfusion experiments, discussed in more detail below, which, although complex, suggests NaB may in fact have some neuroprotective effects, a finding which has also been reported by others (Andoh et al., 1999; Kim et al., 2004; Huang et al., 2007; Kim et al., 2007).

Secondly, it will be necessary to confirm whether the systemic drug manipulation had the anticipated molecular effects; in other words, was the dose of NaB used in the current experiments effective in altering HA in key brain regions of interest. In the experiments presented in this thesis we used a lower dose of NaB than the doses often used in behavioural epigenetic studies (Reolon et al., 2011; Stefanko et al., 2009), making questions regarding the effects of the drug on HA in the brain particularly relevant. The decision to use a dose half the strength of that used in many of published studies was made following pilot work in our laboratory showing adverse effects of this higher dose. Not only did this dose cause distress to the animals upon administration, making it unsuitable for use on welfare grounds, it also led to a period of prolonged hypoactivity following administration, making it inappropriate to use on scientific grounds. Therefore, we opted to use a dose that was well within the range shown by others in the literature to have both behavioural effects and, importantly, effects on histone acetylation in the brain (Fontán-Lozano et al., 2008; Hui et al., 2010; Kwon and Houpt, 2010; Raybuck et al., 2013), but

which didn't produce the adverse effects seen with the higher dose. Of course, it will still be important to establish, in our own hands, whether or not this dose, when given systemically, is effective in altering HA in brain. In fact, we have started to run some preliminary Western blot analyses on samples of systemic NaB treated tissue; however the antibodies used were not sensitive enough to detect any qualitative differences in HA between NaB-treated samples and control tissue and this work is ongoing. For example, we plan to use more sensitive techniques (e.g. ELISA analysis) in the future.

Thirdly, as well as looking for global changes in HA, it will be important to try to ascertain the specific nature of any NaB-induced acetylation marks. Much of the work conducted in this field has relied on one-trial learning paradigms, such as contextual fear conditioning, and it will be important to attempt to identify the specific acetylation marks associated with appetitive instrumental learning. As shown in Table 7.3.1, learning and memory processes have been linked to a number of different acetylation marks. The procedures developed in this thesis mean that, in the future, it will be possible to combine evidence from manipulation studies (i.e. systemic delivery and infusion of HDACis), where behavioural effects of drug administration can be matched to molecular changes, with evidence from correlation studies, such as the one described in Chapter 4.

It will also be possible to further explore any putative involvement of HA in the expression of instrumental behaviour by using the same behavioural paradigm to investigate the effects of different HDACi compounds. Following the burgeoning interest in the therapeutic potential of drug compounds with HDACi properties, there has been a focus on developing more targeted HDACis, and now there are a number of drugs available which block the activity of specific HDAC isoforms (Weïwer et al., 2013). As shown in Table 7.3.1, the role of the various HDAC isoforms in brain function is highly complex and therefore, in the future, any attempts to

explore the epigenetic substrates of learning and memory will need to consider the specificity of the manipulation used.

Acetylation Mark	Acetylating enzyme	Deacetylating enzyme	Learning induced HA changes
H2A (K5 K7)	CBP/p300, HAT1, MYST2	HDAC1-2	Reduced in CBP mutant mice (which show impaired recognition memory; Valor et al., 2011)
H2B (K5, K12, K15, K20)	CBP/p300, GCN5	HDAC1-2 HDAC4-7	Induced after MWM and CFC Reduced in CBP mutant mice (Alarcon et al., 2004; Valor et al., 2011) Induced after CTA learning
H3 (K9, K14, K18, K23, K27, K36)	K9: GCN5/pCAF K14: CBP/p300, GCN5/pCAF K18: CBP/p300, GCN5/pCAF K23/K27: GCN5	HDAC1-2 HDAC8 K9: SIRT1	Induced after LTF in Aplysia (Guan et al., 2002) Induced 1 hour after CFC (Levenson et al., 2004) Induced after CuFC in amygdala (Monsey et al., 2011) Reduced in CBP mutant mice (which show impaired recognition memory; Valor et al., 2011) K9, K14: Induced 1 hour after CFC (Peleg et al., 2010)
H4 (K5, K8, K12, K16, K20)	MYST2 K5, 8, 12: CBP/p300, HAT1 K16	HDAC1-2 K16: SIRT1	K12: Induced after MWM and CFC (Bousiges et al., 2010) Not induced after CuFC or CFC (Levenson et al., 2004; Monsey et al., 2011) Induced after latent inhibition (Levenson et al., 2004) K5, K8, K12: Induced 1 hour after CFC (Peleg et al., 2010)

Table 7.3.1: Summary of key histone marks shown to be altered by long-term memory formation. Abbreviations: CBP - CREB-binding protein, CFC - contextual fear conditioning, CuFC - Cued fear conditioning, MWM - Morris water maze (spatial learning), CTA - conditioned taste aversion, LTF - long term facilitation. Table adapted from Peixoto and Abel (2013).

Similarly, as well as exploring the specific HDACs involved in the regulation of instrumental learning, it will also be interesting to investigate the effects of manipulating histone acetyltransferase (HAT) activity. This approach would enable us to look for potential double dissociations in the behavioural effects of drug compounds with oppositional effects on histone acetylation (i.e. HDACis and HAT inhibitors). Indeed, although to date HAT inhibitors (HATi) have received far less attention than HDACi, several recent studies have been published indicating that drugs with HATi activity can have behavioural effectsMaddox et al. (2013).

Moving forward, there is the potential to move away from HA mechanisms specifically, and modifications to chromatin histone residues in general, to focus on other epigenetic modifications. For example, interest is growing in the role of DNA methyltransferases (DNMTS; enzymes which regulate the methylation of DNA cytosine residues, a form of molecular modification typically associated with genomic silencing McCabe and Caudill, 2005), in learning and memory processes (Miller and Sweatt, 2007; Day and Sweatt, 2010). Furthermore, the existence of DNMT inhibitors with a high degree of bioavailability enables the enzymatic regulation of DNA methylation to be manipulated *in vivo* (Lubin et al., 2008), providing the opportunity to conduct similar experiments to those described here examining the role of DNA methylation processes in relation to the transition from goal-directed to habitual behaviour. It should be emphasised that the epigenetic mechanisms influencing patterns of gene expression in brain, that in turn underlie particular facets of instrumental behaviour, are likely to be highly complex. Indeed, the picture that is emerging, even at this early stage of research, indicates that the molecular mechanisms underlying cognitive functions involve a combination of different epigenetic modifications (Miller et al., 2008). Clearly, dealing with this complexity is a major challenge for future research.

