THE REWARDING AND COGNITIVE EFFECTS OF CORTICOSTERONE IN MICE

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Abstract

Corticosterone is the main glucocorticoid in rodents. Its secretion follows a circadian rhythm and is increased in response to stress. Corticosterone is thought to possess rewarding effects. For example, enhanced corticosterone secretion is associated with the exposure to natural rewards such as food, sex and running, and it has been reported that corticosterone is implicated in drug-seeking behaviour. In addition, rats orally self-administer the steroid hormone and are motivated to nose-poke for stress-like levels of corticosterone, suggesting that the stress hormone has rewarding effects on its own, although this proposal is not supported by conditioned place preference studies. Corticosterone is also found to be critical for learning and memory performance, but it is unclear whether cognitive effects of the hormone are modulated by its rewarding properties. Elevated glucocorticoid levels have been reported to accompany certain psychiatric and cognitive disorders, but it is not clear whether this is an underlying cause or a consequence of these diseases.

The two broad aims of the present thesis were to investigate potential rewarding properties of corticosterone in C57BL/6J male mice and to examine their long-term impacts on cognition. In the first experiment, mice were subjected to a prolonged two-bottle choice test to measure their preference for the stress hormone. Results indicated that mice were motivated to self-administer corticosterone at high doses and this was accompanied by enhanced dopamine turnover in the striatum, suggesting that the rewarding effects of corticosterone involve the mesolimbic dopamine pathway; the activity of which is enhanced by the majority of addictive drugs. Corticosterone self-administration did not impair recognition memory in most groups, but it did improve spatial memory.

Importantly, corticosterone self-administration induced neuroplasticity changes in the hippocampus and frontal cortex, indicating the involvement of these memory processing areas in the development of corticosterone-seeking behaviour. These results suggest that corticosterone self-administration is associated with spatial memory improvement in mice. Therefore, the remainder of the studies aimed to use a stressful natural reward (voluntary wheel running) to extend our understanding of the role of corticosterone in stress-seeking behaviour and its memory-related effects. The

preliminary results revealed that single or repeated 1-hour running sessions increased plasma corticosterone levels. Moreover, metyrapone (a corticosterone synthesis inhibitor) attenuated the running performance of trained mice. In another pilot study, repeated running sessions prevented the improvement of the spatial memory of mice but did not impair their recognition memory compared to non-exercising mice. The final study was undertaken to determine the influences of pharmacological blockade of glucocorticoid or dopamine D2 receptors on the running performance and the exercise-related effects on memory in mice. In contrast to metyrapone, mifepristone (a glucocorticoid receptor antagonist) did not decrease running distances of mice, but it did induce recognition memory impairment in non-exercising mice. These memory deficits were reversed by running, suggesting that exercise-evoked corticosterone increase is involved in the cognitive effects of wheel running. On the other hand, a dopamine D2 receptor antagonist (sulpiride) attenuated the running performance of mice and induced exercise-dependent opposite effects on memory. Overall, the results of this thesis support the hypothesis that corticosterone is rewarding in mice, and that these effects probably enhance certain aspects of cognition.

Publications

Abstracts

Ebada M, Kendall DA, Pardon MC. "Evidence for addictive properties of corticosterone in a self-administration paradigm." Poster presentation at the 2009 British Association of Psychopharmacology summer meeting in Oxford, UK (26 - 29 July).

Ebada M, Kendall DA, Pardon MC. "Molecular mechanisms underlying corticosterone self-administration and its impacts on spatial and recognition memory in mice." Poster presentation at the 2010 British Association of Psychopharmacology summer meeting in Harrogate, UK (25 -28 July).

Ebada M, Kendall DA, Pardon MC**.** "Intermittent voluntary wheel running is rewarding and affects cognition: Role of the stress hormone corticosterone." Poster presentation at the 2011 British Association of Psychopharmacology summer meeting in Harrogate, UK (24 -27 July).

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Abbreviations

ACTH	Adrenocorticotropic hormone (corticotropin)
BDNF	Brain derived neurotrophic factor
BW	Body weight
CPP	Conditioned place preference
CRF	Corticotropin releasing factor
CU/PU	Caudate/Putamen
DOPAC	3, 4-dihydroxyphenylacetic acid
ELISA	Enzyme linked immunosorbent assay
ERK1/2	Extracellular signal regulated kinase 1/2
FL-TrkB	Full length tropomysin-related kinase
GABA	γ aminobutyric acid
GCs	Glucocorticoids
GR(s)	Glucocorticoid receptor(s)
H	Habituation
5HIAA	5-hydroxyindoleacetic acid
HPA axis	Hypothalamic pituitary adrenocortical axis
HPLC	High pressure liquid chromatography
HR rats	High-responder (novelty-seekers) rats
5HT	Serotonin
HVA	Homovanillic acid
IMP	Inosine monophosphate
LI(s)	Location index
LR rats	Low-responder rats
LTP	Long-term potentiation
MIF	Mifepristone
MR(s)	Mineralocorticoid receptor(s)
MRI	Magnetic resonance imaging
NORT	Novel object recognition test
OLT	Object location test
PET	Positron emission tomography
pERK 1/2	Phosphorylated extracellular signal regulated kinase 1/2

RI(s)	Recognition index
Run-Met	Metyrapone-treated running group
Run-Sal	Saline-treated running group
SAT	Spontaneous alternation test
Sed-Sal	Saline-treated sedentary group
Sul25	Sulpiride 25 mg/kg, i.p.
Sul50	Sulpiride 50 mg/kg, i.p.
SYN	Synaptophysin
t-ERK 1/2	Total extracellular signal regulated kinase 1/2
t-TrkB	Truncated tropomysin-related kinase
TrkB	Tropomysin-related kinase

Contents

Chapter 1

General Introduction

1.1 Brain and reward

Over the last decades, the science of reward has progressed markedly. Olds and Milner (1954) were the pioneers in this research field, when they discovered that electrical stimulation in the septal region or lateral hypothalamus reinforced rats to lever-press.

1.1.1 Brain areas mediating reward

The mesolimbic dopamine pathway (Fig. 1.1), which has been found to be activated by natural rewards and abused drugs, is anatomically described as a part of the mesotelencephalic dopamine system, which also includes the mesocortical and mesostriatal dopamine pathways (Gardner and Ashby, 2000). Dopaminergic neurons (A10 group of midbrain dopaminergic neurons) in the mesolimbic system originate mainly from the ventral tegmental area with some other neurons projecting from different sites of the substantia nigra and the

retrorubal dopamine cell field (Gardner and Ashby, 2000), indicating the involvement of the nigrostriatal dopamine pathway in the response to rewards (Wise, 2009). These neurons innervate the nucleus accumbens shell, amygdala, bed nucleus of the stria terminalis, ventral striatum (olfactory tubercles and nucleus accumbens core), lateral septum, lateral hypothalamus, thalamic loci and the diagonal band of Broca (Gardner and Ashby, 2000; Wise, 2009), Fig. 1.1. The mesocortical dopaminergic neurons (A10, mainly, and A9) originate from the ventral tegmental area and project to the cortex including medial, prefrontal, cingulate and entorhinal cortex (Marsden, 2006), Fig. 1.1. On the other hand, mesostriatal dopaminergic neurons (A9), which have been thought to modulate motor function, leave the substantia nigra pars compacta to the dorsal striatum (caudate-putamen) (Marsden, 2006), Fig. 1.1. The major neurotransmitters which regulate the activity of these dopamine pathways are glutamate, GABA, noradrenaline, opioids and endocannabinoids (Marsden, 2006; Koob and Le Moal, 2008; Belujon and Grace, 2011).

1.1.2 Role of dopamine in the rewarding process (dopamine/reward theory)

Exposure to rewards such as food (Hernandez and Hoebel, 1988), sex (Damsma *et al.*, 1992) and drugs of abuse; e.g. amphetamine, cocaine, morphine, nicotine, alcohol (Di Chiara and Imperato, 1988; Volkow *et al.*, 2011) and phencyclidine (McCullough and Salamone, 1992) has been associated with a rise in extracellular dopamine concentration in the nucleus accumbens and striatum. Imaging studies using either single-photon emission computed tomography (Laruelle *et al.*, 1995) or positron emission tomography (PET) / magnetic resonance imaging (MRI) (Drevets *et al.*, 2001) have shown in healthy individuals a positive correlation between euphoria and D-amphetamine-induced dopamine release in the striatum, suggesting that dopamine mediates drugs of abuse-related reward. Drugs of abuse increase synaptic dopamine levels *directly* by blocking dopamine reuptake (cocaine) or by enhancing dopamine release *via* reversing dopamine reuptake (amphetamine) and *indirectly* by controlling the activity of other neurotransmitters; such as GABA, opioids, serotonin, glutamate or endocannabinoids, which modulate the activity of the mesocorticolimbic dopamine system (Tomkins and Sellers, 2001; Koob

and Le Moal, 2008). In contrast, chronic administration of addictive drugs has been found to be associated with a decrease in dopamine release. For instance, PET studies indicated that cocaine addicts exhibited attenuated euphoria and dopamine release in the striatum but increased responses to methylphenidate (a dopamine reuptake inhibitor) in the thalamus, indicating cocaine craving (Volkow *et al.*, 1997). Overall, activation of mesocorticolimbic dopaminergic neurons is involved in the response to primary rewards and abused drugs, but chronic exposure to addictive drugs could be accompanied with reduced activity of the mesolimbic dopamine pathway and enhanced dopamine neurotransmission in other brain areas such as the thalamus, indicating compulsive drug-taking behaviour.

The dopamine theory of reward was introduced by Wise *et al.* (1978). Three hypotheses have been extensively studied to explain the role of dopamine in the rewarding process.

1. The anhedonia hypothesis developed by (Wise, 1982; Wise, 2008) states that dopamine is responsible for *liking* a reward; i.e. dopamine gives a rewarding substance such as food their goodness or hedonic properties. In support of this hypothesis**,** dopamine receptor antagonists such as pimozide were found to attenuate animals' lever-pressing response to access food (Wise *et al.*, 1978). The assumption was, however, contradicted by the fact that schizophrenics present with anhedonia while being treated with dopamine receptor antagonists (neuroleptics). However, neuroleptics were found to treat positive symptoms of schizophrenia rather than the negative symptoms which include anhedonia (Wise, 2008). An important point is that the effects of neuroleptics were interpreted as attenuation of positive reinforcement rather than induction of anhedonia.
2. The incentive (appetitive) salience hypothesis states that dopamine is important for wanting; so dopamine motivates an individual to work for a reward. Indeed, depletion of brain dopamine was associated with decreased motivation for approaching sucrose in rats, despite their ability to discriminate the sweet (hedonic) taste of sucrose *versus* the

bitter (aversive) taste of quinine (Berridge, 1996; Berridge and Robinson, 1998).

3. Finally, the reward prediction hypothesis proposes that dopamine increases in the brain when an individual predicts that he/she will obtain something rewarding (Gluck, 2008). For example, in monkeys first trained to press a lever to receive juice signalled by a cue such as a light or a screen picture, brain dopamine levels were found to rise in response to the presentation of the cue in anticipation of the juice and then to drop after it was drunk (Hollerman and Schultz, 1998; Schultz, 1998).

Altogether, these hypotheses led to the view that dopamine plays a role in the anticipation of rewards, reward conditioning and energizing approach behaviour rather than in mediating hedonic effects of rewards (Ikemoto, 2010).

1.1.3 Dopaminergic neurons and receptors

Dopaminergic neurons are either spontaneously active (population neurons) or inactive (Grace and Bunney, 1983). The active neurons have two different patterns of activity, slow irregular tonic and phasic bursting firing (Grace and Bunney, 1984b; Grace and Bunney, 1984a). The phasic activity has been found to be induced in response to rewards such as abused drugs (Schultz, 1998). A diagrammatic representation of neuronal dopamine synthesis is given in Fig. 1.2.

Dopamine mediates its effects through 2 major classes of G protein-coupled receptors: D1-like receptors (D1, D5) and D2-like receptors (D2, D3, D4), the first class activates and the second class inhibits the adenylyl cyclase/cAMP/ protein kinase A pathway (Kebabian and Calne, 1979; Lu et al., 2006; Marsden, 2006; Lee and Messing, 2008) which activates cAMP response element binding protein (CREB) and dopamine- and cAMP-regulated phosphoprotein (32 kDa) which regulates dopamine signaling (Lee and Messing, 2008), Fig. 1.3.

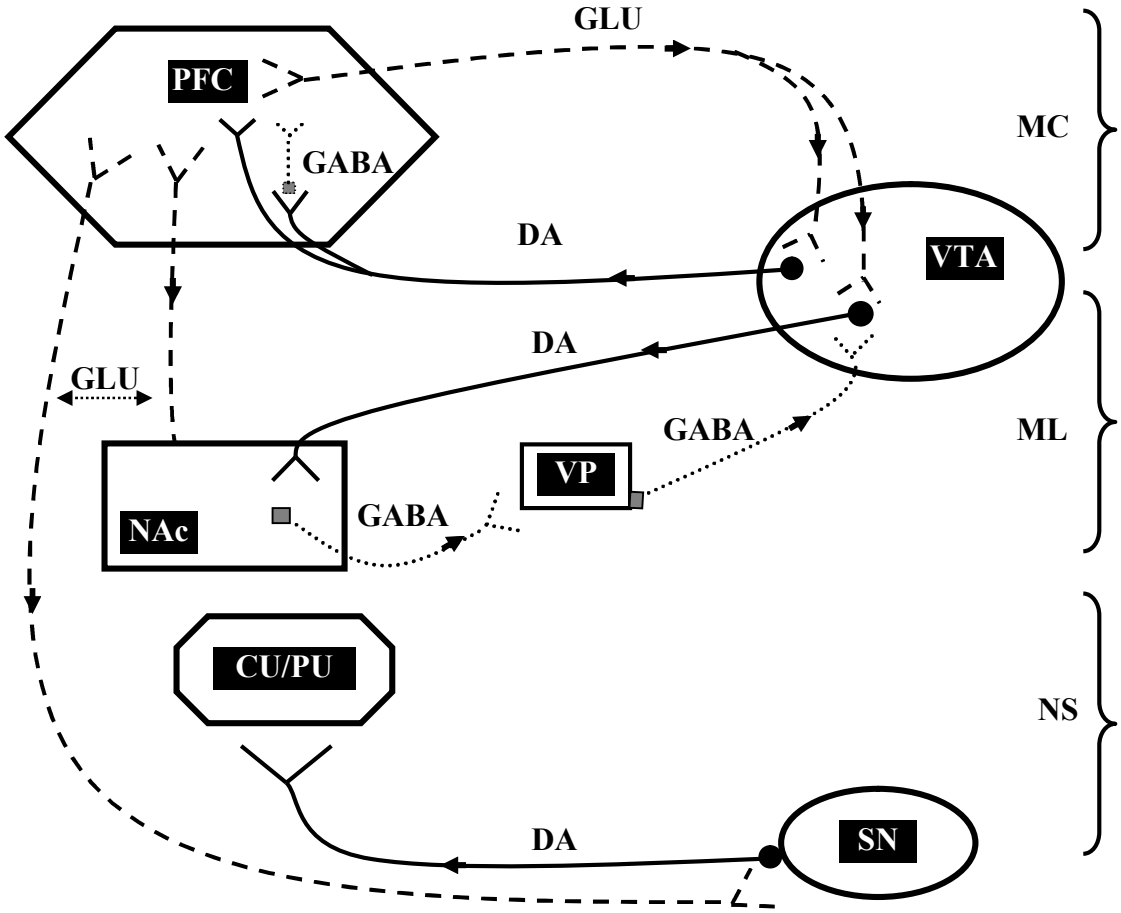

Figure 1.1 Three dopaminergic pathways in the brain: mesolimbic (ML), mesocortical (MC) and nigrostriatal (NS). For ML and MC, dopaminergic neurons (solid lines) project from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and to the prefrontal cortex (PFC). VTA receives inhibitory GABAergic neurons (dotted lines) from NAc *via* the ventral pallidum (VP) and excitatory glutamatergic neurons (divided lines) from PFC. In NS dopamine pathway, the substantia nigra pars compacta sends dopaminergic neurons to the caudate-putamen (CU-PU), and receives glutamatergic neurons from PFC. DA = dopamine, GLU = glutamate. This diagram was adapted from (Marsden, 2006).

D1 and D2 receptors are highly expressed in the nucleus accumbens, caudate-putamen and olfactory tubercle (Marsden, 2006). D2 receptors are expressed at pre- and postsynaptic sites and activation of presynaptic D2 autoreceptors decreases dopamine release (Marsden, 2006). D2 receptors are expressed as two isoforms, D2S (D2 short) and D2L (D2 long), which are encoded by seven exons of D2 receptor genes (Vallone *et al.*, 2000). Moreover, D3 receptors are highly abundant in the limbic system and to a lesser extent in the dorsal striatum (Marsden, 2006). Molecular mechanisms of dopamine D1 and D2 receptors are illustrated in Fig. 1.3. D1 receptors have a lower affinity for dopamine than D2 receptors, and thus their activation increases by dopamine release evoked by phasic dopaminergic neuronal firing (Volkow *et al.*, 2012). In contrast, D2 receptors are activated by the tonic activity pattern of dopaminergic neurons (Volkow *et al.*, 2012). It has been reported that D1 receptors are involved in abused drugs-induced sensitization (Bertran-Gonzalez *et al.*, 2008), but the majority of abused drugs reduce levels of D2 receptors in the striatum (Volkow *et al.*, 2007; Volkow *et al.*, 2009) and this is associated with increased compulsive drug-taking behaviour (Perez *et al.*, 2011).

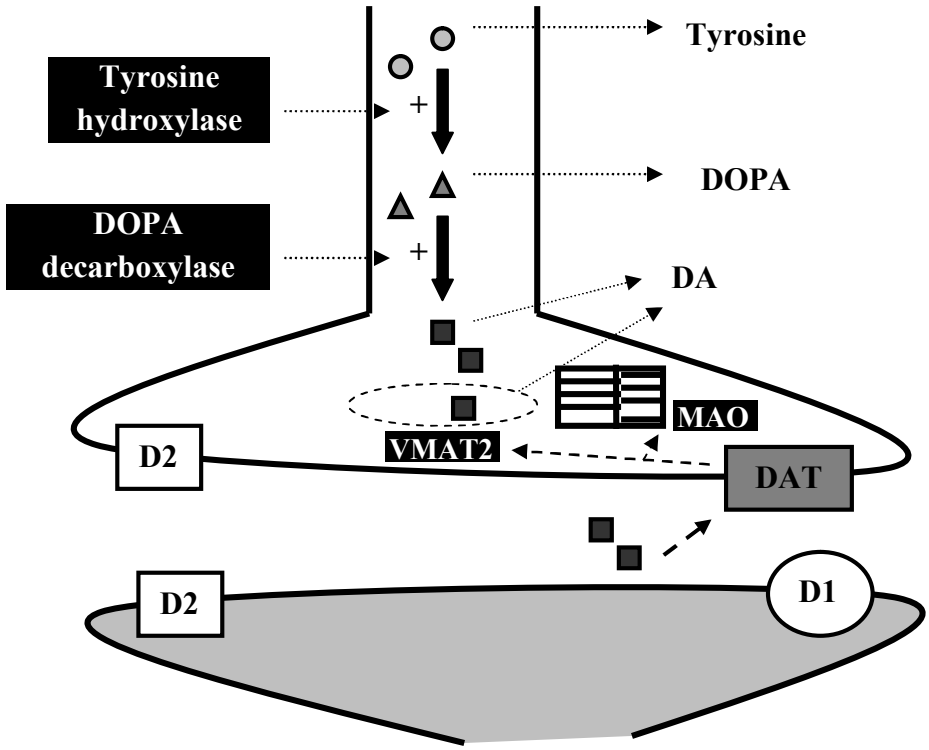

Figure 1.2 Neuronal synthesis and metabolism of dopamine (Marsden, 2006). After its release in the synaptic gap, dopamine acts on its main receptors: postsynaptic D1 receptors and pre- (autoreceptors) and postsynaptic D2 receptors. Reuptake of extracellular dopamine is mediated by dopamine transporters (DAT) into the nerve terminal. Neuronal dopamine is subjected to reuptake by vesicular monoamine transporters (VMAT2) or breakdown by monoamine oxidase (MAO). DA = dopamine.