Class	Type	Neurophysiological, behavioural and cognitive effects	Reference
I	HDAC1	Facilitates memory extinction	Bahari-Javan et al. (2012)
		Increased after contextual fear conditioning	Gupta-Agarwal et al. (2012)
	HDAC2	Moderates associative and spatial learning	Guan et al. (2009); Gräff et al. (2012)
		Decreased after contextual fear conditioning	Gupta-Agarwal et al. (2012)
	HDAC3	Moderates spatial recognition memory	McQuown et al. (2011)
		Reduced following contextual fear conditioning	Gupta-Agarwal et al. (2012)
IIa	HDAC4	Moderates thermotaxic memory in nematodes	Wang et al. (2011b)
		Loss of function impairs associative and spatial learning	Kim et al. (2012)
	HDAC5	Inhibits LTF in Aplysia	Guan et al. (2002)
		Elevated expression in mouse model of schizophrenia with memory deficits	Koseki et al. (2012)
		Reduced following contextual fear conditioning	Gupta-Agarwal et al. (2012)
	HDAC7	Reduced following contextual fear conditioning	Gupta-Agarwal et al. (2012)
	HDAC9	CNV associated with schizophrenia-related cognitive impairments	Tam et al. (2010)
IIb	HDAC6	HDAC6-specific HDACi had no effect on memory	Guan et al. (2009)
IV	HDAC11	Increased following contextual fear conditioning	Gupta-Agarwal et al. (2012)

Table 7.3.2: Summary of the various histone deacetylase isoforms and their studied neurophysiological and behavioural roles. CNV: copy number variant; HDAC: histone deacetylase; HDACi: histone deacetylase inhibitor; LTF: long term facilitation. Table adapted from Gräff and Tsai (2013).

7.4 Manipulating instrumental behaviour with the HDAC inhibitor Sodium Butyrate by direct infusion into prefrontal cortex

Whilst there are clear benefits to non-invasive systemic drug administration, in terms of ease of delivery and possible clinical relevance, as well as the fact that it enables us to dose the whole CNS, questions regarding key brain areas and circuitries require targeted administration of drugs directly into brain. The data presented in Chapter 6 represent an important initial step in the development of an infusion protocol for studying the effects of centrally administered HDACis on instrumental learning. However, again, a number of practical issues highlighted by this study will need to be addressed before we can fully exploit the extensive possibilities offered by such an approach.

Assessing the on- and off-target molecular effects of NaB infusions. As for the systemic work discussed above, it will be important to confirm on-target effects and discount any potential off-target confounds of the microinfusion protocol. Firstly, it will, again, be necessary to address concerns regarding the potential damaging effects of NaB infusions with more extensive molecular analyses. Western blot analyses indicated that infusing NaB into the mPFC had a mixture of neurotoxic and neuroprotective actions. These complex effects need to be investigated further, for example by looking a several other necrosis and apoptosis markers, or by using immunohistochemistry techniques to assess the localization of any up-regulated damage markers in relation to the infusion site. Interestingly, others have shown that NaB has both anti-inflammatory and neuroprotective effects (Andoh et al., 1999; Kim et al., 2004; Huang et al., 2007; Kim et al., 2007). Furthermore, if, as suggested by the

molecular data indicating an upward spread of NaB into the prelimbic cortex, the infusion protocol used here affected the entire mPFC, then the behavioural effects observed would be inconsistent with a damaging effect of NaB. This is because, in contrast to the enhancing effect of NaB on goal-directed responding found in the current study, lesions of the entire mPFC have been shown to block the expression of goal-directed behaviour (Ostlund and Balleine, 2005). Thus, if we assume the infusions affected the entire mPCF (i.e. both IL and PL), as suggested by the Western blot data, the behavioural findings would be more synonymous with a protective effect of NaB, an interpretation which squares with the reduction in Caspase 3 activity seen here in NaB treated tissue, as well as with the work of others (Andoh et al., 1999; Kim et al., 2004; Huang et al., 2007; Kim et al., 2007). .

Secondly, it will be necessary, in future work, to confirm and extend the preliminary evidence, from Western blot analysis, which points to a measurable effect of NaB on acetylation of histone H4 (H4K5/8/12/16ac) in the target region, the infralimbic cortex (IL). Whilst H4K5/8/12/16ac is a commonly studied histone acetylation mark in the neuroscience literature (e.g. Rogge and Wood, 2013), as discussed above, many other acetylation marks have been shown to be altered by learning (see Table 7.3.1).

Once we are satisfied that the NaB microinjection protocol has demonstrable effects on HA in target regions, and does not have significant damaging effects on the brain, we will need to test the extent to which any behavioural effects of the drug infusion can be attributed to changes in HA, as opposed to being caused by general off-target effects on brain function. Indeed, histones are not the only substrates of HDAC enzymes, which can act on acetylated lysine residues of a number of non-histone proteins, including other, mainly metabolic, enzymes, transcription factors and several cytoskeletal proteins (Yuan et al., 2005; Fischer et al., 2007; Sweatt, 2007). One approach could be to reduce HAT activity in the same target regions, for example using infusions of a HAT inhibitor (as discussed above); here, any evidence

of behavioural dissociations using drugs with opposite effects on histone acetylation would help to strengthen arguments attributing behavioural effects on instrumental learning to acetylation changes (Marek et al., 2011; Maddox et al., 2013).

Spatial specificity and brain regional dissociations. By providing a way to manipulate specific molecular processes in discrete brain areas, brain microinfusions have the potential to enable us to parse the molecular mechanisms underlying habit formation by brain area, as well as by learning stage. However, in order to do this, it will be important to assess the extent of the upward spread of NaB indicated by the Western data, for example using more spatial sensitive measures such as immunohistochemistry or radioactive labelling (see Smith and Berridge, 2005 for an example of how this can be done). Upward spread is a common issue in microinfusion experiments and the small size and close proximity of the mPFC subregions of interest here makes it a difficult problem to rectify. However, by establishing the extent to which effects on HA are greater at the infusion site compared to more distal regions, we will be better able to assess how much of an issue this may be in interpreting any behavioural effects.

Furthermore, it will be possible to look for dissociable, region-specific effects by targeting the IL and PL separately, offering a way to ascertain the extent to which behavioural effects can be attributed to molecular changes in the mPFC as a whole as opposed to subregion-specific manipulation effects. Similarly, it will be possible to explore the functional dissociation in striatal subregions also indicated by the lesion work (Yin et al., 2004, 2005; see below for further discussion).

Moreover, in contrast to lesion studies, with microinfusion experiments it is possible to use different drug compounds to target specific molecules, enabling us to investigate any observed effects of NaB infusions to a greater depth. For example, as discussed above, the development of increasingly specific HDACis has enabled others to

attribute behavioural effects to specific HDAC molecules (see Table 7.3.2;McQuown et al., 2011). In addition to validating and verifying any effects of NaB, it will also be possible to target different epigenetic, and even non-epigenetic, molecular pathways. For example, one interesting approach may be to look at the effects of behaviourally penetrant epigenetic manipulations on gene expression as a way of gaining an insight into potentially important downstream consequences of epigenetic modifying drugs (e.g. Bredy et al., 2007). Thus, through the iterative analysis of behavioural and molecular data it will be possible to gain a better understanding of the molecular processes connecting learning experience to the changes in brain function that underlie the switch from flexible, goal directed actions to rigid, relatively automatic habitual responses.

7.5 Moving forward: Designing a striatal microinfusion experiment

As the above discussion makes clear, once we have sufficiently refined the infusion protocol it will be possible to use this basic procedure to investigate a wide range of experimental questions. In particular, the spatial specificity afforded by microinfusions is of great utility, enabling, as it does, the targeted administration of psychoactive drugs into discrete brain areas. Lesion study data indicates that functionally dissociable networks of cortical and subcortical structures are involved in the regulation of instrumental learning (Yin and Knowlton, 2006), and microinfusions provide the opportunity to begin to explore and dissociate the potential role of region-specific epigenetic changes in the transition from goal-directed to habitual behaviour. In addition to mPFC subregions, the dorsal striatum is an obvious target area for future work. The posterior dorsomedial (pDMS) and dorsolateral (DLS) striatum have been shown to have dissociable roles in goal-directed and habitual behaviours respectively

(see Table 6.1.1 for a summary of this lesion work). Therefore, it is possible to envisage a series of infusion experiments which build upon the lesion work by targeting these two striatal subregions with epigenetic modifying drugs, such a NaB, at different stages during training. Whilst the mPFC, in particular the IL, is thought to play more of an executive role in the regulation of instrumental behaviour, gating the relative expression of A-O and S-R associations (Killcross and Coutureau, 2003), the dorsal striatum appears to play a more fundamental role in the acquisition and storage of these associative structures (Yin et al., 2004, 2005). Therefore, one might predict that manipulations targeting these regions are most likely to influence the initial acquisition stage of instrumental learning. Thus, by allowing us to study the interaction of different molecular process, in different brain regions, at different points in training, such experiments have the potential to advance our understanding of the molecular substrates of instrumental learning. By combining such intervention approaches with correlative investigations, such as those described in Chapter 4 of this this thesis, we will begin to gain a clearer idea of the complex neurophysiological pathways underlying the transition from goal-directed to habitual behaviour.

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Appendix A

Additional figures and data for Chapter 3

A.1 Experiment 1

		Session 1		Session 2		Session 3	
		To be Devalued	To be Non-devalued	To be Devalued	To be Non-devalued	To be Devalued	To be Non-devalued
C.R	Box	181.5	181.75	275.13	269.88	351.25	332.25
	Cage	232.14	169	266.71	238.38	338.57	288.25
H	Box	184.88	149	244.5	240.63	269.13	295
	Cage	182.13	142.25	264.13	240	291.13	254.38

(a) Summary of mean lever presses across training session for the different conditions

		Session 1		Session 2		Session 3	
		To be Devalued	To be Non-devalued	To be Devalued	To be Non-devalued	To be Devalued	To be Non-devalued
C.River	Box	527.75	517.75	392.63	394.75	362.25	335.875
	Cage	649.14	475.38	393.29	514.5	314.71	395.5
Harlan	Box	628.25	527	493	401.63	363.63	357
	Cage	574.38	580.5	421.75	470.25	381.75	403.5

(b) Summary of mean magazine entries across training session for the different conditions

Table A.1.1: Summary of mean lever press responses (a) and magazine entries (b) across the training session for the different conditions

A.2 Experiment 2

	Session									
	1	2	3	4	5	6	7	8	9	10
Devalued	153.13	246	309.63	353.75	340	369.63	339.63	416	513	509.25
Non-devalued	192.25	258.88	279.5	367.75	361.13	430.13	384.63	477.75	411.63	502

(a) Lever presses

	Session									
	1	2	3	4	5	6	7	8	9	10
Devalued	524.25	500.5	374.63	360.5	283.88	298.25	262.88	287.88	318.88	303.63
Non-devalued	532.75	456.25	335.5	356.5	282.13	294.88	255.88	328.5	288.25	295.88

(b) Magazine entries

Table A.2.1: Summary of mean lever presses and magazine entries across training session for the different conditions

Appendix B

Additional figures and data for Chapter 4

B.1 Behavioural data

		Session										
		1	2	3	4	5	6	7	8	9	10	**Overall Mean**
Minimal	Array	340.2	425.6	561.6								**442.467**
	qPCR	344.833	542.333	558.167								**481.778**
	Other	293.769	422.846	542.846								**419.821**
	All	316.208	453.292	550.583								**440.028**
Moderate	Array	273.4	324.2	419.0	536.8	535.6	585.6					**445.767**
	qPCR	261.333	342.833	415.5	569.667	602.5	680.167					**478.667**
	Other	241.538	351.462	422.462	523.769	642.462	646.0					**471.282**
	All	253.125	343.625	420.0	537.958	610.208	641.958					**467.813**
Extensive	Array	187.4	266.0	366.6	382.8	438.4	510.6	627.4	698.2	717.6	848.8	**504.380**
	qPCR	205.667	285.167	371.5	436.833	519.833	551.5	498.833	559.5	605.5	7.667	**473.5**
	Other	170.615	220.846	336.308	378.0	406.923	410.462	423.538	547.308	556.615	615.462	**406.608**
	All	182.875	246.333	351.417	393.708	441.708	466.583	484.833	581.792	602.375	685.375	**443.7**

(a) Lever presses

		Session										
		1	2	3	4	5	6	7	8	9	10	**Overall Mean**
Minimal	Array	632.4	405.6	318.8	452.267							**452.267**
	qPCR	690.5	474.5	298.5	487.833							**487.833**
	Other	580.462	468.231	355.846	468.179							**468.179**
	All	618.792	456.750	333.792	469.778							**469.778**
Moderate	Array	664.0	401.0	306.6	339.8	311.6	309.6	388.767				**388.767**
	qPCR	827.167	477.0	375.333	348.0	338.167	354.5	453.361				**453.361**
	Other	751.385	543.0	424.538	372.846	410.538	379.0	480.218				**480.218**
	All	752.125	496.917	387.667	359.750	371.833	358.417	454.451				**454.451**
Extensive	Array	669.2	501.8	499.2	451.4	417.4	439.6	363.2	390.0	384.0	388.0	**450.380**
	qPCR	614.667	441.167	384.833	338.0	371.833	350.167	325.0	330.0	342.333	397.0	**389.5**
	Other	510.385	386.0	368.462	387.538	387.538	381.462	366.462	409.231	372.308	367.077	**393.646**
	All	569.542	423.917	399.792	388.458	389.833	385.750	355.417	385.417	367.250	378.917	**404.429**