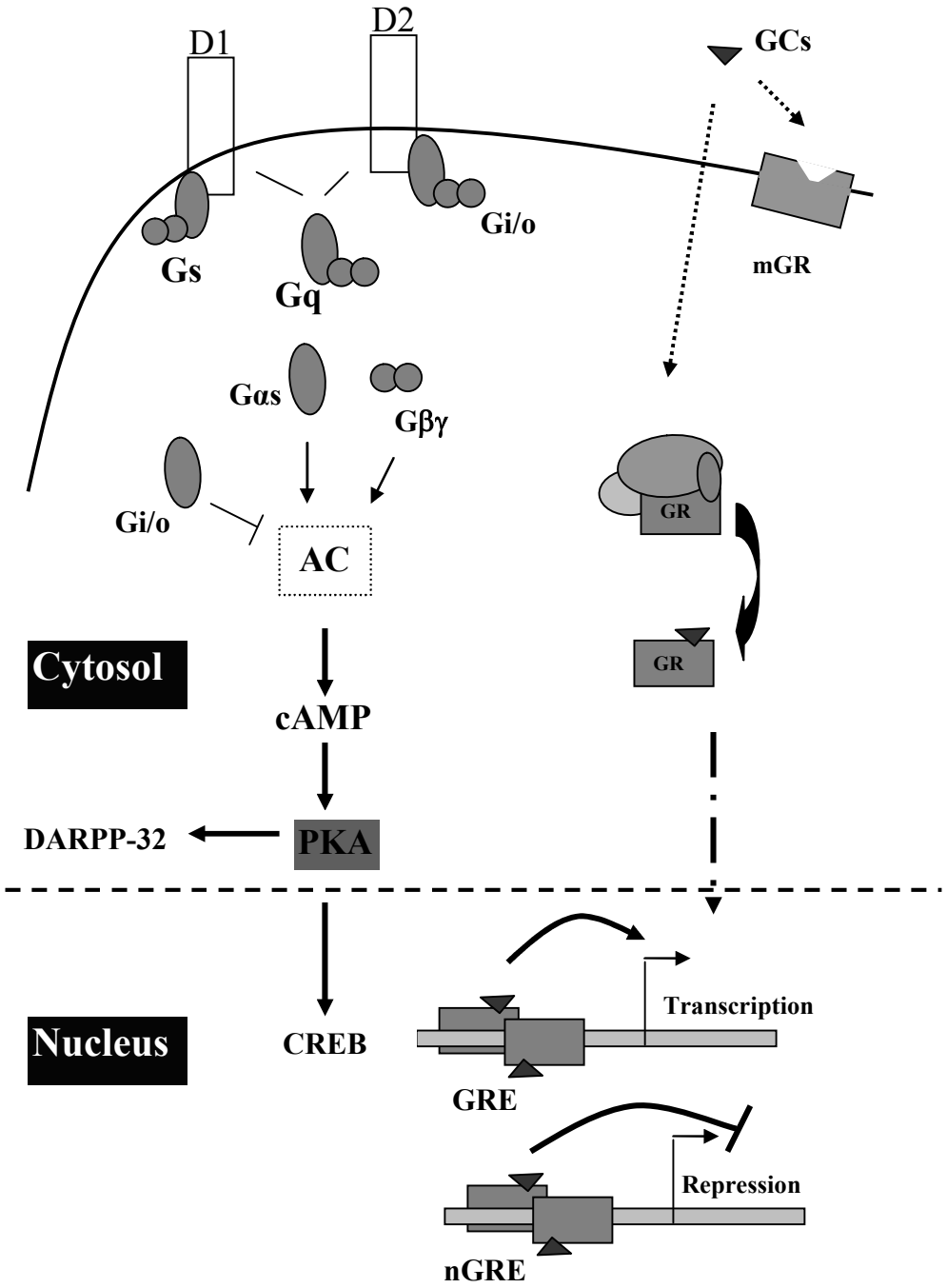

Figure 1.3 Molecular mechanisms of dopamine D1 and D2 (Lee and Messing, 2008) and glucocorticoid receptors (Fink, 2010). G proteins: stimulatory Gs and Gq and inhibitory Gi/o, AC = adenylyl cyclase, PKA = protein kinase A, DARPP-32 = dopamine- and cAMP-regulated phosphoprotein of 32 kDa, CREB = cyclic AMP response element binding protein, GCs = glucocorticoids, GR = glucocorticoid receptor, mGR = membrane glucocorticoid receptor, GRE = glucocorticoid response element, nGRE = negative glucocorticoid response element.

1.1.4 How to verify that a drug or behaviour is rewarding?

Three essential criteria are needed to establish whether a drug or behaviour is rewarding, or not.

1. Individuals should show a clear preference for the drugs or behaviours and self-access them if they have rewarding properties. In pre-clinical studies, several methods can be performed, such as self-administration studies, conditioned place preference (CPP); association between the exposure to a reward and environmental cues, and behavioural sensitization (Ikemoto, 2010). Addicts should also be able to describe the euphoric potential of administered drugs (Laruelle *et al.*, 1995).
2. There should be a clear association between specific brain neurotransmitters and specific reward-related behaviours (Robbins and Everitt, 1996). This can be assessed by using microdialysis (Devine *et al.*, 1993) and voltammetry (Doherty and Gratton, 2007) techniques, which measure the change in the levels of certain neurotransmitters in specific brain areas previously reported to be activated in response to abused drugs. Conversely, the abused drug could be injected in specific brain areas such as the nucleus accumbens (Delfs *et al.*, 1990) to test their involvement in the rewarding effects of the drug. In humans, imaging studies have been used to investigate which areas and neurotransmitters in the brain are associated with drug-induced euphoria (Volkow *et al.*, 2012).
3. The blockade of these secreted neurotransmitters by pharmacological approaches such as antagonism of dopamine receptors (David *et al.*, 2002) or by destroying the dopaminergic neurons using 6-hydroxy dopamine (Berridge and Robinson, 1998) should abolish the expression of their reward-related behaviour.

1.1.5 Drug addiction

Drug addiction is a chronic relapsing disorder induced by rewarding effects of abused drugs in susceptible individuals (10-20 % of drug abusers who have no control on drug intake). According to the Diagnostic and Statistical Manual of Mental disorders fourth edition (DSM-IV), substance-dependents are described

as those demonstrating uncontrolled compulsive drug intake despite negative consequences (Volkow *et al.*, 2012). There are three main characteristics of drug addiction or substance dependence: compulsive seeking for the drug, loss of control when intake is reduced and appearance of negative emotional symptoms such as dysphoria and anxiety upon stopping the drug access (Koob and Le Moal, 2008). Risk factors such as stress, drug access, adolescence, genetic background and addictive properties of the abused drugs have been reported to increase the vulnerability to drug-seeking behaviour (Volkow and Li, 2005; Koob and Le Moal, 2008).

In rodents, increased locomotor activity (behavioural sensitization) following repeated exposure to abused drugs, such as psychostimulants, is an important sign of addiction (Robinson and Berridge, 1993), while continuous drug administration may result in the development of tolerance which is the reduction of drug-induced euphoria with repeated administration of a fixed dose of a drug (Nestler, 1992). Previous research has implicated dopamine in the control of locomotor activity and in abused drugs-related locomotor hyperactivity. For example, intracerebral injections of morphine into the ventral tegmental area of rats selectively increased their locomotor activity, an effect blocked by prior administration of the opioid receptor antagonist, naloxone, or with the antipsychotic D2 receptor antagonist, haloperidol (Joyce and Iversen, 1979). In addition, direct microinjections of cocaine in the nucleus accumbens produced a dose-dependent increase in locomotor activity of rats (Delfs *et al.*, 1990).

Drug addiction develops in consecutive stages: drug taking (feeling euphoric or high), impulsive drug use (feeling aroused and tense just before the administration of the drug), compulsive or repetitive drug use (feeling stressed and anxious, which subsides upon drug intake), dependence, withdrawal (abstinence) stage, and finally relapse or recovery (Koob and Le Moal, 2008)

Imaging studies using PET/MRI have shown that there are four major circuits in the human brain which underlie the development of drug addiction (Volkow *et al.*, 2011; Volkow *et al.*, 2012), Fig 1.4:

1. The reward pathway (the ventral tegmental area, nucleus accumbens and ventral pallidum).
2. Salience attribution and motivation/drive (the medial orbitofrontal cortex, dorsal striatum and motor cortex).
3. Memory, conditioning and habit formation (hippocampus, amygdala, the medial orbitofrontal cortex and dorsal striatum)
4. Executive function and inhibitory control (the prefrontal cortex).

The major neurotransmitters involved in these areas and in drug-seeking behaviour include dopamine, noradrenaline, corticotropin releasing factor (CRF), GABA and glutamate (Volkow *et al.*, 2003; Volkow *et al.*, 2012). In addicts, the activation of reward pathways enhances motivation (impulsivity) to seek abused drugs leading to reduced inhibitory control from the prefrontal cortex on the reward mediating areas ending with impulsivity/compulsivity cycle (Koob and Le Moal, 2008; Volkow *et al.*, 2012).

Taken together, dopamine and dopaminergic receptors are targeted by the majority of abused drugs and primary rewards, in particular the mesocorticolimbic dopamine pathway is involved in the response to rewards. Other areas associated with the limbic system are also required for the conditioning, associative learning, memory processing of rewards and development of addiction.

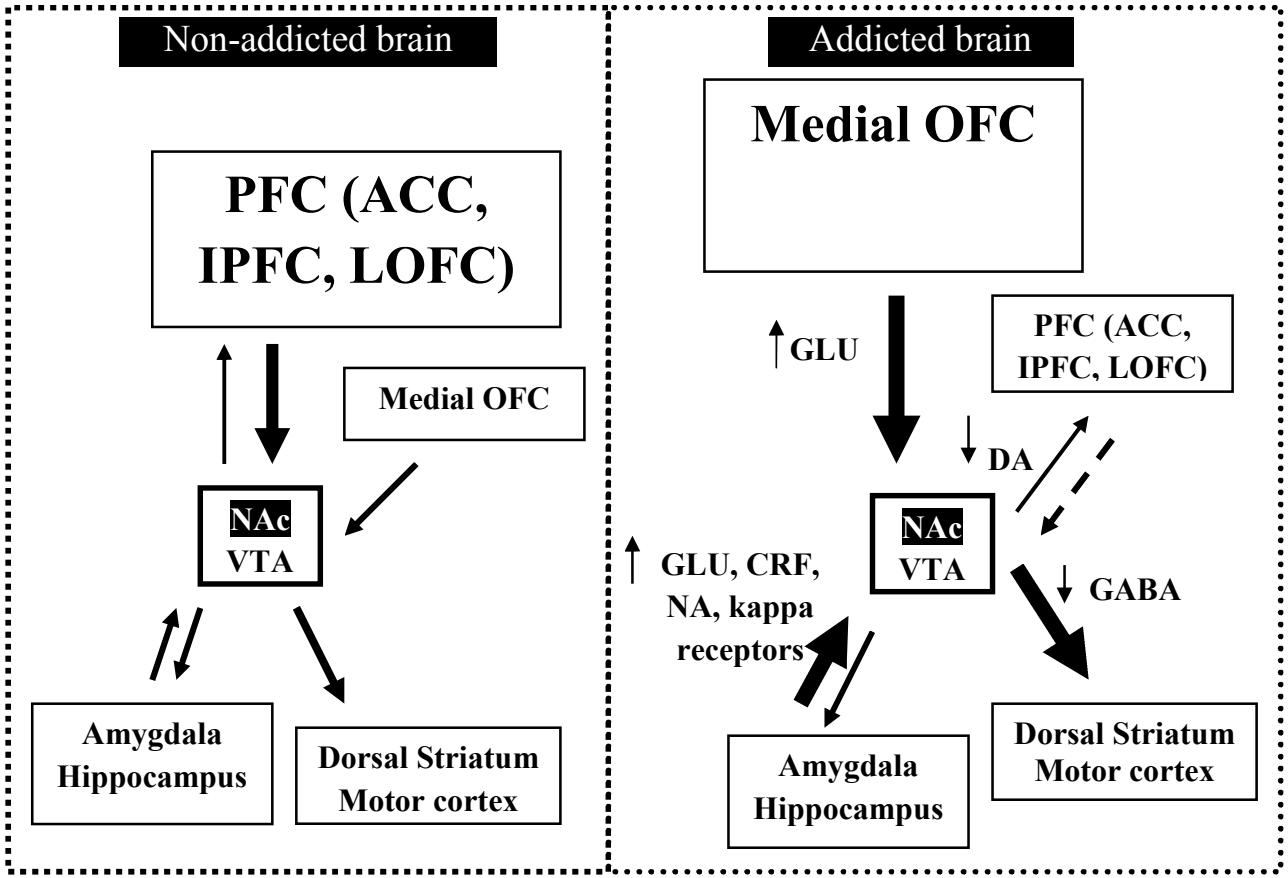

Figure 1.4 Theoretical network of human brain circuits involved in the development of drug addiction. The consequences of addiction on the brain as shown by arrows were proposed by (Volkow *et al.*, 2011) based on imaging studies.

Circuits: 1- Reward (NAc = nucleus accumbens, VTA = ventral tegmental area). 2- Memory and conditioning (hippocampus, amygdala, medial orbitofrontal cortex (OFC) and dorsal striatum). 3- Motivation/drive (medial OFC, VTA, dorsal striatum and motor cortex). 4- Executive function and inhibitory control (PFC = prefrontal cortex which includes ACC = anterior cingulate cortex, IPFC = inferior PFC and lateral OFC).

Neurotransmitters involved are CRF = corticotropin releasing factor, DA = dopamine, GABA, GLU = glutamate, NA = noradrenaline. This figure is adapted from (Volkow *et al.*, 2011).

1.2 Corticosterone

Corticosterone is the main glucocorticoid in rodents. Its secretion by the adrenal glands peaks naturally during the dark (active) phase and reaches a nadir in the light (inactive) phase (Halberg *et al.*, 1958). Corticosterone levels also increase in response to stress (Sapolsky *et al.*, 2000) and abused drugs (Moldow and Fischman, 1987). Endogenous glucocorticoid (GC) secretion is vital for the regulation of metabolism, immune and central nervous system functions (Buckingham, 2006) and plays a role in learning and memory processes (Akirav *et al.*, 2004). GC elevation have been reported in certain hormonal (Fink, 2010), metabolic (Buckingham, 2006), psychiatric (Souetre *et al.*, 1988) and cognitive (Swanwick *et al.*, 1998) disorders. As discussed below, corticosterone is also thought to modulate rewarding effects of abused drugs and may have rewarding properties of its own. The biosynthesis of corticosterone (Fink, 2010) is illustrated in Fig. 1.5.

1.2.1 Stress and hypothalamic-pituitary-adrenocortical (HPA) axis

Stress has a profound impact on living organisms, usually triggered by a stimulus (stressor), to which an organism responds (stress perception). Then, other physiological systems take part in the response to stress, including the immune, endocrine, and central nervous systems (Cacioppo *et al.*, 1998; Sapolsky *et al.*, 2000). The response to stress, acute or chronic, is an evolutionarily adaptive psychophysiological survival mechanism that gives an individual the opportunity to react "fight or flight," and/or to save energy (Cannon, 1914). Hans Selye, who introduced the concept of stress in biology, gave many definitions of stress according to experimental progress. The simplest definition of stress is ''a non specific response of the body to any demand'' (Fink, 2010). Other stress definitions have been extended to involve not just the biological response to stress, but also its impact on cognition and mood status (Fink, 2010).

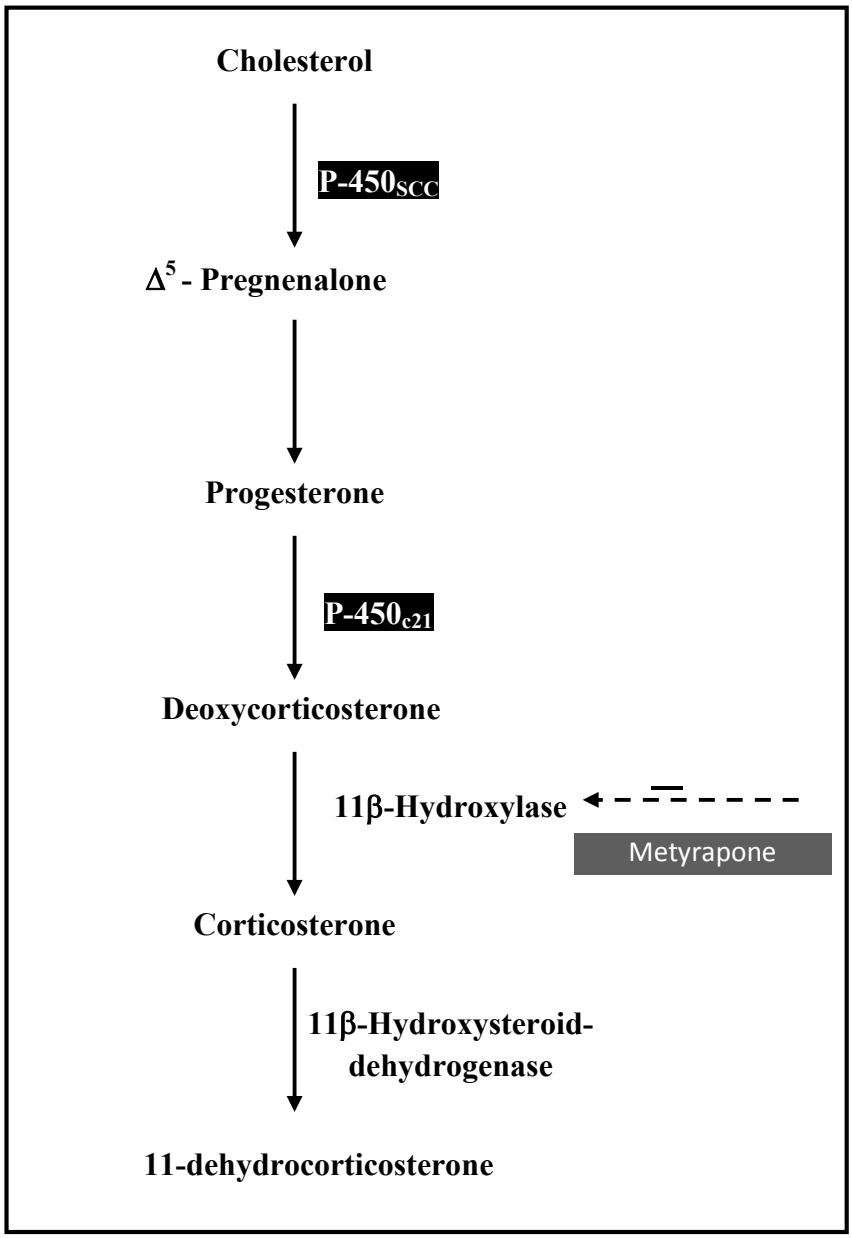

Figure 1.5 Biosynthesis of corticosterone in the adrenal cortex (Fink, 2010). Metyrapone inhibits 11β-hydroxylase which induces the conversion of deoxycorticosterone to corticosterone.

The hypothalamic-pituitary-adrenocortical (HPA) axis is well known as a major component of the stress response (Selye, 1950; Mcewen, 1991; Sapolsky *et al.*, 2000). In addition, it has been documented that stress activates extra-hypothalamic limbic-associated regions including the hippocampus, locus coeruleus (the noradrenaline nucleus), basolateral and central nucleus of the amygdala, bed nucleus of stria terminalis and adrenal medulla which secretes adrenaline (Sapolsky *et al.*, 2000; Belujon and Grace, 2011), and the hippocampus and amygdala are also involved in the regulation of HPA axis activity (Sapolsky *et al.*, 1984; Lowry, 2002). Activation of the HPA axis involves the secretion of CRF, a 41 amino acid hypothalamic peptide (Vale *et al.*, 1981) and arginine vasopressin from parvocellular neurons in the paraventricular nucleus of the hypothalamus, which trigger the production and release of anterior pituitary proopiomelanocortin-derived peptides, adrenocorticotropic hormone (ACTH) and β-endorphin (Vale *et al.*, 1981), Fig. 1.6. ACTH stimulates the adrenal cortex to synthesize and secrete GCs (cortisol in human and corticosterone in rodents) (Goddyer *et al.*, 1976; Buckingham, 2006), Fig. 1.6

Reul and de Kloet (1985) have shown that GC actions in the rat brain are mediated *via* two nuclear receptors: mineralocorticoid receptors (MRs, Type I) and glucocorticoid receptors (GRs, Type II). Whereas MRs are mainly expressed in the septohippocampal complex, GRs are widely distributed in the brain (Reul and de Kloet, 1985). MRs have a 10-fold higher affinity for GCs than GRs (Reul and de Kloet, 1985). Both MRs and GRs work in a harmonized pattern. For example, levels of GCs determine their role in the response to stress starting from a permissive action at basal levels (mediated by MRs), a stimulatory action which enhances physiological effects, a suppressive action (mediated by GRs) which inhibits stress-induced effects, and a preparative action for a subsequent stress exposure (Sapolsky *et al.*, 2000). MRs are activated during inactivity (basal circadian levels of GCs) and become saturated during stress and at circadian peaks when levels of GCs rise sharply, which induce negative feedback effects on the HPA axis activity *via* stimulation of

GRs (Reul and de Kloet, 1985; Dallman *et al.*, 1989; Ratka *et al.*, 1989; Bradbury *et al.*, 1991; Funder, 1994), Fig. 1.6.

It should be noted that the intensity of stress determines the magnitude of corticosterone increase. While stressors such as exposure to cold, ether or cage transfer is associated with an increase in plasma corticosterone levels between 15 and 20 μg/dl (Sapolsky *et al.*, 1985), restraint stress produced higher levels of corticosterone at 40 μg/dl (Sapolsky *et al.*, 1984) in rats. On the other hand, corticosterone (40 mg/kg, sc) produced a high plasma level of corticosterone (68 μg/dl) which decreased to 32 μg/dl 24 hours after the end of 21 days of chronic corticosterone injections (Pavlides *et al.*, 1993).

1.2.2 Pharmacodynamic properties of GRs

GRs are mainly located in the cytosol in an inactive state in combination with chaperone proteins; heat shock protein 90 and 70 (HSP70 and HSP 90) and 56-kDa immunophilin. Also, several studies have shown that membrane GRs exist (Tasker *et al.*, 2006; Roozendaal *et al.*, 2010). When GCs bind to the ligand-binding domain of the cytosolic GRs, the latter dissociate from the chaperone proteins, become active and their DNA binding domain becomes available for interaction with specific DNA regions. The interaction between the GC-GR complex and DNA takes place in the nucleus after translocation of the active complex, Fig. 1.3. Within the nucleus, two molecules of GC-GR complex bind together to form homodimers which bind to positive glucocorticoid response elements (GRE) or negative GRE in DNA, thereby mediating gene transcription or repression, respectively (Fink, 2010), Fig. 1.3. The main difference between nuclear and membrane GRs is that the latter mediate rapid non-genomic effects, while the shortest gene transcription induced by nuclear GRs has been found to be 15 minutes (Hallahan *et al.*, 1973).