(b) Magazine entries

Table B.1.1: Summary of mean lever press and magazine entry data across the three training groups for animals from which tissue was intended for molecular analysis (Array: N=5; qPCR: N=6) and for the remaining animals (Other: N=13)

B.2 Molecular data

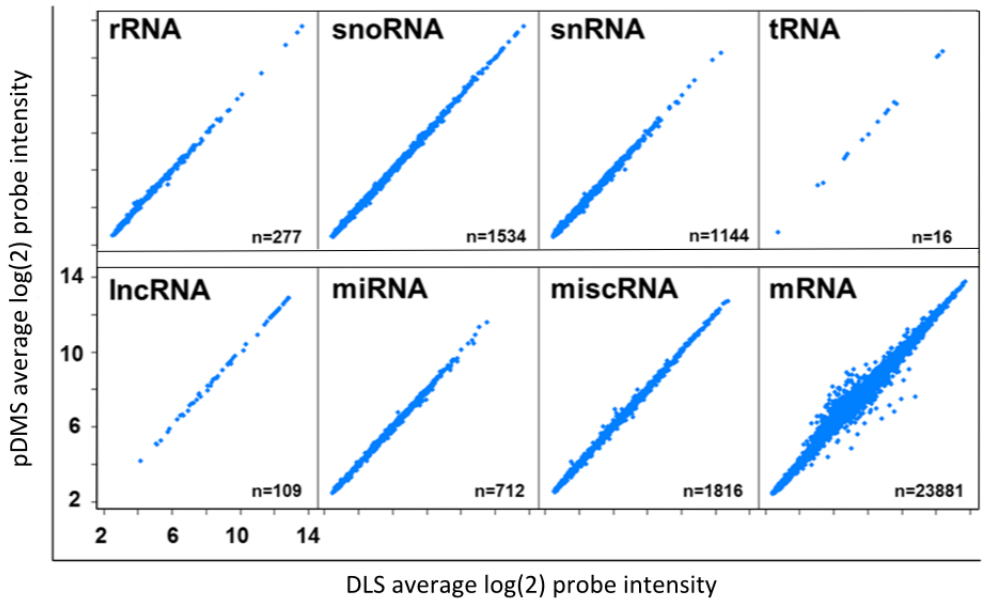


Figure B.1.1: XY intensity plots for different RNA subclasses (based on Affymetrix annotation).

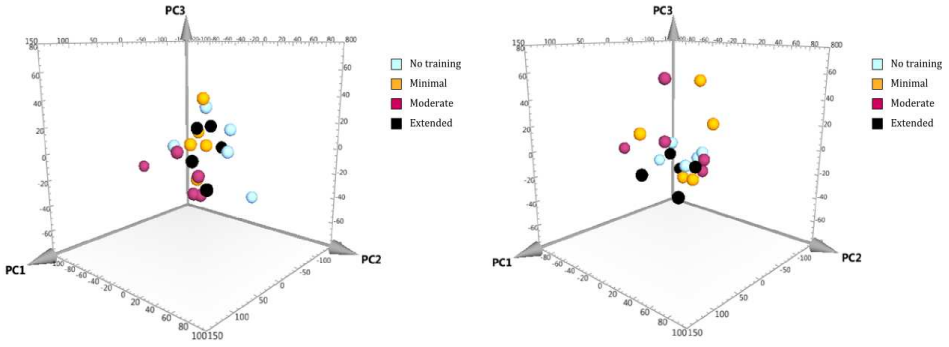


Figure B.2.1: Group-coded PCA plots for DLS (left) and pDMS (right)

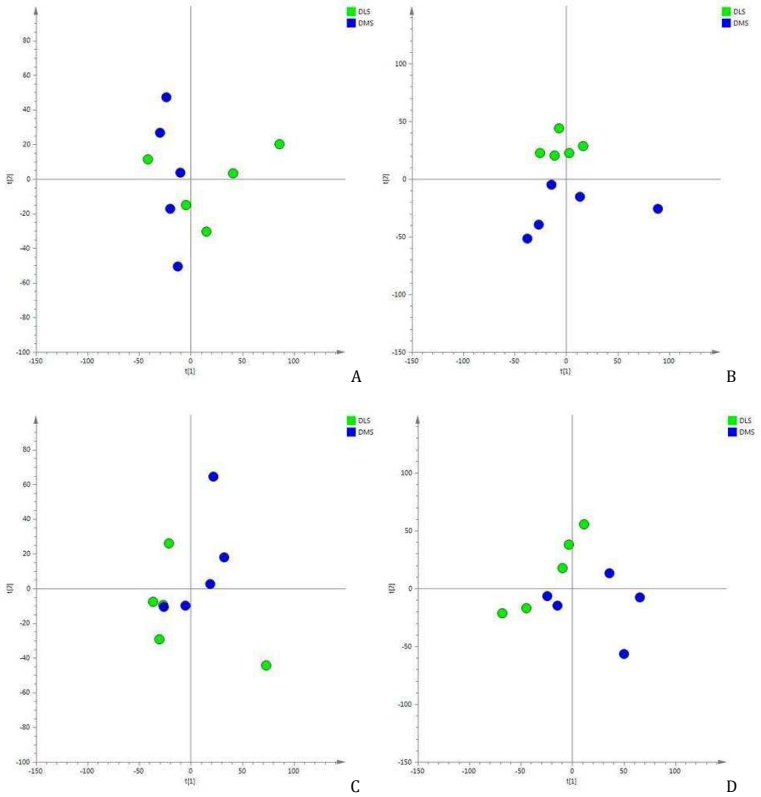
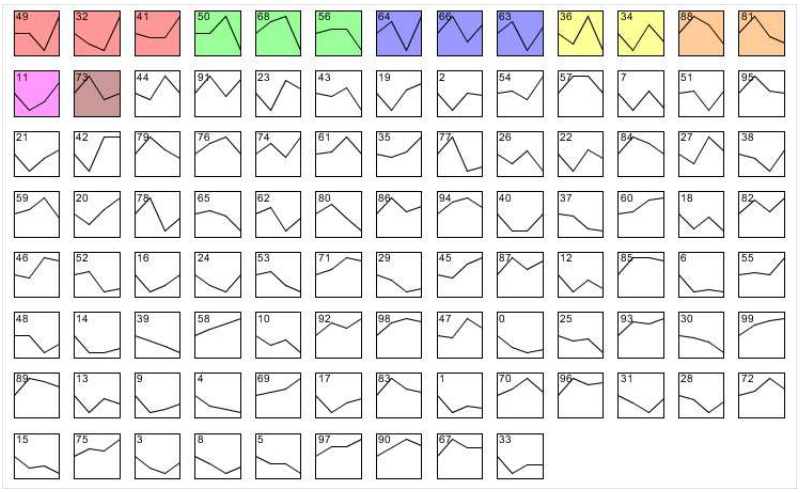
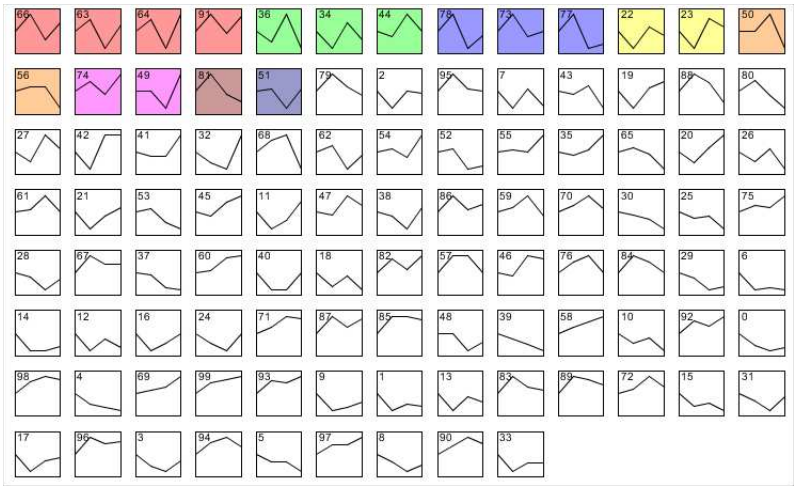


Figure B.2.2: Region-coded PCA plots for individual training groups: A = No training, B = Minimal training, C = Moderate training and D = Extended training.

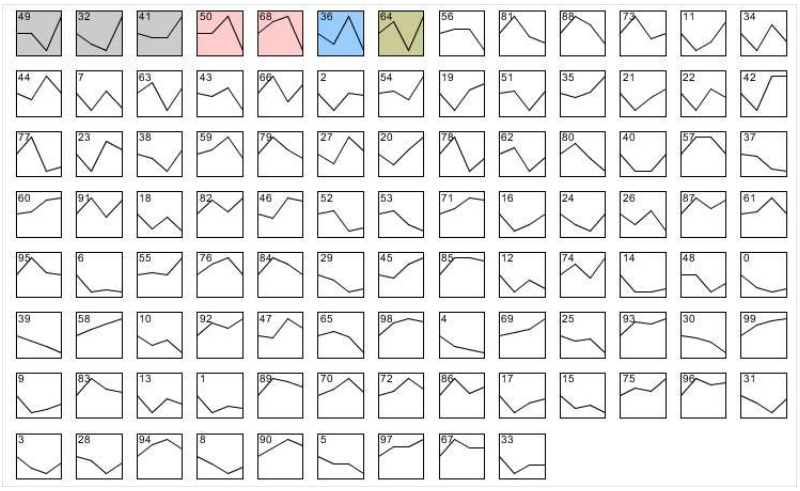


(a) DLS

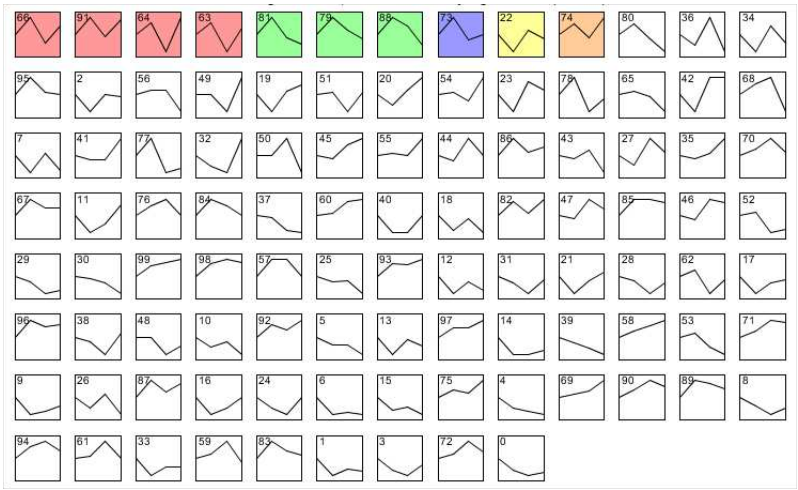


(b) pDMS

Figure B.2.3: All model profiles generated from STEM analysis (predefined n=100) of ALL probes for the DLS (a) and pDMS (b). All significantly enriched profiles (FDR $p<0.01$) have colour. Clusters of profiles (correlation 0.85) have the same colour.

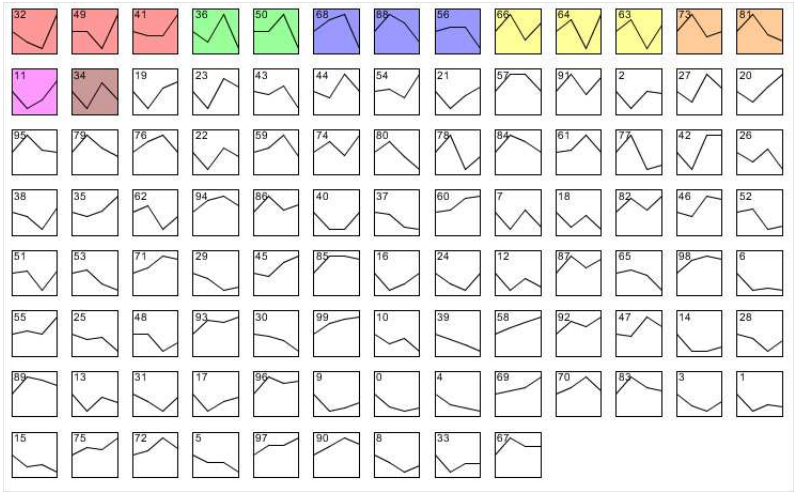


(a) DLS

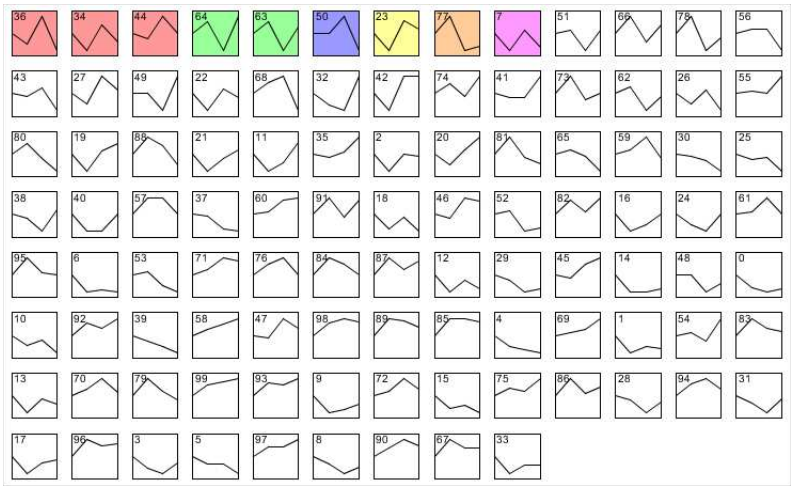


(b) pDMS

Figure B.2.4: All model profiles generated from STEM analysis (predefined n=100) of GO ANNOTATED probes for the DLS (a) and pDMS (b). All significantly enriched profiles (FDR p<0.01) have colour. Clusters of profiles (correlation 0.85) have the same colour.