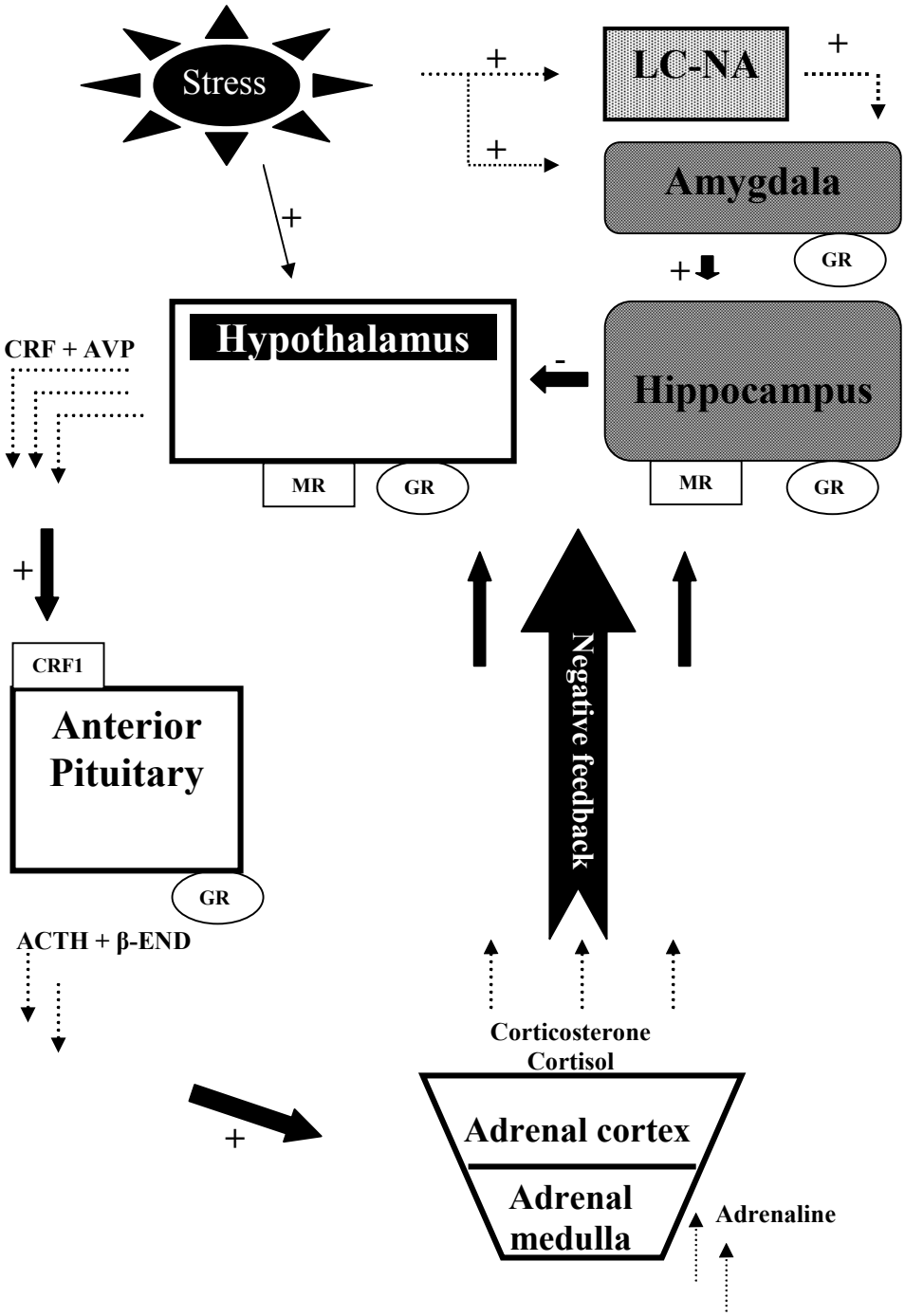

Figure 1.6 Schematic representation of the stress response and negative feedback of glucocorticoids (corticosterone or cortisol) on HPA axis activity and certain extra-hypothalamic tissues; the hippocampus, amygdala, locus coeruleus (LC) and adrenal medulla. ACTH = adrenocorticotropic hormone, AVP = arginine vasopressin, β-END = β-endorphin, CRF = corticotropin releasing factor, CRF_1 = CRF receptor$_1$, NA = noradrenaline.

1.2.3 Impacts of corticosterone on memory

Corticosterone is involved in learning and memory processing (Akirav *et al.*, 2004; Okuda *et al.*, 2004; Barsegyan *et al.*, 2010; Roozendaal *et al.*, 2010): a number of studies regarding stress- or corticosterone-related effects on synaptic plasticity and memory performance are listed in table 1.1. As mentioned earlier, MRs are abundant in the hippocampus (Reul and de Kloet, 1985), which is critical for memory performance (Gould *et al.*, 2002; Broadbent *et al.*, 2004; Heldt *et al.*, 2007). In contrast, GRs are widely expressed in the brain and mainly activated by high corticosterone levels (Reul and de Kloet, 1985), and, therefore, stress-related effects on memory are mediated by the activation of GRs. The intensity of activation of both MRs and GRs controls emotional level and cognitive learning. It was reported that chronic activation of MRs by aldosterone was anxiogenic in rats (Hlavacova and Jezova, 2008). In contrast, activation of MRs was associated with low anxiety levels in mice (Brinks *et al.*, 2007; Rozeboom *et al.*, 2007). Whereas MRs have been found to be essential for explorative behaviour (Brinks *et al.*, 2007), GRs have been reported to be implicated in memory processing such as spatial, recognition and fear memory (Oitzl *et al.*, 1997; Barsegyan *et al.*, 2010; Roozendaal *et al.*, 2010). Thus, activation of MRs and moderate activation of GRs improved memory performance in C57BL/6 mice (Brinks *et al.*, 2007). In addition, moderate corticosterone elevation enhanced spatial (Akirav *et al.*, 2004) and recognition memory (Okuda *et al.*, 2004) in rats.

Increased corticosterone levels have been reported to mediate harmful effects of stress on cognition (McEwen and Sapolsky, 1995; Sandi, 2004). In conjunction with this, repeated corticosterone injections for 3 months decreased neuronal number in the hippocampus (Sapolsky *et al.*, 1985). Long-term potentiation (LTP), long lasting synaptic plasticity which is thought to mediate some forms of learning and memory, in the hippocampal/prefrontal cortex neuronal circuits was depressed in rats after exposure to acute (Rocher *et al.*, 2004) or chronic (Cerqueira *et al.*, 2007) stress. In agreement with the effects of chronic stress on LTP, repeated restraint stress for 3 weeks induced reversible spatial memory impairments in Sprague-Dawley rats (Luine *et al.*, 1994). Moreover, 4 weeks of unpredictable stress impaired spatial working and reference memory in rats

(Cerqueira *et al.*, 2007). Several factors, such as corticosterone levels and duration of exposure have been found to induce different effects on synaptic plasticity and memory. It has been reported that effects of corticosterone on memory follow an inverted U-shaped pattern (Diamond *et al.*, 1992), meaning that moderate corticosterone elevation improves memory even for long time (Pravosudov, 2003). Stress levels of corticosterone (> 20 μg/dl) induced by implanted corticosterone pellets in adrenalectomized Sprague-Dawley rats inversely correlated with the magnitude of primed burst potentiation (PBP), a long-lasting (>30 minutes) physiological model of memory, in the CA1 region of the hippocampus (Diamond *et al.*, 1992). In contrast, low and moderate corticosterone levels showed a direct relationship with the magnitude of primed burst potentiation (Diamond *et al.*, 1992). Whereas hippocampal LTP in Sprague-Dawley rats was reduced 2 hours after a single corticosterone injection, there were no effects after 48 hours (Pavlides *et al.*, 1993). In contrast, chronic corticosterone injections at high doses (40 mg/kg) for 3 weeks impaired hippocampal LTP in Sprague-Dawley rats 48 hours after the last injection (Pavlides *et al.*, 1993). Furthermore, a MR agonist (aldosterone) improved LTP, while a GR agonist (RU28362) impaired LTP in the dentate gyrus, a hippocampal region in which new granule neurons are generated throughout adulthood (Gould and Gross, 2002), of Sprague-Dawley rats (Pavlides *et al.*, 1995). These responses were abolished by treating adrenalectomized rats with MR and GR antagonists, respectively (Pavlides *et al.*, 1995). It was reported that mifepristone (a GR antagonist) abolished acute restraint stress-induced hippocampal LTP impairment in Sprague-Dawley rats *via* attenuating the increased activity of extracellular signal regulated kinase 1 and 2, ERK 1/2 (Yang *et al.*, 2004). In contrast, chronic corticosterone treatment decreased phosphorylated Erk2 levels in the hippocampus and frontal cortex of mice (Gourley *et al.*, 2008). In effect, ERK 1/2 has been found to modulate synaptic plasticity and learning processes (Rodrigues *et al.*, 2004).

In agreement with the chronic effects of corticosterone on LTP, memory performance of animals has been reported to be affected in similar way. Chronic corticosterone treatment (26.8 mg/kg/day, s.c.) for 8 weeks impaired spatial memory of Sprague-Dawley rats in a Y-maze (Coburn-Litvak *et al.*, 2003).

Moreover, corticosterone-implanted rats exhibited poor spatial learning performance in the radial 8-arm maze after 3 months (Endo *et al.*, 1996). However, rats treated with a lower corticosterone dose (10 mg/kg/day) for 8 weeks exhibited no impairment in the platform maze spatial task (Bardgett *et al.*, 1996), indicating no hippocampal LTP impairment (Barnes, 1979). However, this dose was found to induce atrophy in apical dendrites in the hippocampal CA3 region of Sprague-Dawley rats (Woolley *et al.*, 1990; Watanabe *et al.*, 1992). An acute corticosterone injection in the prefrontal cortex impaired delayed alternation in Sprague-Dawley rats (Barsegyan *et al.*, 2010). Also, corticosterone treatment shortly before the Morris water maze test impaired memory retrieval in Sprague-Dawley rats (Roozendaal *et al.*, 2001). In contrast, acute blockade of GRs prior to the probe test of the Morris water maze task improved spatial memory in rats (Oitzl *et al.*, 1998), indicating that elevation of corticosterone shortly before memory testing could impair working memory (Barsegyan *et al.*, 2010). Also, mifepristone (a glucocorticoid receptor antagonist) and metyrapone (a corticosterone synthesis inhibitor) ameliorated recognition memory deficits caused by corticosterone elevation induced by a short period of morphine withdrawal in mice (Mesripour *et al.*, 2008).

It is important to emphasize that corticosterone plays an important role in cognitive performance. Adrenalectomized rats showed spatial memory deficits (Vaher *et al.*, 1994), which could be due to impairment in exploratory activity (Veldhuis *et al.*, 1982). Also, spatial memory was suggested to be improved in running rats *via* corticosterone increase (Hajisoltani *et al.*, 2011). Corticosterone has been found to be involved in recognition memory (Okuda *et al.*, 2004). Moreover, post-training corticosterone injections enhanced consolidation of novel object recognition and location in Sprague-Dawley rats and these effects were mediated *via* GRs (Roozendaal *et al.*, 2010). Also, fear memory was improved by pre-test corticosterone injection in the prefrontal cortex of Sprague-Dawley rats (Barsegyan *et al.*, 2010).

Overall, short-term exposure to moderate levels of corticosterone is required to facilitate learning and improve memory. In contrast, chronic corticosterone

elevation could cause memory deterioration through effects on neural plasticity and survival.

Table 1.1 A number of studies carried out to examine the effects of acute and chronic exposure of stress or corticosterone treatment on synaptic plasticity and memory performance. Underlined references indicate that Sprague-Dawley rats were used. PBP = primed burst potentiation.

		Synaptic plasticity neuronal survival	Memory
Stress	**Acute**	Impaired LTP *(Rocher et al., 2004; Yang et al., 2004)*	Impaired recognition memory with morphine induced withdrawal (Mesripour et al., 2008)
	Chronic	Impaired LTP *(Cerqueira et al., 2007)*	Spatial impairment *after 3 or 4 weeks of restraint (Luine et al., 1994)* or unpredictable *(Cerqueira et al., 2007)* stress.
Corticosterone, MR and GR agonists and antagonists	**Acute**	Inverted U-shaped relationship between corticosterone levels and PBP *(Diamond et al., 1992)* A single high corticosterone dose decreased hippocampal LTP *(Pavlides et al., 1993)* MR activation increased LTP and GR activation decreased LTP in the rat hippocampus *(Pavlides et al., 1995)*	Inverted U-shaped relationship between corticosterone levels and recognition *(Okuda et al., 2004)* or spatial (Akirav et al., 2004) memory. GR blockade improved spatial memory *(Oitzl et al., 1998)* Impairment of delayed alternation *(Barsegyan et al., 2010)* Improvement of inhibitory avoidance *(Barsegyan et al., 2010)* Enhanced consolidation of spatial *(Sandi et al., 1997)*, recognition and location *(Roozendaal et al., 2010)* memory. Memory retrieval impairment *(Roozendaal et al., 2001)*
	Chronic	A high dose for 3 weeks impaired hippocampal LTP *(Pavlides et al., 1993)* Three weeks induced reversible hippocampal atrophy *(Woolley et al., 1990; Watanabe et al., 1992)* Hippocampal neuronal loss after three months *(Sapolsky et al., 1985)*	A high dose (26.8 mg/kg) did not impair spatial working memory after 21days, but impaired it after 8 weeks in a Y-maze *(Coburn-Litvak et al., 2003).* A small dose (10 mg/kg) for 8 weeks produced no spatial memory impairment *(Bardgett et al., 1996)* Three months of moderate corticosterone elevation improved spatial memory *(Pravosudov, 2003)* Three months impaired spatial memory in radial 8-arm maze *(Endo et al., 1996)*

1.3 Rewarding effects of stress and corticosterone

1.3.1 Overview

It has been found that exposure to natural rewards such as food (Merali *et al.*, 1998), copulation (Szechtman *et al.*, 1974) and wheel running (Droste *et al.*, 2003) are accompanied by corticosterone elevation. Moreover, several studies have indicated that corticosterone modulates reinforcing and rewarding effects of abused drugs (Dong *et al.*, 2006; Mantsch and Katz, 2007). The clinical use of GCs has been limited by their numerous adverse effects including psychotic symptoms with persistent euphoria, suggesting that GCs might have rewarding effects (Hall *et al.*, 1979). In addition, euphoria was reported in a patient with Cushing's syndrome (Myhill *et al.*, 2008), an endocrine disorder characterised by elevated cortisol levels (Fink, 2010). Taken together, corticosterone is suggested to have rewarding effects. Previous work showing evidence for corticosterone-related reward is reviewed in the next section.

1.3.2 Rewarding effects of stress

Avoidance is the most common reaction of an organism to stress. However, stress-seeking behaviour has been observed in different occasions. For example, monkeys were trained to lever-press to receive electrical shocks in their tails (Barrett and Spealman, 1978). Also, rats were found to develop place preference to certain forms of mild stress such as frequent handling (Bozarth, 1987). Whereas some Sprague-Dawley rats showed high locomotor activity and sustained corticosterone secretion when exploring novel environments (e.g. High responders, HR rats), others (e.g. low responders, LR rats) avoided exploring new environments and had low basal corticosterone levels (Piazza *et al.*, 1990; Piazza *et al.*, 1991; Rouge-Pont *et al.*, 1993), indicating that GCs could underlie stress-seeking behaviour as increased corticosterone or cortisol secretion is a common feature associated with stress exposure. HR rats have been found to be more sensitive to psychostimulants and exhibit a higher tendency to self-administer these drugs than LR rats (Piazza *et al.*, 1989; Piazza *et al.*, 1990; Piazza *et al.*, 1991), suggesting that elevated basal corticosterone levels in HR rats facilitate drug-seeking behaviour. Interestingly, HR rats displayed higher dopamine release in the nucleus accumbens than LR rats in

response to 10-minute tail-pinch (Rouge-Pont *et al.*, 1993), but this difference in dopamine levels disappeared in adrenalectomized rats (Rouge-Pont *et al.*, 1998).

Stress has been reported to trigger dopamine release in brain areas associated with reward. For instance, painless footshocks increased extracellular dopamine levels in the nucleus accumbens shell of Sprague-Dawley rats (Kalivas and Duffy, 1995). Moreover, the same rat strain exhibited increased dopamine release in the nucleus accumbens when they were subjected to ten-minute tail pinch stress, but adrenalectomized rats exhibited an increase in dopamine release only after corticosterone treatment (Rouge-Pont *et al.*, 1998), suggesting that stress-induced corticosterone secretion enhances dopamine release. Stress has been repeatedly reported to facilitate self-administration of abused drugs (Piazza and Le Moal, 1998). Furthermore, food restriction enhanced behavioural sensitization induced by amphetamine or morphine injections in the nucleus accumbens and the ventral tegmental area of Sprague-Dawley rats, respectively (Deroche *et al.*, 1995). In addition, the positive effects of food restriction on sensitization of mesencephalic dopamine release in response to cocaine was attenuated in Sprague-Dawley rats after being treated with the corticosterone synthesis inhibitor metyrapone (Rouge-Pont *et al.*, 1995).

It is noteworthy that stress and abused drugs have been found to induce common alterations in the brain regions involved in drug-seeking behaviour (Cleck and Blendy, 2008). For instance, enhanced activation of glutamate receptors in midbrain dopaminergic neurons appears to be involved in the synaptic adaptation induced by stress and abused drugs (Saal *et al.*, 2003). Furthermore, modulation of GABA receptor activity in the ventral tegmental area has been implicated in stress-induced dopamine release in the nucleus accumbens (Doherty and Gratton, 2007). Similarly, drugs of abuse modulate GABA neurotransmission in the mesolimbic dopamine pathway (Koob and Le Moal, 2008). Stress-induced reinstatement of drug-seeking behaviour has been suggested to involve activation of the ventral subiculum (the main hippocampus output) / nucleus accumbens pathway *via* the activation of the basolateral amygdala and locus coeruleus (noradrenaline secreting neurons); both of them

regulate glutamatergic activity in the ventral subiculum (Belujon and Grace, 2011). It was found that CRF was involved in footshock-induced cocaine reinstatement (Erb *et al.*, 1998). Stress-evoked corticosterone increase was also reported to promote cocaine self-administration (Mantsch and Katz, 2007).

1.3.3 Corticosterone facilitates acquisition and reinstatement of abused drugs

Stress is implicated in worsening drug addiction (Cleck and Blendy, 2008) and therefore, a considerable body of research has been carried out to investigate the role of corticosterone in drug addiction. Several studies have addressed how addictive drugs alter endogenous corticosterone secretion and others have investigated the impact of elevated corticosterone levels on drug self-administration, conditioned place preference (CPP) and behavioural sensitization. A single cocaine injection in male Sprague-Dawley rats was associated with transient corticosterone elevation (Moldow and Fischman, 1987). In contrast, low basal plasma corticosterone levels were observed in Wistar rats 24 hours after chronic non-contingent or self-administration of cocaine infusions compared to saline-treated rats (Mantsch and Goeders, 2000).

As mentioned earlier, elevated basal corticosterone levels facilitated amphetamine self-administration in HR Sprague-Dawley rats (Piazza *et al.*, 1991). Whereas high doses of cocaine enhanced corticosterone secretion which sufficiently maintained its self-administration, low cocaine doses could not efficiently be self-administered at reduced corticosterone levels (Goeders and Guerin, 1996), indicating the role of corticosterone in drug-seeking behaviour. The reduction of cocaine intravenous self-administration was dose-dependently reversed in adrenalectomized male Sprague-Dawley rats by simultaneous administration of corticosterone in the drinking water at concentrations of 25, 50 or 100 μg/ml (Deroche *et al.*, 1997). Furthermore, intravenous corticosterone injections (0.37 mg/kg) facilitated cocaine-induced reinstatement in adrenalectomized rats (Deroche *et al.*, 1997), indicating that corticosterone underlies stress-induced reinstatement of abused drugs. Nevertheless,

corticosterone elevation was not involved in chronic footshocks-induced escalation of cocaine self-administration in male Sprague-Dawley rats (Mantsch and Katz, 2007), indicating that stress probably modulates drug-seeking behaviour *via* corticosterone dependent and independent mechanisms. Also, administration of corticosterone synthesis inhibitors such as metyrapone or aminoglutethimide attenuated stress-enhanced CPP to morphine in Sprague-Dawley rats (Der-Avakian *et al.*, 2005). Moreover, cocaine- or morphine-induced locomotor hyperactivity was reduced in adrenalectomized Sprague-Dawley rats compared to sham-operated and corticosterone-supplemented rats (Marinelli *et al.*, 1994).

It is noteworthy that the corticosterone administration in drinking water (100 μg/ml) or parenterally (0.37 mg/kg, i.v.) was reported to increase plasma corticosterone levels to ~ 40-60 μg/dl (Deroche *et al.*, 1995; Deroche *et al.*, 1997) similar to those levels induced by electric footshocks (Bassett *et al.*, 1973). This suggests that GR activation is involved in drug-seeking behaviour, as GRs are found to be activated by stress levels of corticosterone (Reul and de Kloet, 1985). For instance, morphine-induced dopamine release in the nucleus accumbens and locomotor hyperactivity, a dopamine-dependent behaviour (Le Moal *et al.*, 1977; Delfs *et al.*, 1990), was reduced by prior administration of the GR antagonists, mifepristone or RU39305, in a dose-dependent manner (Marinelli *et al.*, 1998). In contrast, these morphine-related effects were retained after treating rats with the MR antagonist, spironolactone (Marinelli *et al.*, 1998). Furthermore, mifepristone injected into the hippocampus or the nucleus accumbens of Sprague-Dawley rats impaired morphine-induced CPP in a dose-dependent manner (Dong *et al.*, 2006), suggesting that the behavioural expression of CPP to morphine is modulated by GR activation. In contrast, basal corticosterone levels were sufficient for cocaine reinstatement in Long Evans rats compared to the adrenalectomized group, indicating that the GR involvement in cocaine-seeking behaviour is strain-dependent (Erb *et al.*, 1998). In agreement with this, spironolactone decreased cocaine-induced locomotor hyperactivity but mifepristone attenuated cocaine-self-administration in

C57BL/6J mice (Fiancette *et al.*, 2010), suggesting a role of MRs in cocaine-related unconditioned effects.

In support of behavioural data, there has been some evidence that corticosterone interacts with the mesolimbic reward pathway. In Sprague-Dawley rats, GRs are expressed by mesolimbic dopaminergic neurons (Harfstrand *et al.*, 1986). So, it is expected that mesolimbic dopamine release in response to drugs of abuse and other rewarding stimuli is controlled at least partially by corticosterone. Indeed, *in vitro* application of a GR antagonist and a MR antagonist to cultured embryonic mesencephalic dopaminergic neurons showed that only a GR antagonist decreased potassium-activated dopamine secretion in a dose-dependent manner (Rouge-Pont *et al.*, 1999). This gives some evidence that corticosterone increases dopamine release *via* GRs.

Taken together, the evidence supports the proposal that corticosterone facilitates drug self-administration and conditioning effects likely *via* activation of GRs.

1.4 Does corticosterone have reinforcing and rewarding properties?

Three experiments directly addressed if corticosterone is rewarding, with contradictory results. As discussed below, two studies showed that male Sprague-Dawley rats self-administered corticosterone, but they did not exhibit corticosterone-induced conditioned place preference in the third study.

1.4.1 Self-administration studies

In the first experiment, Piazza *et al.* (1993) addressed whether rats self-administer corticosterone as they do for abused drugs. Rats were subjected to a series of operant intravenous self-administration sessions, a 30-min session per day, in the dark phase. The operant self-administration paradigm tests whether a substance or behaviour acts as a positive reinforcer which is defined as a consequence (A) for a behaviour (B) which increases the probability of this behaviour, i.e. A is the reinforcing properties of a drug and B is the response of the animal such as lever-pressing (Gluck, 2008). In the classical Pavlovian

conditioning, an animal learns an association between a conditioned stimulus (sound or light) and an unconditioned stimulus (rewarding or aversive), but the response of the animal to the conditioned stimulus does not affect the availability of the unconditioned stimulus (Gluck, 2008). In contrast, in instrumental conditioning, such as the operant procedures, the animal has to lever-press to receive a reinforcer (Gluck, 2008).

In the operant procedures, an animal is placed in a computer-controlled chamber which has two holes or two levers (active and inactive). When the animal inserts its nose into the active hole or presses the active lever, a dose of tested substance is then delivered to be consumed within a specified time. Three dose levels of corticosterone; 12.5, 25 and 50 μg, i.v., were tested in these sessions (Piazza *et al.*, 1993). Rats self-administered the two highest doses used, which produced plasma corticosterone levels in the range induced by stress, as the number of injections and nose pokes were significantly high in the corticosterone self-administered group compared to control. Although corticosterone was self-administered, more investigation is required. These sessions took place only in the dark phase for only a 30-minute session over 6 days, which might be insufficient to induce reward at low doses. In the dark phase, rodents are naturally active and corticosterone peaks at this time which may contribute to the self-administration behaviour. Also, rats in this experiment were not tested for reinstatement to corticosterone after a period of withdrawal. This experiment also demonstrated that HR rats, which are stress (novelty) seekers, self-administrated corticosterone at higher rate than LR rats.

In another experiment, the same research group used a two-bottle choice model to confirm that corticosterone could be orally self-administrated in rats (Deroche *et al.*, 1993). Corticosterone was added to drinking water at three different concentrations. Rats self-administered corticosterone at the two highest concentrations (50 and 100 μg/ml) used, as they preferred corticosterone to water, but not at a low concentration (25 μg/ml). The short duration of the choice test, carried out over the dark phase for only 5 days, may not have been sufficient for the development of corticosterone self-

administration at low doses. Another explanation is that corticosterone is self-administered only at doses which produce high plasma levels of corticosterone comparable to those induced by stress. In the same experiment, rats relapsed to the highest dose used after being submitted to a withdrawal period. Additionally, temporary food restriction produced no attenuation of drinking behaviour in rats that had access to the corticosterone solution compared to rats which had access to water only. The latter finding means that even when rats had no access to food, they drank more from the corticosterone solution than from water; rats naturally drink very little amount of water when being food restricted, but they drank a lot in the presence of corticosterone.

Overall, these two experiments have provided clear evidence that corticosterone can be self-administered by rats using the intravenous operant self-administration and the two-bottle choice paradigms, suggesting that it may be rewarding.

1.4.2 Does corticosterone induce conditioned place preference?

One experiment was carried out to investigate the rewarding properties of corticosterone in rats using the CPP test. CPP is a classical conditioning procedure widely used to study the conditioned reinforcing effects of drug and non-drug rewards (Shalev *et al.*, 2002). CPP involves 2 separated compartments which differ in their floor and walls: one compartment is paired with a test drug and the other is paired with its vehicle. In CPP, an animal learns to associate a reward with environmental cues after repeated sessions (conditioning), i.e. the conditioner is a neutral stimuli that cues the rewarding effects of a drug or behaviour which is solely able to increase dopamine levels in the nucleus accumbens of the animal predicting the reward (Volkow *et al.*, 2012), and, thus, a conditioner in CPP is the shape and colour of the floor and walls of one of the two CPP compartments. On the post-conditioning day, the animal has a probe test in a drug-free state and the time spent in each chamber is recorded. If the animal shows a significant preference for a drug-paired chamber, this indicates that the drug is rewarding (Bardo and Bevins, 2000). It has been reported that animals spend more time in the chamber previously paired with an abused drug

such as morphine (Dong *et al.*, 2006) and cocaine (Chen and Xu, 2010) than the vehicle-paired chamber. Furthermore, the nucleus accumbens (Miller and Marshall, 2005) and hippocampus (Ferbinteanu and McDonald, 2001) have been found to be implicated in the development of CPP.

Rewarding effects of corticosterone were tested in CPP in Sprague-Dawley rats (Dietz *et al.*, 2007). Rats were subjected to 8 alternating sessions in which corticosterone at two dose levels (2.5 or 10 mg/kg, i.p.) was paired with the least preferred compartment and saline was paired with the other compartment. Cocaine (12 mg/kg, i.p) was used as a positive control. The outcome of this study showed that corticosterone is neither rewarding nor aversive, since rats did not spend a significantly different time in the compartment paired with corticosterone. In contrast, cocaine induced CPP in HR and LR rats (Dietz *et al.*, 2007). Another study testing the effects of corticosterone at 10 mg/kg on ethanol-induced CPP showed that the stress hormone at this dose did not induce CPP on its own in mice (Brooks *et al.*, 2004). However, this corticosterone dose is very unlikely to be rewarding, since rats self-administered corticosterone at doses which produced stress levels of corticosterone (Piazza *et al.*, 1993) and, generally, mouse doses should be twice as much as rats' to produce the same physiological levels (Freireich *et al.*, 1966; Reagan-Shaw *et al.*, 2008).

Thus, these results are in apparent contradiction with a previous report showing that dexamethsone, a high potency GR agonist, induced CPP in rats (Lebedev *et al.*, 2003) and self-administration studies reporting that corticosterone at stress levels, required to activate GRs, increased nose-poking by rats (Piazza *et al.*, 1993).

The lack of behavioural expression of reward in the above study could be due to methodological factors. CPP has been useful for assessing the rewarding properties of drugs of abuse, as it is inexpensive, rapidly undertaken (Katz and Gormezano, 1979) and animals need no surgery and little training (Bardo and Bevins, 2000). However, major limitations of CPP involve the lack of providing animals with a state of free choice to administer the drug or not (Bardo and Bevins, 2000) as opposed to self-administration. This is critical as it is different

from the way in which drugs are abused by humans. Another limitation of CPP is the confounding potential of novelty seeking on the post-conditioning test day when animals are tested in a drug free state. It was documented that rats prefer novel to familiar contexts (Bardo *et al.*, 1993). Further, it is important to make sure that animals do not prefer one context over another before conditioning (Bardo and Bevins, 2000). Also, CPP is limited with regard to producing pharmacological dose/response relationships (Bardo and Bevins, 2000).

Many arguments have been raised against the outcome of CPP, whether it is due to learning association between a context (conditioned stimulus) and a drug (unconditioned stimulus), or if it is due to rewarding properties of the drug (Beach, 1957; Rossi and Reid, 1976). Moreover, there are a number of drugs which are self-administered but for which CPP failed to show rewarding properties; for example, pentobarbital and phencyclidine (Collins *et al.*, 1984; Marquis *et al.*, 1989; Acquas *et al.*, 1990; Lew and Parker, 1998), suggesting that the reinforcing and rewarding effects of abused drugs are not identical. As stated earlier, reinforcing agents are stimuli whose presentation is contingent upon the response. Reward is a general term which extends to include positive reinforcement, emotions (feelings of well-being) and appetitive characteristics and approach behaviours (Cannon and Bseikri, 2004; Salamone *et al.*, 2007). These differences between the reward and reinforcement probably underscore why some reinforcers do not induce CPP. Whereas drugs are injected at a high rate as bolus injections prior to introducing the animal to the compartment in CPP, the animals self-administer drugs at a much slower rate, and, therefore, the reward pathway may respond differently to the same drug according to the mode of exposure. It was found that self-administration but not yoked (unpredictable) injections of methamphetamine decreased the levels of D1 and/or presynaptic D2 receptors in the substantia nigra, nucleus accumbens and ventral tegmental area (Stefanski *et al.*, 1999). As mentioned earlier, abused drugs have been found to decrease D2 receptor expression in the striatum (Volkow *et al.*, 2007). This suggests that drug self-administration enhances dopamine release, which decreases the expression of dopamine D2 receptors, because the animal could predict the reception of the reward. In contrast, the animal is unable to anticipate the reward with yoked injections of the drug.

Furthermore, the lag interval between drug administration and the entrance to the drug-paired compartment is another important point. For instance, cocaine injections 5 minutes or immediately before the CPP test produced CPP, but cocaine injections 15 min prior to the CPP test produced conditioned place aversion (Ettenberg *et al.*, 1999). In agreement with this, it has been established that addictive drugs induce euphoria after different post-injection times (Volkow *et al.*, 2012).

1.4.3 Intracranial self-stimulation paradigm

Intracranial self-stimulation (ICSS) has been considered as a paradigm to test the abuse potential of a substance (Feltenstein and See, 2008). The extent at which a substance lowers the ICSS threshold is directly proportional to its reinforcing and addictive properties (Feltenstein and See, 2008). Rats treated chronically with corticosterone (40 mg/kg, s.c., for 21 days) became more sensitive to rewarding brain stimulation (Barr *et al.*, 2000).

In summary, rats self administered corticosterone and reinstated this behaviour after an extinction period. Although rats failed to show CPP to corticosterone, further investigation is required to demonstrate rewarding responses to corticosterone or to find out the reason behind corticosterone self-administration. Also, rewarding reactions to corticosterone should be studied in different species and different reward testing paradigms. Until now, corticosterone has shown some evidence of rewarding properties since it was self-administered in rats and lowered the threshold for intracranial self-stimulation.

1.5 Wheel running: a rewarding stimulus which increases corticosterone secretion?

1.5.1 Overview

Wheel running represents a natural stress model which activates HPA axis and can be used to study the rewarding effects of corticosterone**.** In animal studies, exercise can be forced or voluntary. Treadmills and motorized running wheels

are used to undertake forced exercise, but voluntary exercise studies use running wheels. It has been reported that chronic voluntary and forced exercise increases plasma corticosterone levels in mice (Droste *et al.*, 2003; Chang *et al.*, 2008). An essential difference between the forced and voluntary exercise is that the use of voluntary wheel running allows the recording of the motivation of each mouse to run, but mice exercising on treadmills run the same distances regardless of whether they are motivated or not. Importantly, forced exercise has been found to induce adverse physiological adaptations similar to those induced by chronic stress (Moraska *et al.*, 2000) and, technically, the animal should repeatedly receive mild footshocks to encourage running (Hattori *et al.*, 1994). Overall, voluntary wheel running is more appropriate than treadmill exercise to test the involvement of corticosterone in the motivation of mice to run. Below, previous work studying the rewarding, cognitive and HPA axis activating effects of voluntary wheel running is reviewed.

1.5.2 Voluntary wheel running is a motivated behaviour

1.5.2.1 Behavioural evidence for motivational effects of voluntary wheel running

A considerable amount of work has demonstrated that voluntary wheel running is rewarding (Brene *et al.*, 2007). Outbred strains such as Long-Evans rats (Iversen, 1993) and Wistar rats (Belke and Wagner, 2005) lever-pressed to get access to running wheels. Also, Sprague-Dawley rats (Lett *et al.*, 2000; Lett *et al.*, 2001) and Wistar rats (Belke and Wagner, 2005) exhibited CPP for the after-effects of wheel running. Moreover, low-running inbred Fischer rats displayed CPP induced by the after-effects of 6-week wheel running (Greenwood *et al.*, 2011). In addition, Sprague-Dawley rats demonstrated a continuous increase in the running activity over weeks (Fediuc *et al.*, 2006). Similarly, C57BL/6N mice having unlimited access to running wheels kept increasing the running distances and did not develop habituation to the running behaviour (Droste *et al.*, 2003). Other inbred mouse strains varied in their motivation to run ranging from CBA as a high-running strain to DBA/2 as a low-running strain (Festing, 1977; Brene *et al.*, 2007). As was the case with abused drugs, animals from the same strain showed variable motivation to run.

For instance, C57BL/6J mice (Klaus *et al.*, 2009) and Long-Evans rats (Tarr *et al.*, 2004) showed great variability in the running distances per fixed time.

It is worthy pointing out that wheel running has been reported to modulate drug-seeking behaviour. For example, cocaine self-administration in female rats was reduced by concurrent wheel running compared to a no-wheel access condition, suggesting that reinforcing properties of cocaine can be substituted for by wheel running (Cosgrove *et al.*, 2002). Moreover, cocaine-primed relapse in female rats was attenuated by prior or concomitant access to wheel running (Zlebnik *et al.*, 2010). In contrast, ethanol-withdrawn rats demonstrated enhanced ethanol-induced relapse after 2-weeks of wheel running (Werme *et al.*, 2002a). Similar to cocaine, female C57BL/6J mice which had access to 10% ethanol in the two-bottle choice while undergoing intermittent periods of wheel running increased their running performance when alcohol was withdrawn, but their running activity was reduced when ethanol was reintroduced, suggesting that rewarding effects of wheel running substituted for ethanol-associated reward (Ozburn *et al.*, 2008).

1.5.2.2 The involvement of dopamine in the rewarding effects of voluntary wheel running

Several studies have suggested various mechanisms underlying the motivation to run. Dopamine and its receptors have been implicated in running behaviour. First of all, treadmill exercise has been reported to increase dopamine levels in the nucleus accumbens (Freed and Yamamoto, 1985; Wilson and Marsden, 1995) and/or the striatum (Hattori *et al.*, 1994) of Sprague-Dawley, Wistar and Long-Evans rats after a 20-minute running session. Moreover, chronic unlimited wheel running increased mRNA levels for tyrosine hydroxylase, the rate limiting enzyme for dopamine biosynthesis, in the ventral tegmental area, but decreased mRNA levels for D2 receptors in the nucleus accumbens core of Fischer rats (Greenwood *et al.*, 2011).

Interestingly, the running distances of Long-Evans rats, which lever-pressed for accessing wheel running, correlated negatively with potassium-evoked

dopamine release in the nucleus accumbens core and dorsal striatum, indicating depletion of dopamine stores with chronic running (Tarr *et al.*, 2004). For instance, mice bred as a high-running strain were less sensitive to SCH 23390, a D1 receptor antagonist, than the control mouse line (Rhodes and Garland, 2003), suggesting that low dopamine levels in high-running animals are probably associated with modified dopamine receptor sensitivity. Furthermore, high-running rats exhibited greater dopamine release in the nucleus accumbens after a 20-minute running session than their low-running littermates (Wilson and Marsden, 1995), suggesting that reduced dopamine release in the nucleus accumbens core of high-running rats probably motivated them to run more to enhance dopamine release (Tarr *et al.*, 2004). MPTP (a dopamine depleting agent used to induce Parkinsonism-like symptoms) decreased wheel running activity of C57BL/6 mice compared to saline-treated animals, suggesting that dopamine is required for wheel running performance (Leng *et al.*, 2004). In contrast, methylphenidate (a dopamine reuptake inhibitor) enhanced running activity in mice (Rhodes and Garland, 2003). Overall, these findings suggest that dopamine regulates the motivation for running behaviour.

1.5.2.3 Other mechanisms involved in the rewarding effects of voluntary wheel running

A number of studies have shown that the opioidergic system is involved in the rewarding effects of wheel running. Whereas high-running inbred Lewis rats exhibited high mRNA levels for the endogenous opioids dynorphin and enkephalin, low-running inbred Fischer rats did not show increased levels of endogenous opioids in the medial caudate-putamen after long-term unlimited wheel running (Werme *et al.*, 2000). In contrast, both rat strains showed the same effects on dynorphin mRNA levels in the medial caudate-putamen in response to chronic cocaine treatment (Werme *et al.*, 2000), suggesting that wheel running could be addictive. Moreover, exercising Fischer rats exhibited increased mRNA levels for delta opioid receptors in the nucleus accumbens shell and kappa opioid receptors in the nucleus accumbens core compared to non-exercising rats (Greenwood *et al.*, 2011). On the other hand, naloxone attenuated CPP induced by the after-effects of wheel running in Sprague-

Dawley rats (Lett *et al.*, 2001). In addition, inbred BalB/c mice did not show CPP for wheel running after naloxone treatment which also postponed the acquisition of this motivated behaviour, compared to the control group (Vargas-Perez *et al.*, 2008). Overall, this suggests that endogenous opioid peptides and receptors play a significant role in the rewarding effects of wheel running.

Other work has shown that endogenous cannabinoids could have a role in wheel running-related reward. It was found that plasma levels of endocannabinoids such as anandamide increased following 1-hour of intense cycling in men (Heyman *et al.*, 2011), and there was a positive correlation between levels of anandamide and cortisol (Heyman *et al.*, 2011). It was reported that corticosterone injection rapidly increased anandamide levels in the hippocampus, amygdala and hypothalamus of Sprague-Dawley rats (Hill *et al.*, 2010). As endocannabinoids have been shown to enhance dopamine release in the nucleus accumbens (Maldonado *et al.*, 2006), this suggests a role of GCs in the rewarding effects of exercise *via* elevation of endocannabinoids.

Similar alterations in the expression of certain markers of plasticity induced by abused drugs have been reported to occur with wheel running. It has been reported that natural rewards (Wallace *et al.*, 2008) and abused drugs (Nestler, 2008) enhance the expression of Δ FosB (a transcription factor) in the nucleus accumbens. Compared to other Fos family members, Δ FosB is very stable and, thus, when it is accumulated in response to chronic exposure to abused drugs and other rewards, it persists for a long time, indicating long-lasting plasticity changes (Nestler *et al.*, 1999). As with drugs of abuse, wheel running has been found to enhance expression of Δ FosB in the nucleus accumbens of Lewis (Werme *et al.*, 2002b) and Fischer (Greenwood *et al.*, 2011) rats. Conversely, transgenic mice over-expressing Δ FosB in the striatum demonstrated enhanced running activity (Werme *et al.*, 2002b).

1.5.3 Effects of wheel running on HPA axis activity

Physical exercise is a stressor which increases the secretion of catecholamines and GCs to meet metabolic needs and to maintain homeostasis. Cortisol levels in men were elevated after cycling (Heyman *et al.*, 2011) and repeated 1-hour treadmill exercise sessions increased corticosterone levels in C57BL/6J mice (Chang *et al.*, 2008). Furthermore, the baseline circadian corticosterone peak in C57BL/6 mice (Droste *et al.*, 2003; Otawa *et al.*, 2007) and the nadir in corticosterone levels in C57BL/6 mice (Adlard and Cotman, 2004) were enhanced after 2-4 weeks of unlimited wheel running. Similarly, Wistar rats showed increased corticosterone levels after 10 days of continuous running (Hajisoltani *et al.*, 2011). Also, high-running Lewis rats demonstrated an increase in corticosterone levels at their nadir after 23 days of continuous wheel running (Makatsori *et al.*, 2003). In contrast, Sprague-Dawley rats exhibited an initial rise in circadian corticosterone levels which gradually decreased until no significant differences were found between running and non-running groups after the fourth week of unlimited wheel running (Fediuc *et al.*, 2006; Sasse *et al.*, 2008).

HPA axis adaptation has been reported in response to chronic wheel running in Sprague-Dawley rats (Fediuc *et al.*, 2006) and C57BL/6N mice (Droste *et al.*, 2003). This was supported by finding a lack of change in plasma ACTH levels in running mice or rats in response to different stressors such as novelty, forced swimming (Droste *et al.*, 2003) and immobilization stress (Fediuc *et al.*, 2006), although there was an increase in corticosterone levels. The rise in corticosterone levels without an increase in ACTH in response to stress can be explained as chronic wheel running upregulated enzymes involved in the adrenal corticosterone synthesis. It was reported that C57BL/6J mice running for 2 weeks exhibited enhancement of the mRNA expression for steroidogenic acute regulatory protein, the rate limiting step in corticosterone synthesis, and this was suggested to underlie chronic exercise-induced corticosterone elevation without a concomitant ACTH increase (Otawa *et al.*, 2007). Also, chronic exercise-induced corticosterone elevation did not affect hippocampal levels of GRs (Droste *et al.*, 2003; Fediuc *et al.*, 2006; Chang *et al.*, 2008). On the other hand, long-term wheel (Droste *et al.*, 2003) and treadmill (Chang *et al.*, 2008)

running decreased hippocampal levels of MRs in C57BL/6 mice and this was found to be involved in exercise-induced neurogenesis improvement in the dentate gyrus (Chang *et al.*, 2008).