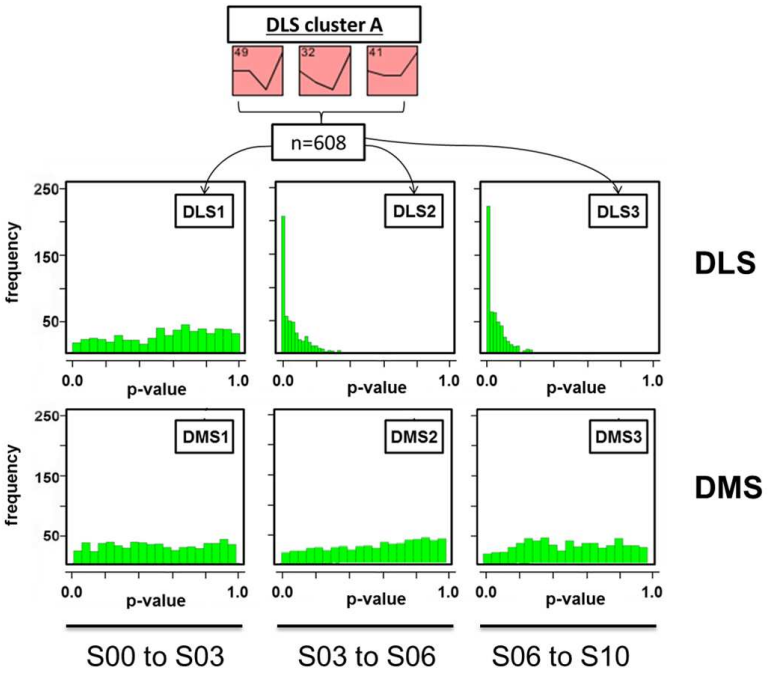


(a) DLS

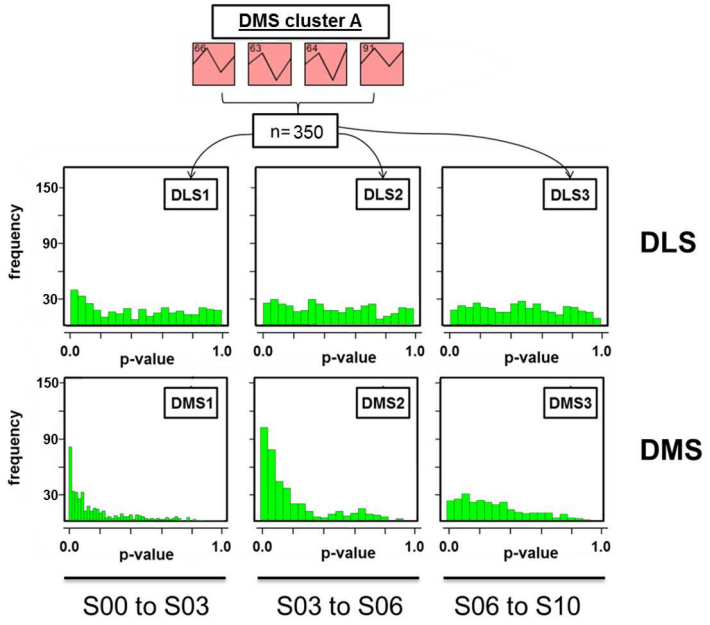


(b) pDMS

Figure B.2.5: All model profiles generated from STEM analysis (predefined n=100) of ncRNA probes for the DLS (a) and pDMS (b). All significantly enriched profiles (FDR $p<0.01$) have colour. Clusters of profiles (correlation 0.85) have the same colour.



(a) Histograms for DLS profile cluster A (profile n=3, total number of unique probes = 608; histogram breaks n=20)



(b) Histograms for pDMS profile cluster A (profile n = 4, total number of unique probes = 350; histogram breaks n=30)

Figure B.2.6: Frequency histograms over moderated p-vales for each pair-wise comparison for the DLS and pDMS clusters with the largest number of probes.

	Fold Change	p-value	FDR
DLS1	**-1.1602822**	**0.017038672**	**0.81331522**
pDMS1	0.0919588	0.843954186	0.99898365
DLS2	0.7258992	0.126306639	0.93190023
pDMS2	-0.0727826	0.876184971	0.9998205
DLS3	-0.3480496	0.457888434	0.9323118
pDMS3	0.0845744	0.85633665	0.998736

(a) Cartpt (CART propeptide). Selected as DLS1 candidate

	Fold Change	p-value	FDR
DLS1	-0,1402766	0,728218729	0,98697987
pDMS1	0,2411312	0,50527626	0,99898365
DLS2	**-1,4614502**	**0,000238792**	**0,22949695**
pDMS2	-0,0790618	0,82661589	0,9998205
DLS3	1,245693	0,001341836	0,4946177
pDMS3	0,3840484	0,290935145	0,998736

(b) Tbr-1 (T-box, brain, 1). Selected as DLS2 candidate and top-ranked OPLS-DA (REGION) candidate.

	Fold Change	p-value	FDR
DLS1	0.0705792	0.719692444	0.98894617
pDMS1	0.1221294	0.535341896	0.99898365
DLS2	**0.653488**	**0.0019094**	**0.51475136**
pDMS2	0.1167204	0.553478066	0.9998205
DLS3	**-0.7123948**	**0.000821891**	**0.4488287**
pDMS3	-0.1790888	0.364837709	0.998736

(c) Stx1a (Syntaxin 1 brain). Selected as DLS2 and DLS3 candidate

Table B.2.1: Limma data for genes selected for qPCR technical verification of differentially regulated probes in the DLS.

	Fold Change	p-value	FDR
DLS1	-0.4510146	0.095063613	0.87919382
pDMS1	**-0.8651992**	**0.00225239**	**0.96055183**
DLS2	-0.328606	0.219714505	0.93941729
pDMS2	0.0699922	0.79173124	0.9998205
DLS3	0.1523656	0.566108383	0.9466385
pDMS3	-0.409932	0.127940575	0.998736

(a) Arc (Activity-regulated cytoskeleton-associated protein). Selected as IEG and pDMS1 candidate

	Fold Change	p-value	FDR
DLS1	0.1523656	0.566108383	0.9466385
pDMS1	**-0.409932**	**0.127940575**	**0.998736**
DLS2	0.1523656	0.566108383	0.9466385
pDMS2	-0.409932	0.127940575	0.998736
DLS3	0.1523656	0.566108383	0.9466385
pDMS3	-0.409932	0.127940575	0.998736

(b) Egr-1 (Early growth response protein 1). Selected as IEG and pDMS1 candidate.