1.5.4 Effects of wheel running on cognition

Exercise has been found to counteract aging-related cognitive decline (Colcombe and Kramer, 2003; Erickson and Kramer, 2009) and to protect against Alzheimer's dementia (Laurin *et al.*, 2001; Larson *et al.*, 2006; Yuede *et al.*, 2009; Nation *et al.*, 2011). Voluntary wheel running has been repeatedly used to study the influences of exercise on learning and memory in rodents. After chronic unlimited wheel running, young C57BL/6 mice demonstrated enhanced memory performance; in the Morris water maze, and improved hippocampal LTP and neurogenesis (van Praag *et al.*, 1999; van Praag *et al.*, 2005). Neurogenesis is a complex process in which new neurons are generated *via* proliferation of immature precursor cells into newborn cells which are differentiated into granule neurons (Dayer *et al.*, 2003). Also, aged C57BL/6 mice exhibited enhanced spatial learning after 45 days of wheel running (van Praag *et al.*, 2005). Moreover, it was reported that mice with Alzheimer's-like deficits running in the wheel for 5 months showed improved learning ability in the Morris water maze and decreased β amyloid plaques expression in the hippocampus and frontal cortex compared to the non-exercising group (Adlard *et al.*, 2005). In contrast, wheel running for 3 weeks improved spatial learning of aged mice with Alzheimer's-like deficits (Nichol *et al.*, 2007) and decreased inflammation but had no effects on β amyloid plaque load (Parachikova *et al.*, 2008), indicating that the duration of exercise regulates its effect on Alzheimer's-related pathology.

Importantly, the intensity and duration of exercise could produce distinct effects on cognition. For instance, high-running rats exhibited long-term (24 and 72 hour retention test) recognition memory deficits (Garcia-Capdevila *et al.*, 2009). In contrast, low-running rats outperformed high-running and sedentary littermates in the long-term recognition memory test (Garcia-Capdevila *et al.*,

2009). Furthermore, continuous wheel running for 24 days reduced hippocampal neurogenesis in the dentate gyrus, compared to non-exercising rats (Naylor *et al.*, 2005). On the contrary, either short-term running for 9 days or reduced running distances (30-50 % less than high-running performance) for 24 days showed enhanced neurogenesis in the dentate gyrus compared to uncontrolled running for 24 days (Naylor *et al.*, 2005). Adrenal hypertrophy, thymus involution and high baseline corticosterone elevation (indicators of chronic stress) were also observed in rats undergoing long-term unlimited wheel running compared to restricted running rats (Naylor *et al.*, 2005), and this could underlie the negative effects of extensive running on hippocampal neurogenesis. However, others have reported neurogenesis improvement and corticosterone elevation in C57BL/6J mice running on treadmills (Chang *et al.*, 2008), contradicting the proposal that increased corticosterone with extensive running underscores reduced neurogenesis (Naylor *et al.*, 2005). It has been established that enhanced neurogenesis is associated with memory improvement (Dupret *et al.*, 2007), suggesting that a reduction in neurogenesis could prevent exercise-induced cognitive enhancement. In agreement with this, it was reported that 24 hours of wheel running enhanced neurogenesis and this was associated with improved spatial (van Praag *et al.*, 1999) and short-term recognition memory performance (Lafenetre *et al.*, 2010).

It is critical to emphasize that limited access to wheel running, less than 3 hours in the dark phase, was found insufficient to enhance neurogenesis (Holmes *et al.*, 2004). In contrast, the increase in the running performance of exercising mice by a reward did not enhance hippocampal neurogenesis of C57BL/6J mice compared to non-rewarded running mice (Klaus *et al.*, 2009), suggesting that exercise has a therapeutic window for its cognition-enhancing effects, and that there is a ceiling (threshold) level of exercise that must be reached to show cognitive improvement but extensive running could impair memory performance.

There are multiple exercise-induced effects which collectively contribute to the impact of exercise on cognition. As stated earlier, wheel running has been found to enhance neurogenesis in the dentate gyrus (van Praag *et al.*, 2005; Lafenetre

et al., 2010) and hippocampal LTP (van Praag *et al.*, 1999), both are involved in the cognitive improvement. Also, it has been reported that wheel running increases levels of brain derived neurotrophic factor (BDNF) in the hippocampus (Adlard and Cotman, 2004; Liu *et al.*, 2009; Lafenetre *et al.*, 2010), and this is not only implicated in exercise-related cognitive effects, but it has also been thought to mediate its antidepressant effects (Sartori *et al.*, 2011). Moreover, exercise improves brain circulation and metabolism (Vissing *et al.*, 1996; McCloskey *et al.*, 2001). Interestingly, a recent study demonstrated that spatial memory improvement induced by 10 days of unlimited wheel running was attenuated in metyrapone-treated and adrenalectomized rats, suggesting that corticosterone is involved in exercise-dependent memory enhancement (Hajisoltani *et al.*, 2011). Overall, all these exercise-related effects are suggested to act together to modulate its effects on learning and memory performance.

As mentioned previously, treadmills could be used as an alternative for wheel running to study effects of exercise on cognition in rodents. However, it has been reported that each of these exercise modes differentially affects animal's performance in various memory tests. For instance, whereas running in the wheel or on treadmills enhanced spatial memory of BalB/c mice in the Morris water maze, only mice running on treadmills demonstrated improved performance in the one-trial passive avoidance task (Liu *et al.*, 2009). Nevertheless, wheel running enhanced rats' performance in the contextual fear conditioning (Baruch *et al.*, 2004), suggesting that the effects of wheel running on fear memory could be task-dependent. Chronic treadmill running has been reported to improve neurogenesis in the dentate gyrus of C57BL/6J mice (Chang *et al.*, 2008). It has also been found that forced exercise using treadmills increased bromodeoxyuridine, a marker of proliferating progenitor cells, (BRDU)-labelled cells in the dentate gyrus compared to rats which voluntarily ran the same distances in the wheel (Leasure and Jones, 2008). Furthermore, mice running on treadmills for 4 weeks exhibited an increase in levels of BDNF and TrkB in the hippocampus (Liu *et al.*, 2008) and amygdala (Liu *et al.*, 2009); the later underscored the enhancement of mice's performance in the passive avoidance task compared to other mice running in the wheels. Overall, exercise-

induced memory improvement appears to depend on the exercise protocol and memory tasks. Whereas mice running in the wheel exhibited enhancements in spatial learning and hippocampal BDNF levels, and this effect lasted for 1-2 weeks after the end of exercise (Berchtold *et al.*, 2010), mice exercising on treadmills demonstrated spatial memory improvement and a transient rise in their hippocampal BDNF levels (Chang *et al.*, 2008), suggesting that wheel running has longer-lasting effects on cognition.

To sum up, wheel running is rewarding and elevates corticosterone levels, and, therefore, this mode of exercise provides an appropriate model (stressful natural reward) to study the role of corticosterone and GRs in the motivation to run and the influence of chronic running on memory performance.

1.6 Strain differences in vulnerability to addictive drugs

Compared to other strains such as DBA/2J and CBA/J, C57BL/6J mice have shown excellent spatial learning and memory performance (Hyde *et al.*, 1998; Nguyen *et al.*, 2000a). Also, the C57BL/6J strain has been commonly used as a background for genetically modified strains, and, thus, this permits a linkage between certain memory deficits and specific genetic alterations. For example, C57BL/6 mice have been used to generate transgenic mouse models of Alzheimer's disease (Green *et al.*, 2006; Jeong *et al.*, 2006). Furthermore, C57BL/6J mice have been used in operant self-administration studies (Fiancette *et al.*, 2010), the two-bottle free choice test (Phillips *et al.*, 1998) and the CPP test (Orsini *et al.*, 2005): all of these paradigms include some aspects of learning (Bozarth, 1987; Bardo and Bevins, 2000; Tordoff, 2007). Overall, the C57BL/6J mouse strain is appropriate for studying the influence of corticosterone-related reward on cognition.

It has been reported that C57BL/6J mice are more sensitive than other inbred strains such as DBA/2J to addictive drugs. For example, C57BL/6J mice were more prone to develop CPP to morphine and cocaine than DBA/2J mice (Orsini *et al.*, 2005; Solecki *et al.*, 2009). In fact, low reinforcing doses of morphine in C57BL/6J mice were not self-administered by DBA/2J mice (Elmer *et al.*,

2010). Consistent with this, morphine enhanced brain reward stimulation in C57BL/6J mice but attenuated it in DBA/2 mice (Elmer *et al.*, 2010). It has been found that differences in the expression of endogenous opioids in the nucleus accumbens underlie the sensitivity to morphine-induced CPP between the two strains (Gieryk *et al.*, 2010). With regard to psychostimulants, whereas amphetamine induced CPP in C57BL/6J mice, DBA/2J mice exhibited conditioned place aversion to amphetamine (Cabib *et al.*, 2000). Nevertheless, stress induced by food restriction facilitated amphetamine-induced CPP in DBA/2J (Cabib *et al.*, 2000). Similar to morphine and amphetamine, nicotine induced CPP in C57BL/6J mice (Scott and Hiroi, 2010). Furthermore, amphetamine induced locomotor hyperactivity at low doses in C57BL/6J mice compared to DBA/2J mice.

A considerable number of studies have reported that C57BL/6J mice are motivated to self-administer addictive drugs with different mechanisms of actions. For example, C57BL/6J mice lever-pressed to self-administer cocaine, and extinguished (withdrawn) mice reinstated cocaine-seeking behaviour (Fuchs *et al.*, 2003; Ward *et al.*, 2009). Moreover, C57BL/6J mice self-administered midazolam, a benzodiazepine, in the two-bottle choice test (Tan *et al.*, 2010). C57BL/6J mice have also been widely used in alcohol preference studies employing the two-bottle choice test (Crabbe and Phillips, 2004; Melendez *et al.*, 2006). In addition, a single injection of cocaine induced locomotor hyperactivity and accumulation of ΔFosB in the dorsal striatum and nucleus accumbens of C57BL/6J mice (Lhuillier *et al.*, 2007). On the other hand, repeated cocaine injections induced behavioural sensitization and upregulation of ΔFosB and phosphorylated cyclic response element binding protein in the nucleus accumbens (Lhuillier *et al.*, 2007). One important point is that mifepristone was reported to attenuate the reinforcing effects of cocaine in C57BL/6J mice, suggesting that GRs in this strain could play a role in self-administration of abused drugs (Fiancette *et al.*, 2010). Overall, the C57BL/6J mice are an appropriate strain in which to self-administer addictive drugs and to study their impact on cognition.

1.7 Study hypothesis

Previous research over the last decades has advanced our knowledge of the role of corticosterone in modulating self-administration of abused drugs and its interaction with reward pathways. In addition, corticosterone as a neurogenic index of stress may contribute, at least partially, to stress-seeking behaviour. These findings led us to consider the probability that corticosterone is rewarding and addictive. Few experiments have addressed this question and shown evidence for corticosterone self-administration in rats. However, it is unknown whether mice would also self-administer corticosterone, and whether rewarding effects of the stress hormone could enhance memory performance.

1.7.1 Objectives of the thesis

- The first objective of this work was to investigate whether corticosterone was rewarding and addictive in male C57BL/6J mice, through establishing corticosterone self-administration in the two-bottle free choice test.
- The second objective was to investigate whether corticosterone self-administration or exercise-evoked corticosterone elevation improved the cognitive performance of corticosterone or exercise withdrawn mice, respectively.
- Thirdly, it was of interest to study whether chronic corticosterone self-administration altered the expression of key proteins of neuroplasticity in the hippocampus and frontal cortex.
- Lastly, as the striatum is involved in drug-seeking behaviour, levels of dopamine, serotonin and their metabolites in the striatum were assessed in order to establish whether corticosterone self-administration by mice was accompanied with long-lasting neurochemical changes in the reward pathway.

Chapter 2

Investigation of rewarding properties of corticosterone and their effects on cognition in the mouse

2.1 Introduction

The overall aim of the experiments described in this chapter was to investigate the rewarding and addictive properties of corticosterone and their impact on cognition. Drug addiction is defined as a chronic relapsing disorder characterized by compulsive drug-seeking behaviour which leads to intoxication, dependence and adverse withdrawal symptoms (Koob and Le Moal, 2005; Koob and Le Moal, 2008). Seeking drug administration after withdrawal is defined as relapse (reinstatement) and is considered as a valid marker of addiction (Stewart, 2000). In the present study, the two-bottle choice test/oral self-administration model was undertaken to test the development and reinstatement of corticosterone self-administration. There are several standard models that are widely used to evaluate rewarding properties of drugs; for example, conditioned place preference (CPP), intracranial self-stimulation and

self-administration paradigms. Among the latter models, the intravenous drug self-administration paradigm is effectively used to measure abuse potential (Collins *et al.*, 1984) and is a standard alternative for CPP (Bardo and Bevins, 2000). Self-administration paradigms are choice models in which an animal is allowed to choose to access a drug without enforcing the animal to administer the drug in contrast to CPP. In the operant intravenous self-administration model, the reinforcing properties of a drug are determined by manipulating several factors: I- The drug dose. II- The cost of responding; the fixed ratio and progress ratio schedules which are changed according to the rates of the animal's response, such as lever presses or nose pokes, to receive a dose of the tested drug (Kelleher and Goldberg, 1975; Krasnegor, 1978). III- Second-order schedule in which drug-paired stimuli act as conditioned reinforcers to maintain the animal's response (Koob and Le Moal, 2008). The two-bottle choice model is another self-administration paradigm which is simple and reliable as animals have unlimited access to a reward-testing substance, thereby simulating the free access environment of drugs abused by humans. This test is extensively used to examine rodents' preference for different tasting solutions and nutrients (Tordoff and Bachmanov, 2002), and it has been frequently used in ethanol preference studies (Phillips *et al.*, 1998; Carroll *et al.*, 2006). The two-bottle position is alternated every day to control for side preference to ensure that the animal can discriminate the rewarding solution, thereby increasing the validity of the method (Bachmanov *et al.*, 2002; Tordoff and Bachmanov, 2002).

In the present study, a major issue was to help mice to discriminate the corticosterone solution which is tasteless. A preliminary study, involving the use of the two-bottle choice test (unpublished data), was carried out to examine corticosterone self-administration and results showed that mice drank extensively but equally from the corticosterone solution and water bottles, suggesting that they could not recognise the taste of the hormone easily or that corticosterone stimulated the drinking behaviour. Inosine monophosphate (IMP) was, therefore, used as a flavouring agent to allow mice to distinguish between the solutions in the two bottles. From the literature, IMP was thought to be an

ideal flavour because it was tested in the same mouse strain (C57BL/6J) as was used in the present experiment (Tordoff, 2007), it is water soluble and has no sodium content which may compromise the choice behaviour as glucocorticoids were found to increase fluid intake when sodium was added to the drinking fluid (Thunhorst *et al.*, 2007). The preference for IMP was examined in C57BL/6J mice using the two-bottle choice test and a dose/response curve showed that the behavioural detection threshold was at a concentration of 1 mM (Tordoff, 2007). Therefore a 2 mM IMP concentration was chosen for which C57 mice showed 60% preference without altering the normal daily fluid intake (Tordoff, 2007). This ensured that the mice could discriminate the flavour without developing a strong preference to it, which would confound the acquisition of corticosterone self-administration.

Corticosterone is well known to influence cognition and memory processing. A number of studies have shown that chronic stress or high plasma corticosterone levels have deleterious influences on memory and synaptic plasticity, whereas moderate stress-induced corticosterone secretion is beneficial for spatial memory and hippocampal plasticity (Diamond *et al.*, 1992; Akirav *et al.*, 2004; Rocher *et al.*, 2004; Cerqueira *et al.*, 2007). This depends on plasma levels of corticosterone and duration of exposure to corticosterone (Diamond *et al.*, 1992; Pavlides *et al.*, 1993; Bardgett *et al.*, 1994; Coburn-Litvak *et al.*, 2003). However, it is interesting to investigate whether corticosterone has the same widely accepted influences on learning and memory when it is self-administered, and therefore whether its rewarding properties modulate the impact on cognition. Spatial memory and recognition memory of mice were examined to determine impacts of chronic oral corticosterone self-administration on cognition. Spatial working memory was evaluated using the spontaneous alternation test in a T-maze and the object location test, and the novel object test was used to evaluate recognition memory.

To investigate whether corticosterone self-administration was accompanied by synaptic plasticity changes, western immunoblotting was used to measure levels

of key proteins regulating brain plasticity including synaptophysin (SYN), brain-derived neurotrophic factor (BDNF), tropomysin-related kinase B (TrkB), and phosphorylated extracellular signal-regulated kinase 1 and 2 (pERK1/2) in the hippocampus and frontal cortex, two brain regions which are critical for working memory, learning and addiction (Asaad *et al.*, 1998; Asaad *et al.*, 2000; Volkow *et al.*, 2003; Bekinschtein *et al.*, 2007).

SYN, a presynaptic vesicle-associated membrane protein, is a 38 kDa glycoprotein (Jahn *et al.*, 1985; Wiedenmann and Franke, 1985). It is implicated in the exocytosis of neurotransmitters from synaptic vesicles (Valtorta *et al.*, 2004) and, therefore, SYN has been used as a classical marker of synaptogenesis (Mazer *et al.*, 1997; Thome *et al.*, 2001; Vawter *et al.*, 2002). Furthermore, it is believed to play a role in learning and memory (Thome *et al.*, 2001; Nithianantharajah and Murphy, 2008; Schmitt *et al.*, 2009). Interestingly, SYN expression in the hippocampus and nucleus accumbens of Sprague-Dawley rats was positively correlated with their performance in CPP for amphetamine (Rademacher *et al.*, 2006), suggesting it may be involved in drug addiction.

BDNF is a polypeptide neurotrophin which is required for neuronal survival (Ghosh *et al.*, 1994) and neuroplasticity (Thoenen, 1995; Kafitz *et al.*, 1999). Moreover, it has been found that BDNF is required for learning and memory (Hall *et al.*, 2000; Bekinschtein *et al.*, 2007; Heldt *et al.*, 2007). Also, BDNF has been reported to modulate self-administration of abused drugs such as cocaine (Graham *et al.*, 2007). Mature BDNF is synthesized when pre-proBDNF, a BDNF precursor, is cleaved to proBDNF and then cleaved by plasmin or metalloproteinases to the mature form (Cunha *et al.*, 2010)**.** BDNF mediates its actions mainly *via* its high affinity receptor, full length (FL) TrkB, but it can also bind to truncated (t) TrkB and the p75 neurotrophin receptor (p75NTR) which is mainly activated by proBDNF, a precursor of mature BDNF (Teng *et al.*, 2005). Truncated-TrkB is a degradation form of FL-TrkB which lacks tyrosine kinase domain essential for downstream signalling (Klein *et al.*,

1993). Thus, t-TrkB can act as a negative regulator of BDNF mediated effects when BDNF/t-TrkB complex forms heterodimers with FL-TrkB to function as a clearance receptor (Haapasalo *et al.*, 2002). Eventually, proBDNF and p75NTR can also regulate mature BDNF/FL-TrkB signalling (Cunha *et al.*, 2010). Activation of FL-TrkB receptors switches on three major downstream signalling pathways: phospholipase CϒΥ, phosphatidylinositol 3-kinase and extracellular signal regulated kinase / mitogen-activated protein kinase (ERK/MAPK) (Cunha *et al.*, 2010).

ERK is a member of the mitogen-activated protein kinases (MAPK) which contribute to learning, memory formation, neuroplasticity and neuroadaptation (Mazzucchelli and Brambilla, 2000; Rodrigues *et al.*, 2004). Two ERK isoforms have been identified in mammalian nerve cells: ERK1 and ERK2 (Zhai *et al.*, 2008). Previous research has revealed that multiple upstream signalling pathways can activate ERK1/2 by phosphorylation: (1) neurotrophins including BDNF, nerve growth factor, basic fibroblast growth factor and NT-3, (2) drugs whose actions are mediated *via* activating G protein-coupled receptors such as dopamine, glutamate, opioids and cannabinoids and (3) activation of calcium channels by drugs such as nicotine or the excitatory neurotransmitter glutamate (Lee and Messing, 2008). ERK1/2 is widely expressed in the brain and is particularly abundant in mesocorticolimbic dopaminergic neurons (Zhai *et al.*, 2008). A considerable body of research supports the role of ERK1/2 in drug-seeking behaviour and reinstatement of most abused drugs such as cocaine, amphetamine, opioids, endocannabinoid and alcohol (Valjent *et al.*, 2004; Zhai *et al.*, 2008). ERK2 seems to be more important for behavioural expression of drug addiction than ERK1 (Mazzucchelli *et al.*, 2002; Li *et al.*, 2008).

Dopamine is believed to be a major reward neurotransmitter in the brain. It is implicated in the rewarding effects of most abused drugs including cocaine, amphetamine, morphine and nicotine (Yokel and Wise, 1975; De Wit and Wise, 1977; Devine *et al.*, 1993; Lacosta and Roberts, 1993; Glick *et al.*, 1998).

Mesocorticolimbic dopaminergic neurons project from the ventral tegmental area mainly to the nucleus accumbens and frontal cortex, and they are believed to mediate rewarding effects of addicted drugs (Wise, 1998). The nigrostriatal dopamine pathway, which is traditionally considered to be critical for motor function, has also been shown to play a role in reward-related effects (Wise, 2009). One important objective was, therefore, to measure levels of monoamines including dopamine, 5HT and their metabolites in the striatum of mice by means of HPLC.