	Fold Change	p-value	FDR
DLS1	-0.5986996	0.037152647	0.85655028
pDMS1	**-0.9371804**	**0.001716532**	**0.92973393**
DLS2	-0.1497038	0.591746773	0.98031597
pDMS2	0.0150326	0.956966109	0.9998205
DLS3	0.1467088	0.599153047	0.9508644
pDMS3	-0.2961464	0.291519068	0.998736

(c) Egr-2 (Early growth response protein 2). Selected as IEG and pDMS1 candidate

Table B.2.2: Limma data for genes selected for qPCR technical verification of differentially regulated probes in the pDMS

Appendix C

Additional figures and data for Chapter 5

C.1 Systemic Pilot experiment

Eight Lister hooded rats (Charles River, Margate, Kent, UK) were given an IP injections of either NaB (1.2 g/kg; n = 4) or saline (0.9%; n = 4) and monitored for two hours post injection. NaB-treated animals were given a pain and distress scores (based on the scoring system developed by Lloyd and Wolfensohn (1998)) at regular intervals post-injection. The average scores across the two hours are shown in Figure C.1.1. After two hours the rats' showed no signs of pain and distress (i.e. all received Pain and Distress score of 0) and were placed in locomotor activity chambers (described in section 2.2.2) for 30 minutes. Analysis of total beam break data using a two factor between subjects ANOVA, showed that NaB animals were hypoactive relative to saline-treated animals even two hours post injections (Main effect of HDACi group: $F(1, 6) = 38.718$ $p = .001$; see Figure 5.1.3 in Chapter 5).

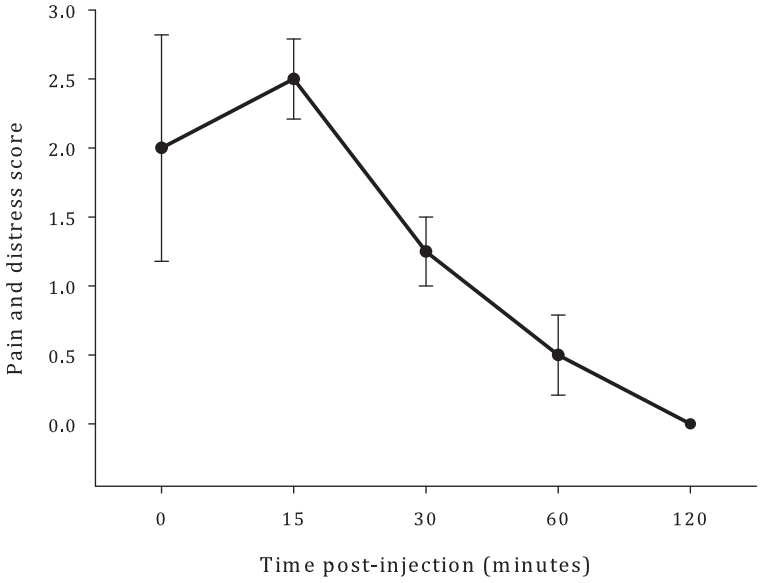


Figure C.1.1: Average pain and distress scores for NaB-treated animals

C.2 Data for CRF session (Experiment 1)

A 2-factor ANOVA revealed that there were no differences in lever press levels during the continuous reinforcement session in Experiment 1 ($F[1,27] = 0.482$, $p > .05$; see Figure C.2.1), indicating that differences between the HDACi groups in lever press response rates across the RI30 training sessions were not the result of pre-exsisting differences in the propensity to lever press.

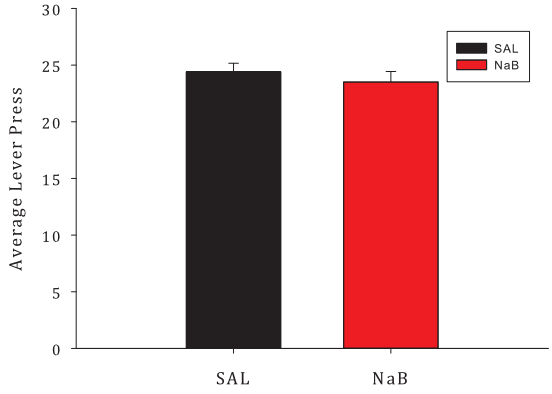


Figure C.2.1: Mean lever press responses made during the CRF training session

C.3 Data for RI30 training sessions

	Session 1		Session 2		Session 3	
	To be *Devalued*	To be *Non-devalued*	To be *Devalued*	To be *Non-devalued*	To be *Devalued*	To be *Non-devalued*
To be *SALINE*	273	223.38	357.88	297.878	475.88	403.25
To be *NaB*	228.75	200.25	318.88	255.88	313.13	379

(a) Lever presses

	Session 1		Session 2		Session 3	
	To be *Devalued*	To be *Non-devalued*	To be *Devalued*	To be *Non-devalued*	To be *Devalued*	To be *Non-devalued*
To be *Saline*	592	534.25	422.63	393.63	295.63	285.25
To be *NaB*	576.75	622.38	355.75	411.38	244	300.5

(b) Magazine entries

Table C.3.1: Summary of mean lever press responses (a) and magazine entries (b) across the training sessions for the different conditions for Chapter 5, Experiment 1.

	Session 1		Session 2		Session 3	
	To be Devalued	To be Non-devalued	To be Devalued	To be Non-devalued	To be Devalued	To be Non-devalued
To be SALINE	275.38	260.63	336.75	293.5	397.38	387.88
To be NaB	290	224.5	357	318	438.13	386.75

(a) Lever presses

	Session 1		Session 2		Session 3	
	To be *Devalued*	To be *Non-devalued*	To be *Devalued*	To be *Non-devalued*	To be *Devalued*	To be *Non-devalued*
To be *Saline*	613.75	510.38	363	351.25	311.38	306.5
To be *NaB*	562.25	504.38	345.88	350.88	273.75	254.63

(b) Magazine entries

Table C.3.2: Summary of mean lever press responses (a) and magazine entries (b) across the training sessions for the different conditions

	Session 1		Session 2		Session 3	
	To be Devalued	To be Non-devalued	To be Devalued	To be Non-devalued	To be Devalued	To be Non-devalued
To be SALINE	288.625	228.375	302.875	305.125	363.25	377.75
To be NaB	230.125	248.75	315.5	347	358.25	411.625

(a) Lever presses

	Session 1		Session 2		Session 3	
	To be *Devalued*	To be *Non-devalued*	To be *Devalued*	To be *Non-devalued*	To be *Devalued*	To be *Non-devalued*
To be *Saline*	611	563.38	339.5	351.5	280.63	279.63
To be *NaB*	596.75	528.5	396.5	340.25	347	299.75

(b) Magazine entries

Table C.3.3: Summary of mean lever press responses (a) and magazine entries (b) across the training sessions for the different conditions.