2.2 Objectives

- To determine if mice would self-administer and reinstate corticosterone in the two-bottle free choice test using IMP as a flavouring agent to allow mice to discriminate the hormone-containing bottle.
- To evaluate the impact of chronic corticosterone self-administration on mice's spatial memory performance by employing the spontaneous alternation and object location tests.
- To investigate the effects of chronic corticosterone self-administration on mice's recognition memory.
- To use western immunoblotting to measure levels of key proteins mediating neuronal plasticity including SYN, BDNF, TrkB and pERK1/2 in the hippocampus and frontal cortex of mice to determine whether behavioural changes are associated with changes in the level of expression of these proteins.
- To investigate the effects of corticosterone self-administration on the mesolimbic reward pathway through measuring, by HPLC, the levels of dopamine, serotonin and their metabolites in the striatum of mice.

Chapter 2 - Part 1

Acquisition and reinstatement of corticosterone self-administration in mice

2.3 Purpose

This part of the experiment was designed to test the development, maintenance and reinstatement of oral corticosterone self-administration in mice using the two-bottle choice model.

2.4 Materials and methods

2.4.1 Animals

Forty six 12-week-old male C57BL/6J mice were purchased from Charles River UK and housed in groups of three per cage. After one week, the animals were individually caged and left undisturbed for one week. Standard environmental conditions including temperature (20 - 22 °C), humidity (55 - 60 %), ventilation and 12:12 light dark cycle (lights on at 7:00 am) were maintained until the end of the experiment. Food and water were available *ad libitum*. After acclimatization, water bottles were replaced by one or two drinking tubes according to experimental progress. The body weight (BW) of mice was recorded weekly at the time of cage change. Animal care and welfare in this experiment were in compliance with the UK Animal Scientific Procedures Act 1986 under project licence 40/2715. Five mice died during the course of the experiment; veterinary advice was sought, and it was concluded that corticosterone treatment was not the cause of death, although we could not establish the cause of death. These mice were excluded from any statistical analysis of data.

2.4.2 Drinking solutions

2.4.2.1 Corticosterone solution

Corticosterone, 4-pregnene-11β,21-diol-3,20-dione, (500 mg per vial, Sigma-Aldrich UK), was dissolved in 20 ml of absolute ethanol using a sonic probe and then diluted with distilled water to give a final concentration of 250 μg/ml. The ethanol concentration in the diluted corticosterone solution was 1 % (Lee *et al.*, 2010). The dose of corticosterone consumed was calculated every day for each animal.

The dose of corticosterone ingested by each mouse was calculated every day according to this equation:

Daily corticosterone dose (mg/kg BW animal) =

$$\frac{\text{Intake from corticosterone solution (ml)} \times 0.25^{*} \times 1000^{\#}}{\text{Animal BW (g)}}$$

* Concentration of corticosterone solution (0.25 mg/ml)

# denotes 1000 g (1 kg) animal BW.

The corticosterone solution was gradually diluted for any of the mice whose dose exceeded 60 mg/kg/day until its daily corticosterone intake returned below this dose.

2.4.2.2 Vehicle for corticosterone solution

20 mls of absolute ethanol stock solution was sonicated under the same conditions as were used to dissolve corticosterone. Then, the absolute ethanol was diluted with distilled water to a final 1% concentration before filling the drinking tubes. Ethanol intake was calculated according to the following equation:

Ethanol intake (g/kg BW animal) = ($1000^{\#}$ X (A/100) X 0.789)/B

- A is the daily number of mls drunk from the corticosterone solution or ethanol solution (control-vehicle) when ethanol is used at 1%.
- B is the animal BW (g).
- 0.789 is specific gravity of ethanol.
- # denotes 1000 g (1 kg) animal BW.

2.4.2.3 Preparation of inosine monophosphate (IMP) solution

One gram of IMP (inosine 5′-monophosphate from Saccharomyces cerevisiae, Sigma-Aldrich UK) was dissolved in 40 ml distilled water as a stock solution

which was used to flavour distilled water or the corticosterone solution at a final concentration of 2 mM (696 μg/ml).

2.4.3 Construction of the drinking tubes

Drinking tubes were built for the purpose in-house (School of Biomedical Sciences, Medical Engineering Unit) according to protocols described by Bachmanov *et al.* (1996) and Bachmanov *et al.* (2002). Graduated 30 ml pipettes were used whose conical openings were cut to give the same diameter at both ends. Silicone stoppers were used to close one end. The second opening was closed by the same stoppers in which an internal hole was made to introduce a spout. A ball was added inside each spout to help minimizing leakage from the drinking tube when the animals were not drinking. Spouts of the two tubes were inserted into the cage through a perforated metal sheet attached to the front side of the cage lid to prevent the mice from chewing the silicone stoppers.

2.4.4 The two-bottle free choice test

In the two-bottle choice model, each individually housed mouse was provided with two drinking tubes, one for the control solution and the other for the test solution. Fluid intake from each tube was recorded daily. The use of drinking tubes helped to minimise the wasting of the drinking solutions since mice drank up to 10 ml a day and the solutions needed to be renewed twice a week. In the two-bottle choice test, each mouse was provided with two drinking tubes of water during the habituation phase and with the relevant test and control solutions (see table 2.1) during the testing phases. Also, spillage was controlled through the use of three drinking tubes attached to empty cages which were placed in the same rack as the experimental animals. The average fluid loss from these tubes was calculated every day and accounted for spillage and was then deducted from the amount of fluid drunk by each mouse. This resulted in accurate drug dose calculation. Weighing tubes to know how much the animal

had drunk over the past 24 hours was seen as a more sensitive measure than using the graduations on the drinking tubes.
The weight of drinking tubes was recorded every day, to calculate the difference from the previous day as described below:

The weight of fluid drunk from one tube =

Weight of this tube on the previous day – (weight of this tube today + spillage)

Then 1 g water (the main vehicle) is equal to 1 ml.
Drinking tubes containing corticosterone and/or IMP were covered with opaque paper since corticosterone is light-sensitive.

Table 2.1 The control and test solutions for each of the five experimental groups during the acquisition, relapse and switching phases of the two-bottle choice test. * =1 and # =3 mice died before the end of the experiment but post-mortem examination excluded corticosterone as a cause of death. These mice were excluded from any statistical analysis.

Choice/Group	Acquisition		Relapse (reinstatement)		Switching	
	Control	**Test**	**Control**	**Test**	**Control**	**Test**
A (n = 8) Control-Flavour	*Water*	*IMP*	*Water*	*IMP*	*Water*	*IMP*
B (n = 8) Control-Vehicle	*Water*	*Ethanol 1%*	*Water*	*Ethanol 1%*	*Water*	*Ethanol 1%*
C* (n = 9)	*Water*	*Unflavoured corticosterone*	*Water*	*Unflavoured corticosterone*	*Water*	*Unflavoured corticosterone*
D# (n = 7)	*IMP*	*Unflavoured corticosterone*	*IMP*	*Unflavoured corticosterone*	*Water*	*Flavoured corticosterone*
E* (n = 9)	*Water*	*Flavoured corticosterone*	*Water*	*Flavoured corticosterone*	*IMP*	*Unflavoured corticosterone*

2.4.5 Experimental design

The timeline of the experiment is presented in Fig. 2.1.

2.4.5.1 Habituation phase

One week after being individually housed, mice were given access to 2 drinking tubes containing tap water for 10 days. This experimental period was required to give mice the opportunity to learn that they could drink from the two tubes, to monitor their normal daily fluid intake, and to monitor side preference. On the last two days of the habituation phase, mice underwent the spontaneous alternation test in a T-maze to evaluate their baseline spatial working memory performance prior to being randomly allocated to the 5 experimental groups as illustrated in table 2.1.

2.4.5.2 Pre-choice test induction phase

For 3 days, mice were only given access to the acquisition test solution delivered in one drinking tube to help them experience the taste of their test solution.

2.4.5.3 Acquisition of corticosterone self-administration

Mice had unlimited access to two drinking tubes for 3 weeks; one containing the control solution and the other containing the test solution, as described in table 2.1. Although one group in which mice rapidly self-administered corticosterone; starting from the first week, when paired with IMP (group D), the acquisition phase was extended for two weeks, as a relatively long lag was probably required for mice in other groups (different choices) to exhibit a significant preference for corticosterone. Also, this permitted to investigate chronic effects of corticosterone-related reward on memory. To control for the side preference, the position of the test and control tubes was switched every day; for half of each group, the test solution was first given on the right side, and then on the left side for the rest of the group.

2.4.5.4 Withdrawal phase I

After 3 weeks of acquisition, the two-bottle choice test was stopped. The two drinking tubes were replaced by one drinking tube filled with tap water (withdrawal I). As the total fluid intake of corticosterone-treated mice rapidly dropped to the control levels on the first day of withdrawal, this period lasted only for a week. To investigate the effects of the three-week treatment on cognitive performance, mice were retested in the T-maze one day after the cessation of the corticosterone treatment.

2.4.5.5 Reinstatement of corticosterone self-administration

Mice were again given access to the same test and control solutions as during acquisition for a 2-day period, see Table 2.1 for details of relapse. This phase of the choice test was required to investigate the addictive potential of corticosterone.

2.4.5.6 Switching phase

This phase was carried out to investigate whether mice would sustain significant corticosterone self-administration after exchanging the flavour paired to their solutions. This phase was added to the experiment because we unexpectedly observed an initial aversion for IMP in the control-flavour group (water *versus* IMP) which could affect corticosterone self-administration. Switching was carried out by exchanging flavoured solutions for unflavoured ones between groups D and E. One day before the switching phase, each mouse had access to only one drinking tube which contained the test solution of the switching phase. Then, the two-bottle choice test was carried out for 5 days to examine the effect of switching. Throughout Chapter 2, each group will be identified by its choice offered during acquisition and relapse, but switching will be indicated when it is relevant.

2.4.5.7 Withdrawal phase II and behavioural assessment

Drinking was monitored for another five days, during which each animal had access to only one drinking tube with tap water. Mice's spontaneous alternation performance was again evaluated in the T-maze. Then, mice were habituated to the open field arenas 24 hours before being subjected to the novel object recognition test. On the following day, the mice underwent the object location test, to investigate the long-term effect of corticosterone treatment on spatial memory.

2.4.6 Blood and tissue collection

Mice were left undisturbed for 48 hours before being sacrificed by cervical dislocation. Trunk blood was collected in heparinised tubes for the measurement of plasma corticosterone concentrations. Plasma was isolated from blood samples after centrifugation at 3000 g at 4 °C for 10 minutes. The brains were removed, and the hippocampus, frontal cortex and striatum were rapidly dissected on ice. In brief, each brain, in its ventral view, was fixed by forceps, and the hypothalamus was removed. After adjusting the brain in its dorsal view, the olfactory bulb was removed before the frontal cortex on both sides of the brain was excised. Then, the internal side of cortex was carefully exposed; by using the scalpel to cut through the middle line between the two hemisperes, to separate a curved band of cortex (the hippocampus) from surrounding tissue. Finally, the striatum which is a part of the basal ganglia was dissected. Plasma and brain tissues were kept in the freezer at – 80 °C for further investigation.

2.4.7 Determination of plasma corticosterone levels (ng/ml)

Circulating plasma corticosterone levels were determined using 96 well plate enzyme-linked immunoassays (ADI-900-097, *Enzo Life*, USA) according to the manufacturer's instructions. The sensitivity of the assay was 27 pg/ml: the intra-assay and inter-assay coefficients of variation were 7.7% and 9.7%, respectively. All samples were run in duplicate and the plate was read at 405

nm and correction made at 580 nm using SoftMax® Pro V 4.0 (Life Sciences Edition).

2.4.8 Statistical analysis

Data are presented as mean ± SEM. Three-way ANOVAs with repeated measures (time X bottle X group) were used to analyse fluid intake (ml) during the two-bottle choice test, and two-way ANOVAs with repeated measures (time X group) were used to analyse fluid intake (ml) during the withdrawal phases, corticosterone doses (mg/kg), ethanol intake (g/kg) and changes in BW (g). The between subject factor was groups to compare total fluid intake or changes in BW (5 levels), corticosterone doses (3 levels) or ethanol intake (4 levels). Within subject factors were time; including weeks of acquisition (three levels), days of relapse (two levels) or days of switching (five levels), and bottles (two levels) to analyse the preference for the test solution in each experimental group, or changes in BW after completing each experimental phase (5 levels). During the acquisition phase, data were averaged on a weekly basis because minor changes were seen within each week. Plasma corticosterone levels were analysed by a one way ANOVA with groups (5 levels) as the between subject factor. Post-hoc planned comparisons and paired sample *t*-tests were used when significant main effects were obtained. An effect was considered significant when a P value was ≤ 0.05. The SPSS and InVivoStat software was used to perform statistical analysis in all experiments presented in the thesis. InVivoStat software was mainly used to run ANOVAs with repeated measures as it calculates P values for the between subject factors on each time point.

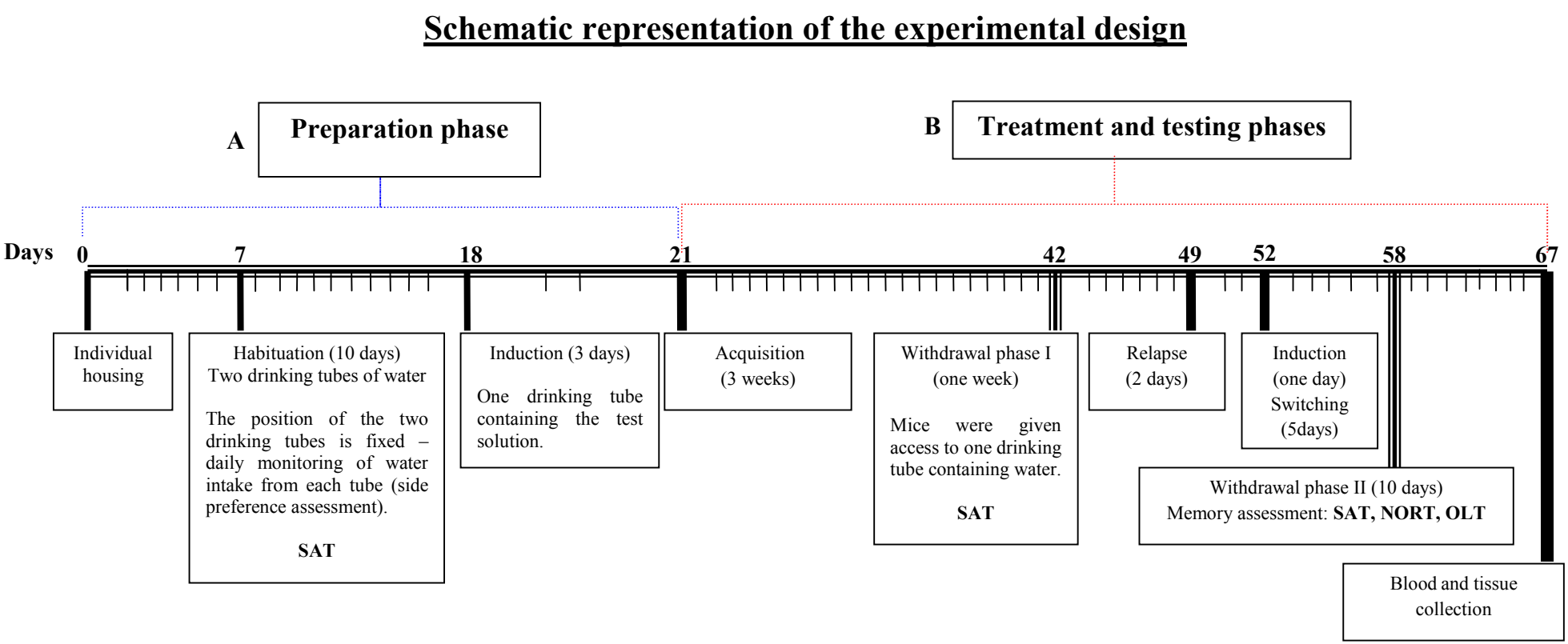

Figure 2.1 Experimental design comprised: (A) Preparation phase for the habituation to the two-bottle choice testing, (B) Treatment & bottle choice testing phases for the investigation of oral corticosterone self-administration. During the three phases (acquisition, relapse and switching) of the two-bottle choice test, each mouse was provided with two drinking tubes (one for the test solution and the other for the control solution). The position of the two drinking tubes was alternated every day during the testing phases. Fluid intake was recorded daily to calculate the doses of corticosterone and/or ethanol ingested by each mouse. The concentration of corticosterone solutions was adjusted when a mouse's daily intake exceeded 60 mg/kg for health reasons. SAT: spontaneous alternation test, NORT: novel object recognition test, OLT: object location test.

2.5 Results

2.5.1 The two-bottle free choice test

2.5.1.1 Fluid intake from the control and test solutions

Summary of the main statistical effects for the factors: bottle, time and time X bottle interaction in each group during acquisition, relapse and switching is presented in table 2.2.

2.5.1.1.1 Did mice self-administer vehicle or flavour?

2.5.1.1.1.1 Group A (control-flavour): water *versus* IMP

Mice from the control-flavour group exhibited significant avoidance towards the IMP solution during the three weeks of acquisition ($F_{(1,7)}$ = 10.76, P = 0.013). Paired sample *t*-tests detected significant avoidance to the IMP solution during the first (P = 0.042) and third (P = 0.004) weeks of acquisition, Fig. 2.2A. However, this initial avoidance of IMP disappeared during the relapse and switching phases, table 2.2.

2.5.1.1.1.2 Group B (control-vehicle): water *versus* ethanol 1%

Mice from the control-vehicle group did not show preference for any of the solutions during the 3 phases: acquisition, relapse and switching, table 2.2. However, there was a significant time effect during the switching phase ($F_{(4,28)}$ = 8.90, P = 0.00009), and post-hoc paired sample *t*-tests indicated a significant difference between ethanol and water intake (t = 2.97, P = 0.021) on the second day of switching only, Fig 2.2B.

Table 2.2 Summary of the main statistical effects for the following factors: bottle, time and time X bottle interaction, for each of the five groups (n = 7-9) of male C57BL/6J mice over the three phases of the two bottle choice test: acquisition, relapse and switching. Data were analysed using three-way ANOVAs with repeated measures. Statistical significance is highlighted in bold. # indicates groups whose solutions were exchanged during the switching phase.

Groups	Factors	Acquisition (weeks) W1, W2, W3	Relapse (days) D50, D51	Switching (days) D53-D57
Water *versus* IMP (control-flavour, n = 8)	Bottle	**$F_{(1,7)}$ = 10.76, P = 0.013**	$F_{(1,7)}$ = 0.35, P = 0.57	$F_{(1,7)}$ = 0.07, P = 0.80
	Time	$F_{(2,14)}$ = 0.62, P = 0.55	$F_{(1,7)}$ = 1.57, P = 0.25	$F_{(4,28)}$ = 1.50, P = 0.23
	Time X bottle interaction	$F_{(2,14)}$ = 0.83, P = 0.46	$F_{(1,7)}$ = 4.65, P = 0.07	$F_{(4,28)}$ = 0.78, P = 0.55
Water *versus* ethanol 1% (control-vehicle, n = 8)	Bottle	$F_{(1,7)}$ = 1.02, P = 0.35	$F_{(1,7)}$ = 1.05, P = 0.34	F(1,7) = 2.55, P = 0.15
	Time	$F_{(2,14)}$ = 0.31, P = 0.74	$F_{(1,7)}$ = 4.64, P = 0.07	**$F_{(4,28)}$ = 8.90, P = 0.00009**
	Time X bottle interaction	$F_{(2,14)}$ = 0.35, P = 0.71	$F_{(1,7)}$ = 0.51, P = 0.50	$F_{(4,28)}$ = 0.91, P = 0.47
Water *versus* unflavoured corticosterone (n = 9)	Bottle	**$F_{(1,8)}$ = 5.50, P = 0.047**	**$F_{(1,8)}$ = 26.33, P = 0.001**	**$F_{(1,8)}$ = 7.43, P = 0.026**
	Time	$F_{(2,16)}$ =1.00, P = 0.39	$F_{(1,8)}$ = 2.54, P = 0.15	$F_{(4,32)}$ = 1.98, P = 0.12
	Time X bottle interaction	$F_{(2,16)}$ = 0.62, P = 0.55	**$F_{(1,8)}$ = 8.11, P = 0.022**	$F_{(4,32)}$ = 1.13, P = 0.36
IMP *versus* unflavoured corticosterone # (n = 7)	Bottle	**$F_{(1,6)}$ = 52.78, P = 0.0003**	**$F_{(1,6)}$ = 63.22, P = 0.0002**	$F_{(1,6)}$ = 3.94, P = 0.09
	Time	**$F_{(2,12)}$ = 6.26, P = 0.014**	$F_{(1,6)}$ = 0.71, P = 0.43	**$F_{(4,24)}$ = 2.83, P = 0.047**
	Time X bottle interaction	**$F_{(2,12)}$ = 9.25, P = 0.004**	$F_{(1,6)}$ = 0.14, P = 0.72	$F_{(4,24)}$ = 0.42, P = 0.79
Water *versus* flavoured corticosterone # (n = 9)	Bottle	$F_{(1,8)}$ = 2.39, P = 0.16	$F_{(1,8)}$ = 0.32, P = 0.59	**$F_{(1,8)}$ = 23.71, P = 0.001**
	Time	$F_{(2,16)}$ = 1.52, P = 0.25	$F_{(1,8)}$ = 0.21, P = 0.66	$F_{(4,32)}$ = 2.32, P = 0.08
	Time X bottle interaction	$F_{(2,16)}$ = 0.70, P = 0.51	$F_{(1.8)}$ = 3.35, P = 0.10	$F_{(4,32)}$ = 1.64, P = 0.19

2.5.1.1.2 Group C: Did mice self-administer corticosterone in the absence of IMP?

In the absence of IMP, although mice showed an overall significant preference for the unflavoured corticosterone solution over water during acquisition ($F_{(1,8)}$ = 5.50, P = 0.047), paired sample *t*-tests did not reveal any significant bottle effect on individual weeks (P >0.05, in all case), Fig. 2.2C. This suggests that mice had difficulty in recognising the unflavoured corticosterone solution when it was paired with water. However, the preference for the corticosterone solution increased with time and persisted during the relapse ($F_{(1,8)}$ = 26.33, P = 0.001) and switching ($F_{(1,8)}$ = 7.43, P = 0.026) phases, Fig. 2.2C. Also, there was a significant day X bottle interaction during relapse ($F_{(1, 8)}$ = 8.11, P = 0.022), reflecting increased corticosterone intake and decreased water intake with repeated days, table 2.2. Paired sample *t*-tests revealed a significantly higher intake from the unflavoured corticosterone solution than from water during relapse (P_{D50} = 0.008, P_{D51} = 0.001) and two separate days of switching (P_{D53} = 0.015, P_{D55} = 0.018), indicating the inability of mice in this group to easily recognise the stress hormone without a flavour, Fig. 2.2C.

2.5.1.1.3 Did the flavour facilitate corticosterone self-administration and did mice retain the preference for corticosterone after switching flavoured for unflavoured solutions?

2.5.1.1.3.1 Group D (IMP *versus* unflavoured corticosterone and water *versus* flavoured corticosterone before and after the switching phase, respectively)

Mice which had a choice between IMP and unflavoured corticosterone during acquisition drank almost exclusively from the corticosterone solution ($F_{(1,6)}$ = 52.78, P = 0.0003). During acquisition, there were also significant effects of weeks ($F_{(2,12)}$ = 6.26, P = 0.014) and week X bottle interaction ($F_{(2,12)}$ = 9.25, P = 0.004), reflecting an increase in the preference for corticosterone and a decrease in the intake of IMP with time. Paired sample *t*-tests showed a significant preference for corticosterone during all 3 weeks of acquisition (P_{W1} = 0.0003, P_{W2} = 0.001, P_{W3} = 0.001), Fig. 2.2D.

High intake from the unflavoured corticosterone solution was retained during the relapse phase ($F_{(1,6)} = 63.22$, $P = 0.0002$), table 2.2, and paired sample *t*-tests showed significant intake from unflavoured corticosterone during the two days ($P_{D50} = 0.002$, $P_{D51} = 0.00002$), Fig 2.2D. After switching there was, however, no significant preference for flavoured corticosterone over water ($F_{(1,6)} = 3.94$, $P = 0.09$) and no significant time X bottle interaction effect ($F_{(4,24)} = 0.42$, $P = 0.79$), but there was a significant time effect ($F_{(4,24)} = 2.83$, $P = 0.047$), table 2.2, reflecting the effects of switching to the new choice. Although paired sample *t*-tests did not show a significant preference for flavoured corticosterone on individual days ($P_{D53} = 0.14$, $P_{D54} = 0.20$, $P_{D55} = 0.12$, $P_{D56} = 0.056$, $P_{D57} = 0.09$), mice's total fluid intake remained significantly high compared to both control groups ($P = 0.005$), and they consumed high doses of corticosterone, as they did during the acquisition and relapse periods, Fig 2.3.

2.5.1.1.3.2 Group E (water *versus* flavoured corticosterone and IMP *versus* unflavoured corticosterone before and after switching, respectively)

Mice's intake of the flavoured corticosterone solution was not significantly different from their intake of water during the acquisition and relapsing phases, table 2.2. In contrast, mice exhibited a remarkable preference for the unflavoured corticosterone solution over IMP after switching ($F_{(1,8)} = 23.71$, $P = 0.001$), table 2.2. Paired sample *t*-tests revealed a significant preference for unflavoured corticosterone over IMP during the first three days of switching ($P_{D53} = 0.03$, $P_{D54} = 0.002$, $P_{D55} = 0.0002$), Fig. 2.2E.

2.5.1.2 Total fluid intake

Total fluid intake remained unchanged, between 2.5 to 3.2 ml/day, throughout the experiment for both control groups, Fig 2.2A and Fig 2.2B. Three-way ANOVAs with repeated measured revealed significant differences between groups for the total fluid intake during acquisition ($F_{(4,36)} = 11.16$, $P < 0.001$), relapse ($F_{(4,36)} = 11.44$, $P < 0.001$) and switching ($F_{(4,36)} = 8.40$, $P < 0.001$). Also, there were significant time (weeks or days) effects during acquisition ($F_{(2,72)} =$

6.85, P = 0.002) and switching ($F_{(4,144)}$ = 5.85, P <0.001) but not during the relapse ($F_{(1,36)}$ = 0.77, P = 0.39). Moreover, there was a significant time X group interaction effect during acquisition ($F_{(8,72)}$ = 3.48, P = 0.002), reflecting a gradual rise in the total fluid intake of two corticosterone-treated groups which began to self-administer corticosterone during the acquisition phase.

For group C, mice which had a choice between water and unflavoured corticosterone, post-hoc planned comparisons showed a significant increase in their total fluid intake, which peaked at 4.94 ml/day (1.5 times control levels), compared to the control-vehicle group starting from the second week (P_{W2} = 0.005, P_{W3} = 0.006) during acquisition, and it went up to 8.86 ml/day (~3 times control levels) during relapse (P_{D50} = 0.003, P_{D51} <0.001) and switching (P_{D53}, P_{D54}, P_{D55} and P_{D57} <0.001, P_{D56} = 0.003) compared to the control-vehicle group, Fig. 2.2C.

Group D in which mice's choice was IMP *versus* unflavoured corticosterone showed a significant rise in the total fluid intake from the first week and peaked at 7.55 ml/day (~2.5 times control levels) during acquisition (compared to the control-flavour group: P_{W1} = 0.059, P_{W2} and P_{W3} <0.001; the control-vehicle group: P_{W1} = 0.048, P_{W2} and P_{W3} <0.001) and 6.53 ml/day (~2 times control levels) during relapse (P_{D50} and P_{D51} < 0.001) compared to the two control groups. After switching, mice's total fluid intake remained high and peaked at 7.23 ml/day (~2.5 times control levels) compared to both control groups (the control-flavour group: P_{D53} = 0.006, P_{D54} = 0.008, P_{D55} = 0.002, P_{D56} = 0.061, P_{D57} = 0.008; the control-vehicle group: P_{D53} = 0.007, P_{D54} = 0.007, P_{D55} = 0.002, P_{D56} = 0.059, P_{D57} = 0.009), Fig. 2.2D.

On the other hand, group E in which mice had the choice between water and flavoured corticosterone showed an increase in total fluid intake only during the second day of relapse compared to the control-flavour (P_{D51} = 0.032) and the control-vehicle (P_{D51} = 0.032) groups, Fig. 2.2E.

During habituation, none of the groups, except the control-vehicle group, showed side preference but this factor was in any case controlled for by daily counterbalancing the position of the control and test solutions in each group. The average total fluid intake during the habituation in all groups was between 3-4 ml per day. During the withdrawal phases, the total fluid intake of mice in corticosterone-treated groups dropped markedly from the first day of both withdrawal phases, compared to their fluid intake on the last day (Day 41) of acquisition (paired sample *t*-tests: P_{groupC} = 0.01, P_{groupD} = 0.013, P_{groupE} = 0.0001) and on the last day (Day 57) of switching (paired sample *t*-tests: P_{groupC} = 0.006, P_{groupD} = 0.013, P_{groupE} = 0.04). For withdrawal I (after acquisition), a two-way ANOVA with repeated measures showed that there was no significant main effect of groups on total fluid intake ($F_{(4,36)}$ = 1.18, P = 0.34), but there were significant main effects of time ($F_{(4,144)}$ = 3.91, P = 0.005) and time X group interaction ($F_{(16,144)}$ = 2.63, P = 0.001), reflecting the gradual decrease in the total fluid intake over withdrawal days. During withdrawal II (after switching), no significant differences in the total fluid intake were found between all groups ($F_{(4,36)}$ = 0.63 , P = 0.65), but significant main effects of time ($F_{(2,72)}$ = 20.93, P <0.001) and time X group interaction ($F_{(8,72)}$ = 2.99, P = 0.006) were found, and this indicated a gradual decline of the total fluid intake of corticosterone-treated groups.

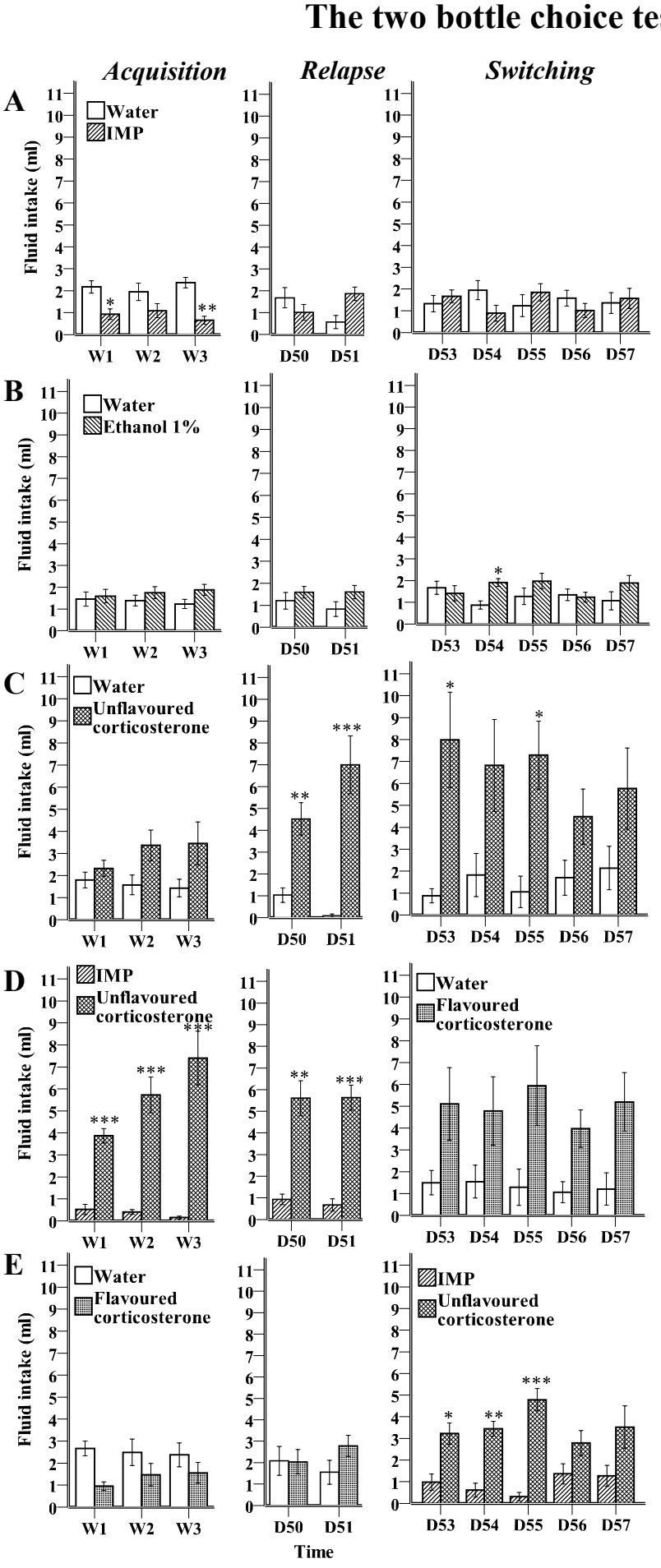

Figure 2.2 The two-bottle choice test. Fluid intake (ml) from the two bottles in the 5 groups (A, B, C, D and E) of male C57BL/6J mice (n = 7-9) during the three phases of the choice test. Data are expressed as mean ± SEM. *P<0.05, **P<0.01 and ***P<0.001 compared to the control solution, the bar on the left. After acquisition, mice underwent one week withdrawal which involved access to one bottle of water. On Day 52, mice had access to one bottle of the new test solution according to the switching phase, see table 2.1.

2.5.2 Dose of corticosterone ingested (mg/kg)

During acquisition, the two way ANOVA with repeated measures showed a significant main group effect for corticosterone intake ($F_{(2, 22)}$ = 16.14, P <0.001) but no significant effects of time ($F_{(2,44)}$ = 2.09, P = 0.14) or time X group interaction ($F_{(4,44)}$ = 0.39, P = 0.82). Post-hoc planned comparisons showed that mice whose choice was IMP *versus* unflavoured corticosterone (P_{W1} and P_{W2} <0.001, P_{W3} = 0.003) or water *versus* unflavoured corticosterone (only during the second week, P_{W2} = 0.041) ingested higher doses of corticosterone than mice which had a choice between water and flavoured corticosterone, Fig. 2.3. Also, mice whose unflavoured corticosterone was offered *versus* IMP ingested more corticosterone than mice which had the choice between water and unflavoured corticosterone (P_{W1} = 0.016, P_{W2} = 0.028, P_{W3} = 0.022). During relapse, the intake of corticosterone differed significantly between groups ($F_{(2,,22)}$ = 5.42, P = 0.012) and, overall, increased over the 2 days ($F_{(1,22)}$ = 5.87, P = 0.02), but there was no significant time X group interaction effect ($F_{(2,22)}$ = 2.79, P = 0.083). Similarly, mice which had a choice between water and flavoured corticosterone consumed lower doses of corticosterone than mice which had unflavoured corticosterone solution paired with water (P_{D50} = 0.052, P_{D51} = 0.004) or with IMP (P_{D50} = 0.009, P_{D51} = 0.050), Fig. 2.3. Lastly, there were no significant differences in corticosterone intake between groups after switching ($F_{(2,,22)}$ = 0.79, P = 0.46) and no time X group interaction effect ($F_{(8,88)}$ = 0.71, P = 0.68), but there was a significant time effect ($F_{(4,88)}$ = 4.44, P = 0.003), Fig. 2.3. Planned comparisons showed that mice whose choice was water *versus* unflavoured corticosterone significantly ingested more corticosterone only on Day 53 (P = 0.023) than mice which had IMP *versus* unflavoured corticosterone after switching, group E.

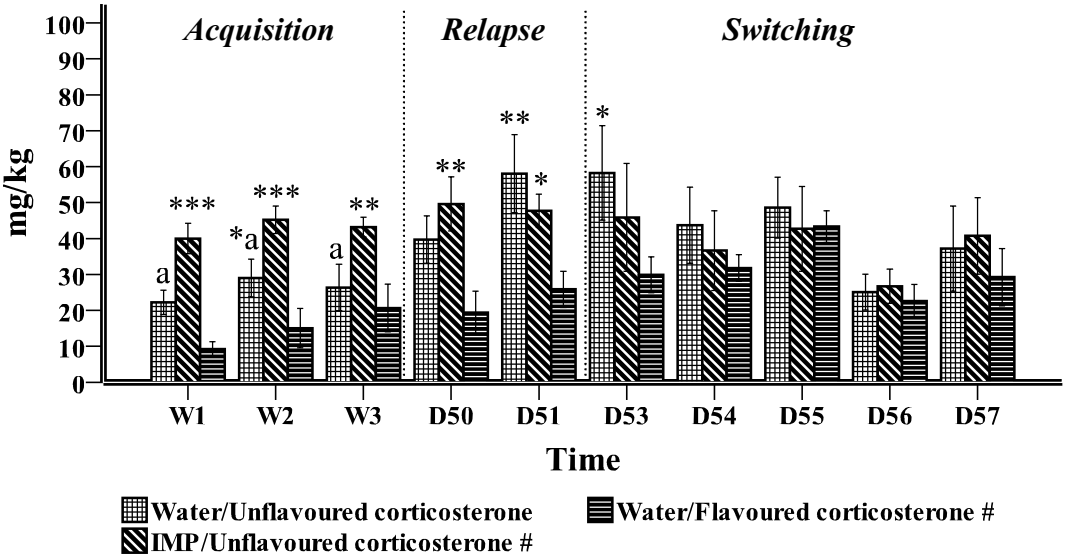

Figure 2.3 Mean corticosterone doses (mg/kg) orally self-administered over the three phases of the two-bottle choice test: acquisition (3 weeks), relapse (2 days) and switching (5 days). Three groups of male C57BL/6J mice (n = 7-9) had access to the corticosterone solution which was flavoured or not with IMP and paired with water and IMP, respectively. Data are expressed as means ± SEM. Significant differences between groups were determined by post-hoc planned comparisons: *P ≤0.05, **P <0.01 and ***P <0.001 compared to (water *versus* flavoured corticosterone), ^{a}P <0.05 compared to (IMP *versus* unflavoured corticosterone). # is for groups whose flavoured and unflavoured solutions were exchanged during the switching phase.

2.5.3 Ethanol consumption (g/kg) during the two-bottle choice test

During acquisition, the two way ANOVA with repeated measures showed significant main effects of groups ($F_{(3, 29)} = 17.70$, $P < 0.001$) and time ($F_{(2,58)} = 5.11$, $P = 0.009$), but no significant time X group interaction effect ($F_{(6,58)} = 1.44$, $P = 0.22$) was found. Post-hoc planned comparisons indicated that mice which had a choice between IMP and unflavoured corticosterone ingested significantly higher doses of ethanol, reaching ~ 2 g/kg during the third week of acquisition compared to the control-vehicle group ($P_{W1} = 0.006$, $P_{W2} < 0.001$, $P_{W3} < 0.001$), Fig. 2.4. In contrast, the other two corticosterone-treated groups did not exhibit significant ethanol intake during the acquisition phases, compared to the control-vehicle group ($P > 0.05$, in all case). Significant main effects of group ($F_{(3, 29)} = 8.13$, $P < 0.001$), time ($F_{(1,29)} = 6.39$, $P = 0.017$) and time X group interaction ($F_{(3,29)} = 3.04$, $P = 0.045$) were detected during relapse. Planned comparisons showed that mice which had a choice between water and unflavoured corticosterone ($P_{D50} = 0.019$, $P_{D51} < 0.001$) or IMP and unflavoured corticosterone ($P_{D50} = 0.003$, $P_{D51} = 0.003$) ingested significantly higher doses of ethanol during relapse than the control-vehicle group, Fig. 2.4. During switching, ethanol intake differed significantly between groups ($F_{(3,29)} = 3.36$, $P = 0.032$) and with time ($F_{(4,116)} = 4.93$, $P = 0.001$), but the time X group interaction effect was not significant ($F_{(12,116)} = 0.93$, $P = 0.52$). Mice whose unflavoured corticosterone solution was paired with water ingested higher ethanol doses, which peaked at 2.28 g/kg, than the control-vehicle group ($P_{D53} < 0.001$, $P_{D54} = 0.006$, $P_{D55} = 0.004$, $P_{D56} = 0.101$, $P_{D57} = 0.061$), Fig. 2.4. Also, mice which had a choice between water and flavoured corticosterone after switching ingested more ethanol than the control-vehicle group on Day 55 ($P = 0.034$).

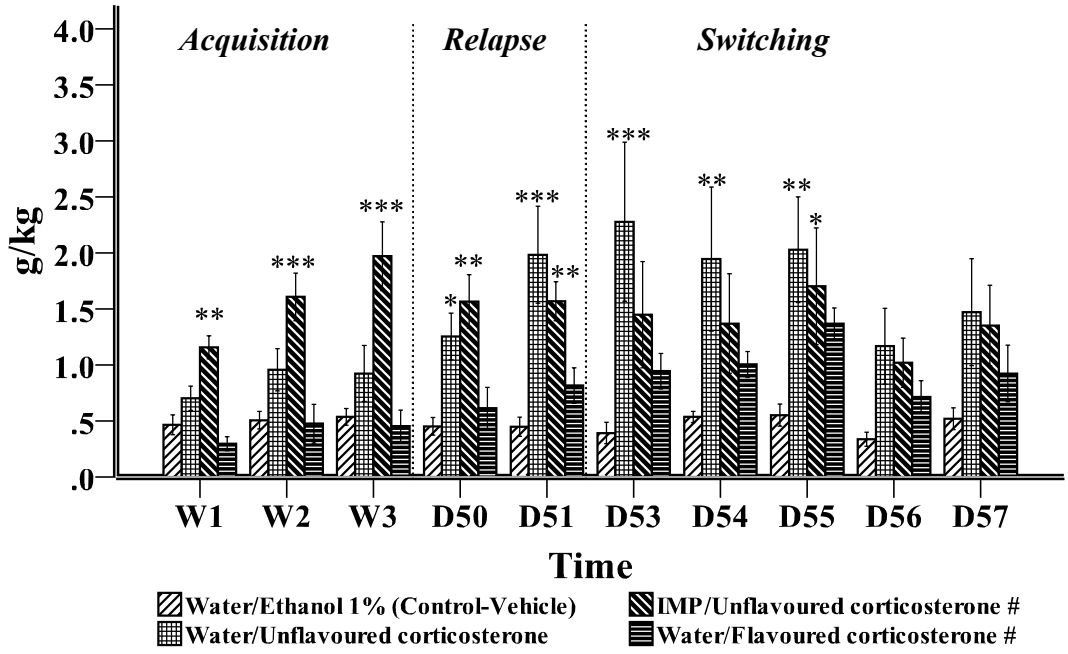

Figure 2.4 Average ethanol intake (g/kg) in male C57BL/6J mice (n = 7-9/group) during the acquisition (3 weeks), relapse (2 days) and switching (5 days) phases of the two-bottle choice test. Data are expressed as means ± SEM. Two-way ANOVAs with repeated measures followed by post-hoc planned comparisons indicated these significant effects: *P <0.05, **P <0.01 and *** P <0.001 compared to the control-vehicle (water *versus* ethanol 1%). # is for groups whose solutions were exchanged during the switching.