Appendix D

Additional figures and data for Chapter 6

D.1 NaB infusions into the infralimbic cortex alter histone acetylation in the medial prefrontal cortex

Brain tissue, taken from Lister hooded rats (Harlan, UK) which had received infusions of either NaB (n = 3) or Saline (n = 3), targeting the infralimbic (IL) cortex, was analysed by Western blotting with antibodies for H4K5/8/12/16ac. This analysis indicated an increase in H4 acetylation in NaB treated samples. However, this increase was detectable in both the IL and the more dorsal prelimbic region, pointing to an upward spread of the drug from the infusion site (see Figure D.1.1).

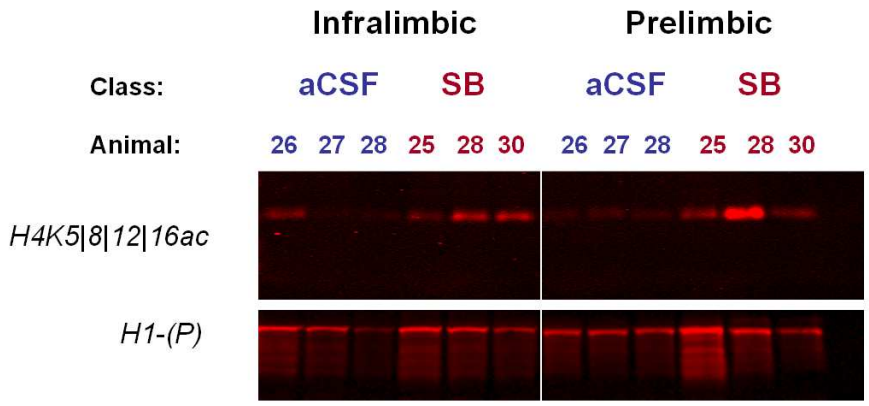
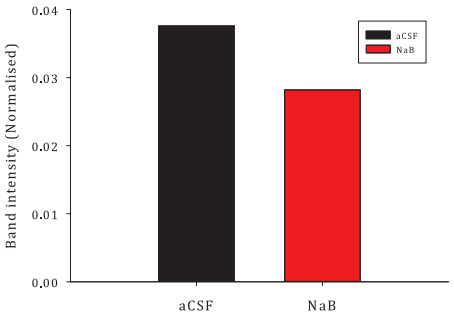


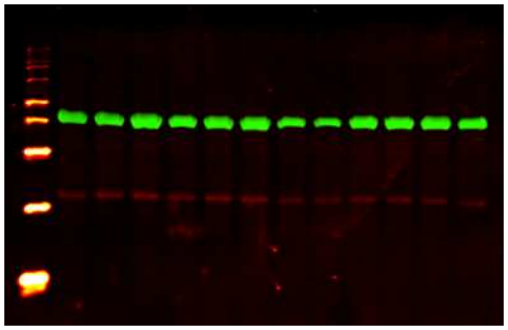
Figure D.1.1: Image of Western blot gel with antibodies for H4K5/8/12/16ac and H1-phosphorylation (H1-[p]; used here as housekeeping control).

D.2 NaB infusions into the infralimbic cortex have complex effects on molecular signals of cellular damage

Western blot analysis of NaB- (n = 6) and saline-infused (n = 6) IL tissue samples with antibodies for cleaved caspase-3 (Asp175; Cell Signaling Technology, Danvers, MA) and phosphorylated histone H2AX (Anti-gamma H2A.X [phospho S139]; Abcam, Cambridge, UK) as markers of apoptosis and necrosis respectively. This analysis revealed a reduction Caspase-3 cleavage (t (10) = -2.296, p = .007; see Figure D.2.1) and an increase in phosphorylated histone H2AX ($t(10)$ = 3.374, p = .045; see Figure D.2.2) in NaB-treated samples relative to controls.



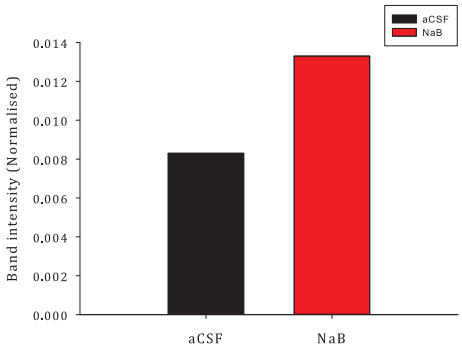
(a) Band intensity data for aCSF and NaB treated samples (normalised to alpha-tubulin housekeeping control)



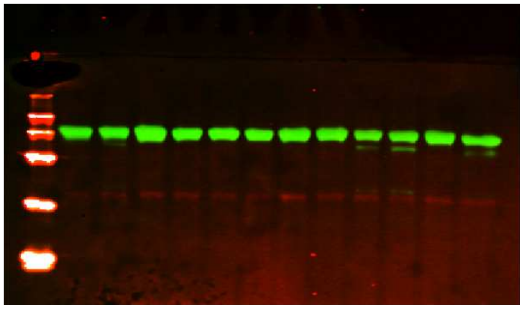
(b) Gel image for aCSF (wells 0-5) and NaB (wells 6-11) treated samples. Red bands show cleaved caspase-3 levels and green bands show alpha-tubulin levels (used here as a housekeeping control).

Figure D.2.1: Results for Western blot analysis using antibody for cleaved caspase-3.

D.3 Behavioural data



(a) Band intensity data for aCSF and NaB treated samples (normalised to alpha-tubulin housekeeping control)



(b) Gel image for aCSF (wells 0-5) and NaB (wells 6-11) treated samples. Red bands show phosphorylated histone H2AX levels and green bands show alpha-tubulin levels (used here as a housekeeping control).

Figure D.2.2: Results for Western blot analysis using antibody for phosphorylated histone H2AX.

	Session 1		Session 2		Session 3	
	To be *Devalued*	To be *Non-devalued*	To be *Devalued*	To be *Non-devalued*	To be *Devalued*	To be *Non-devalued*
To be *aCSF*	127.5	133.333	235.375	219.667	312.125	259.5
To be *NaB*	123.429	135.25	188	195.75	227.714	258.125

(a) Lever presses

	Session 1		Session 2		Session 3	
	To be *Devalued*	To be *Non-devalued*	To be *Devalued*	To be *Non-devalued*	To be *Devalued*	To be *Non-devalued*
To be *aCSF*	585.125	795.167	402.125	548.5	326.125	424.333
To be *NaB*	618.286	609.5	391.857	424.125	332.857	360.375

(b) Magazine entries

Table D.3.1: Summary of mean lever press responses (a) and magazine entries (b) across the training sessions for the different conditions