2.5.4 Body weight (BW)

Animal body weight (grams) was recorded weekly throughout the experiment. Weight gain or loss was calculated as the difference in BW at the following time points: before habituation and after 3 days of induction of acquisition (Day7 to Day21), after acquisition (Day 21 to Day 42), after withdrawal I (Day 42 to Day 49), after relapse and switching (Day 49 to Day 56), after withdrawal II (Day 56 to Day 63). The two way ANOVA with repeated measures showed no overall significant main effects of groups ($F_{(4,36)} = 1.12$, $P = 0.36$), but there were significant time ($F_{(4,144)} = 130.93$, $P < 0.001$) and time X group interaction ($F_{(16,144)} = 14.44$, $P < 0.001$) effects. After habituation to the two-bottle choice test, post-hoc planned comparisons revealed no difference in BW between groups ($P > 0.05$, in all case). In contrast, 3 weeks of acquisition of corticosterone self-administration resulted in a significant increase in BW of mice compared to the control-flavour (group D and E, $P < 0.001$) and the control-vehicle ($P < 0.001$, in all case) groups, Fig 2.5. At the end of withdrawal I and just before testing relapse of corticosterone self-administration, significant weight loss was observed in all groups treated with corticosterone compared to the corresponding control groups ($P < 0.001$, in all case), Fig 2.5. The three corticosterone-treated groups regained weight after relapse and switching ($P_{groupC} = 0.006$ compared to the control-vehicle group, P_{groupD} and $P_{groupE} < 0.001$ compared to both control groups), Fig 2.5. Similar to withdrawal I, the body weight of corticosterone-treated mice dropped after withdrawal II compared to the control groups ($P < 0.001$, in all case), Fig. 2.5.

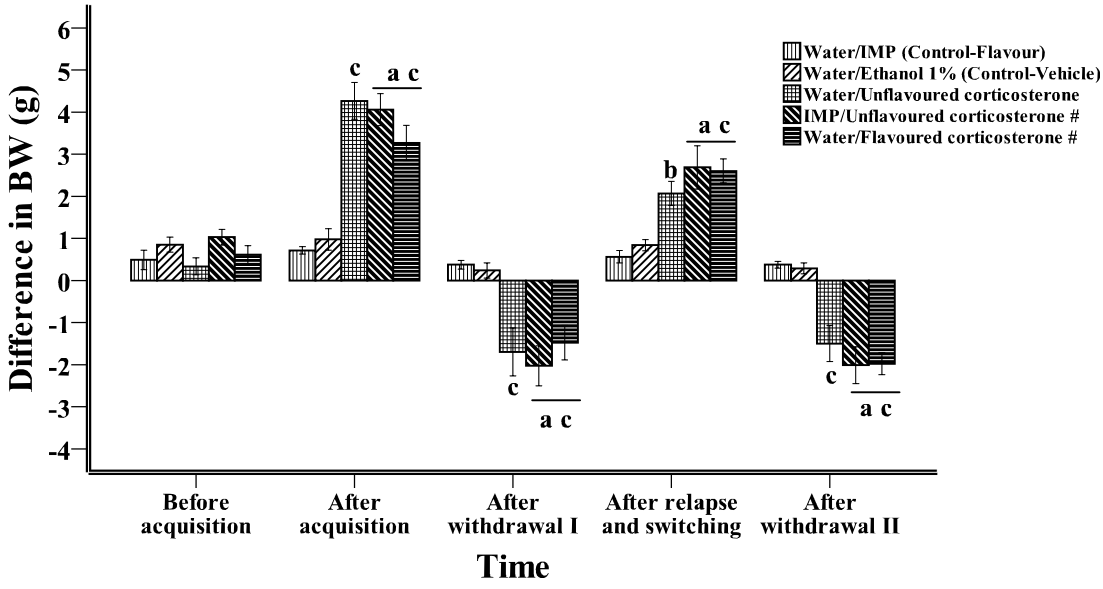

Figure 2.5 Differences in BW (g) as a result of oral corticosterone self-administration. Differences in BW (g) of mice (n = 7-9/group) at the following time points: before acquisition, after acquisition, after withdrawal I, after relapse and switching and after withdrawal II. Data are expressed as mean ± SEM. Post-hoc planned comparisons showed these significant effects: $^{a}P < 0.001$ compared to the control-flavour group (water *versus* IMP), $^{b}P < 0.01$ and $^{c}P < 0.001$ compared to the control-vehicle group (water *versus* ethanol 1%). # indicates the groups whose solutions were exchanged during the switching phase.

2.5.5 Plasma corticosterone levels (ng/ml)

Trunk blood was collected 10 days after the cessation of the two-bottle choice test for the measurement of plasma corticosterone levels. There were no differences in circulating levels of corticosterone between corticosterone self-administered groups and their respective controls ($F_{(4,35)} = 0.49$, $P = 0.74$), Fig. 2.6.

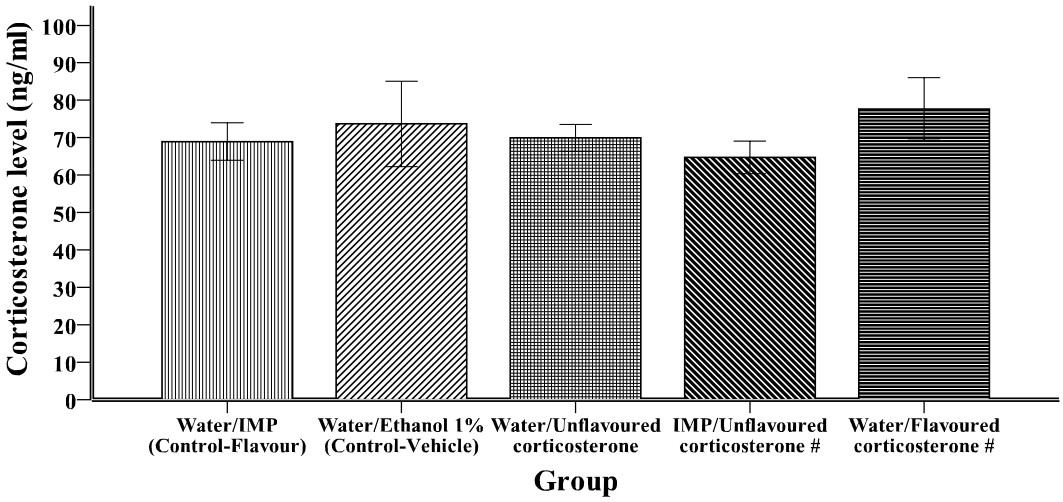

Figure 2.6 Mean plasma corticosterone levels (ng/ml) in the 5 groups of male C57BL/6J mice (n = 7-9) measured 10 days following the cessation of the two-bottle choice test. Data are expressed as means ± SEM. # indicates groups whose two solutions of choice were exchanged during the switching.

2.6 Discussion

The purpose of this phase of the experiment was to test the hypothesis that mice orally self-administer corticosterone. We found that mice acquired oral corticosterone self-administration and ‘‘extinguished mice’’ reinstated oral corticosterone self-administration. Throughout the two-bottle choice test, mice consumed different doses of corticosterone according to the two choices offered and consequently their ethanol (the vehicle) intake was also different. Nevertheless, plasma corticosterone levels were not significantly different between corticosterone naïve and treated groups after a 10 day corticosterone-free period. Interestingly, all corticosterone-treated groups showed weight gain during corticosterone treatment and weight loss during withdrawals.

Side preference was investigated during habituation and controlled daily by counterbalancing and exchanging the location of the two bottles for each mouse. This was important to ensure the validity of the method (Bachmanov *et al.*, 2002; Tordoff and Bachmanov, 2002), and that the difference in the fluid intake between the two bottles was independent of the bottle location. Also, the mice’s total fluid intake was between 3 to 4 ml/day on most days in all groups before acquisition. Since corticosterone is tasteless, IMP was used to flavour the test or the control solutions during the two-bottle choice test, in order to enable mice to distinguish between the two solutions and to facilitate corticosterone self-administration. IMP was used at a concentration of 2 mM for which C57 mice demonstrated 60 % preference over water in the two-bottle choice test (Tordoff, 2007). In contrast, our results showed that there was an initial mild avoidance to IMP. The reason for this is unclear, since we used the same mouse strain as previously reported to have no such aversion (Tordoff, 2007). Given the initial avoidance to IMP, which may facilitate or prevent corticosterone self-administration, it was necessary to extend the two-bottle choice test for 5 days (switching) after the 2-day relapse testing phase to verify that the preference or aversion to corticosterone was flavour-independent.

The results of the study suggest that corticosterone is rewarding as it was self-administered by all corticosterone-treated groups during some or all

experimental phases. In the absence of IMP, mice showed, overall, a small but significant preference for unflavoured corticosterone solution during acquisition, but post-hoc paired sample *t*-tests failed to show a significant preference for unflavoured corticosterone *versus* water over the 3 weeks. Obviously, this suggests that mice had difficulty in acquiring corticosterone self-administration when the two solutions were provided without an IMP cue. The use of IMP, which has an umami taste (Rong *et al.*, 2005), as a control solution facilitated the acquisition of unflavoured corticosterone self-administration due to the initial aversion to the flavour, but switching data showed that corticosterone-dependent mice still self-administered corticosterone when it was flavoured with IMP, reinforcing the hypothesis that self-administration is driven by rewarding properties of the hormone. In addition, mice which did not initially self-administer corticosterone when flavoured with IMP developed a preference for the unflavoured hormone solution when it was paired with IMP during the switching phase, and this was in accordance with the disappearance of the avoidance to IMP in the control-flavour group. Also, it is important to emphasize that the total fluid intake of corticosterone-treated mice did not differ from control-vehicle or control-flavour groups during withdrawals and extinguished mice, which previously showed a significant preference for unflavoured corticosterone over IMP or water, markedly relapsed to self-administer the unflavoured corticosterone solution after withdrawal I.

Thus, these results are consistent with previous work showing that rats self-administered corticosterone using an operant intravenous self-administration protocol and the two-bottle choice test (Deroche *et al.*, 1993; Piazza *et al.*, 1993) and that extinguished rats reinstated oral corticosterone self-administration (Deroche *et al.*, 1993). Similarly, mice reinstated corticosterone self-administration during relapse, as they showed a marked preference for the corticosterone solution over the other choice (water or IMP), and their total fluid intake increased significantly compared to the control groups. It is believed that relapse is a valid marker of addiction (Stewart, 2000). Moreover, food-restricted rats drank more from the corticosterone solution than other rats

which had access to water only (Deroche *et al.*, 1993), since rats naturally drink only a small amount of water when food is not available; this means that corticosterone enhanced drinking behaviour independent of food, and this suggests that corticosterone is rewarding. Importantly, results revealed that mice exhibited a significant preference for the corticosterone solution when their daily intake of the stress hormone was above 30 mg/kg, suggesting that GR activation is crucial for corticosterone self-administration. In agreement with that, Sprague-Dawley rats self-administered corticosterone at doses which produced high plasma corticosterone levels comparable to those induced by stress (Piazza *et al.*, 1993). In the present study, when unflavoured corticosterone was paired with IMP during acquisition, mice showed marked oral corticosterone self-administration and ingested doses of corticosterone over 40 mg/kg on average, peaking at ~ 50 mg/kg. Results clearly indicated that when daily corticosterone intake was as high as 40 mg/kg, corticosterone self-administration became more evident, suggesting that it is dose-dependent. In the current study, the selected concentration of corticosterone (250 µg/ml) solution was thought to be ideal to rapidly elevate plasma corticosterone levels depending on the dose consumed in the two-bottle choice test. For instance, C57BL/6 mice which had access to corticosterone (100 µg/ml) in drinking water demonstrated an increase of corticosterone levels after 4 weeks of treatment (Karatsoreos *et al.*, 2010; Lee *et al.*, 2010). Also, corticosterone-treated mice showed thymus and adrenal atrophy (Karatsoreos *et al.*, 2010). Despite high corticosterone intake, our results showed no statistical alterations in plasma corticosterone levels of corticosterone naïve and treated groups. The lack of difference in corticosterone levels could be attributed to the relatively long withdrawal II (10 days) interval between stopping of the two-bottle choice test and blood collection. For example, rats which were injected with corticosterone (40 mg/kg/day, s.c., for 21 days) showed high plasma levels of corticosterone similar to those induced by immobilization stress, but elevated corticosterone levels returned to control levels within 48 hours (Sapolsky *et al.*, 1985; Pavlides *et al.*, 1993). Also, rats which were subjected to restraint stress showed high corticosterone levels which decreased to the control values within 4 hours (Sapolsky *et al.*, 1984). Similarly, rats implanted with corticosterone

pellets for 1 week had high corticosterone levels compared to the controls, but the elevated corticosterone levels returned to the control level 1 week after removing the pellets (Fernandes *et al.*, 1997). Altogether, our results are consistent with previous work, and this suggests that consuming high doses of corticosterone chronically, as in the present study, is not associated with long-lasting dysregulation of the HPA axis. Indeed, the purpose of measuring plasma corticosterone levels was to verify whether HPA axis function was disrupted, as chronic exposure to high levels of corticosterone has been found to impair the negative feedback regulation of the HPA axis activity, resulting in high basal levels of the hormone (Makino *et al.*, 2002). Rats chronically exposed to stressors including immobilization exhibited high plasma corticosterone levels and GR downregulation in the hippocampus but reduced GR levels were restored after a week of recovery, and this was accompanied by normalisation of corticosterone levels (Sapolsky *et al.*, 1984). Taken together, corticosterone self-administration and relapse were only observed for high doses of corticosterone, suggesting the proposal that rewarding properties of corticosterone are mediated by low affinity GRs which are mainly activated by high plasma levels of corticosterone (Reul and de Kloet, 1985) produced by stress or circadian peaks (Selye, 1950).

An important observation is that corticosterone intake was accompanied by an increase in mice's BW, but mice lost weight during the withdrawal phases when they no longer had access to corticosterone. The change in animal BW which accompanies corticosterone treatment has been used as a marker for effective oral corticosterone administration. Previous research, albeit on birds, indicated that oral corticosterone treatment (20 mg/l in the drinking water) decreased chickens' BW after 1 week (Shini *et al.*, 2009), but treatment with chronic ACTH increased corticosterone levels and BW of laying hens (Mumma *et al.*, 2006). It was suggested that ACTH increased BW of laying hens through increasing water content of muscle through some unknown mechanisms (Mumma *et al.*, 2006). On the other hand, C57BL/6J mice showed no significant change in their BW after 4 weeks of corticosterone availability in drinking water (100 μg/ml) which doubled their plasma corticosterone levels

compared to the control group (Lee *et al.*, 2010). In contrast, C57BL/6 mice showed weight gain and increased food consumption after chronic corticosterone ingestion offered in drinking water (100 μg/ml) in 1% ethanol (Karatsoreos *et al.*, 2010). The effects on BW could be attributed to corticosterone altering electrolyte balance. It was found that dexamethasone (a GR agonist) plus deoxycorticosterone (a MR agonist) increased urine excretion and intake of water and sodium from saline solution but decreased BW of rats (Thunhorst *et al.*, 2007). In the present study, corticosterone-treated mice had no access to saline but their food was a source of sodium. This suggests that mice consumed more food to increase their sodium intake and consequently BW increased. The inconsistencies between our results and those previously reported need further investigation as the amount of food pellets consumed by mice was not weighed. The increased total fluid intake which was seen in two corticosterone-treated groups was very unlikely to have resulted in the observed weight gain after acquisition or switching, as one of groups (water *versus* flavoured corticosterone) showed a similar increase in BW to the other corticosterone-treated groups but no rise in total fluid intake compared to the control groups even after switching when mice in this group significantly self-administered the unflavoured corticosterone solution.

In conclusion, these findings emphasize that corticosterone does not only facilitate the rewarding effects of natural rewards and drugs of abuse, but also *per se* it has reinforcing and rewarding properties.

Chapter 2 - Part 2

How does corticosterone affect cognition when it is rewarding?

2.7 Aims

A main aim of this experiment was to investigate how corticosterone self-administration could affect 2 cognitive processes: spatial and recognition memory. Spatial memory was evaluated using the spontaneous alternation and novel object location tests, while the novel object recognition test was used to evaluate recognition memory.

2.8 Materials and methods

2.8.1 The spontaneous alternation test (SAT) in a T- maze

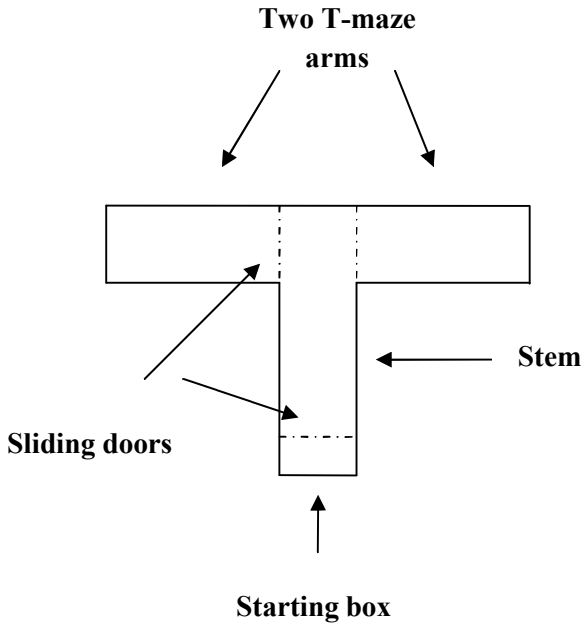

Figure 2.7 Diagrammatic representation of the T-maze apparatus

The T-maze test uses the strong tendency of rodents to explore a new arm of a maze after the other arm has been visited shortly beforehand (Montgomery, 1952). The apparatus is composed of three main parts; the starting box (6 cm X 7.5 cm), stem and two identical arms (41.6 cm long), which are closed by vertical sliding doors. The walls of the T-maze are made of transparent Perspex which allowed an animal to use different cues present in the room such as a lab

book, a clock, etc…to choose to enter the arm unexplored during the previous trial. The test was carried out in a sound attenuated room illuminated with fluorescent lighting. The test was undertaken during the light phase and groups were counterbalanced throughout the test day. In brief, an animal was placed in the starting box for 5 sec, and then the door to the stem was opened allowing the animal to move freely through the stem and the two arms for about 1-minute habituation. Then, the animal was returned back to the starting box and was subjected to a series of 9 consecutive trials. In each trial, the door to the stem was opened after 5 sec, and the animal entered one of the two arms (free choice). Once the animal entered one arm, the door to that arm was closed for 15 sec as well as the door to the other arm. Then, the animal was returned back to the starting box by gently pushing its back for the following trial. The equipment was cleaned with 20 % ethanol between animals to remove olfactory cues. Mice were tested in the T-maze paradigm before acquisition and during the two withdrawal phases.

The percentage of correct alternation for each mouse was calculated according to this equation:

$$\text{The percentage of correct alternations} = \frac{\text{Number of alternations}}{\text{Maximum alternation (8)}} \times 100$$

Memory unimpaired rodents significantly alternate between the two arms above 50 % (Montgomery, 1952; Dember and Fowler, 1958)**.**

2.8.2 Novel object recognition and location tests

2.8.2.1 Habituation

Before performing the memory test, habituation is required to make mice familiar with empty open-field arenas in order to direct their exploratory behaviour mainly toward the test objects during the memory tests. It has been reported that mice have to be habituated to the open field arena to learn about the objects during the acquisition trial (Stefanko *et al.*, 2009). Also, the open

field has been used to assess motor activity and anxiety-like behaviour (Choleris *et al.*, 2001), as these factors could interfere with memory performance. Mice were individually habituated to the test arenas 24 hours before the novel object recognition test. In accordance with Howlett *et al.* (2004), mice individually underwent a 30-minute habituation session during the light phase using open field Perspex arenas (30 x 35 x 25 cm) with transparent sides and grey bottom. Sessions were tracked using a camcorder attached to the ceiling of the testing room which was illuminated by fluorescent lights. The number of defecations was counted for each mouse, and arenas were cleaned using 20 % ethanol between mice to remove any olfactory cues. The total distance travelled, the distance travelled in the centre or the time spent in the centre was automatically tracked using the Ethovision software (Noldus, Netherlands). The percentage of distance travelled and time spent in the centre was calculated relative to the total distance travelled and the total time spent in the arena, respectively. The total distance travelled, percent distance travelled and percent time spent in the centre were used to investigate the group differences in locomotion and anxiety-like behaviour, respectively, prior to the memory tests as these processes may interfere with object exploration.

2.8.2.2 The novel object recognition test (NORT)

This task depends on the high tendency of mice to explore a novel object for a longer time than a familiar one (Ennaceur and Delacour, 1988). The test was carried out one week after the end of corticosterone treatment to evaluate the effects of chronic corticosterone self-administration on recognition memory. Twenty four hours after the habituation session, mice were subjected to two trials, 10 minutes each, separated by a four-hour inter-trial interval (Howlett *et al.*, 2004), Fig 2.8. In the first trial (acquisition), each mouse was left to explore one object (a varnished wooden triangular or spherical block); to help mice use the spatial strategy as well as familiarity to recognise the novel object. During the second trial (retention), the mouse explored two objects, the same object explored during the first trial (familiar object) and another object of a different shape (novel object). Sessions were recorded using a camcorder attached to the

ceiling of the testing room which was illuminated by fluorescent lights. The mouse movement in the arena was tracked using the Ethovision software (Noldus, Netherlands). The arenas and objects were cleaned with 20 % ethanol between sessions to remove any olfactory cues. The exploration times for the familiar and novel objects were manually and blindly scored twice for each mouse, and if the second score of a given mouse differed by 10% from the first score, a third scoring was done for this mouse and the 2 closest values were averaged for statistical analysis. The mouse was considered as exploring an object when it approached and directed its nose toward the object within a distance of 1 cm. The time spent touching or sitting on an object while the mouse's nose was not directed toward it was not included in the exploration time. The position of objects in the arenas was counterbalanced to control for location preferences. Recognition index (RI) was calculated according to the following equation:

$$RI = \frac{\text{Novel object exploration time X 100}}{\text{Novel object exploration time + Familiar object exploration time}}$$

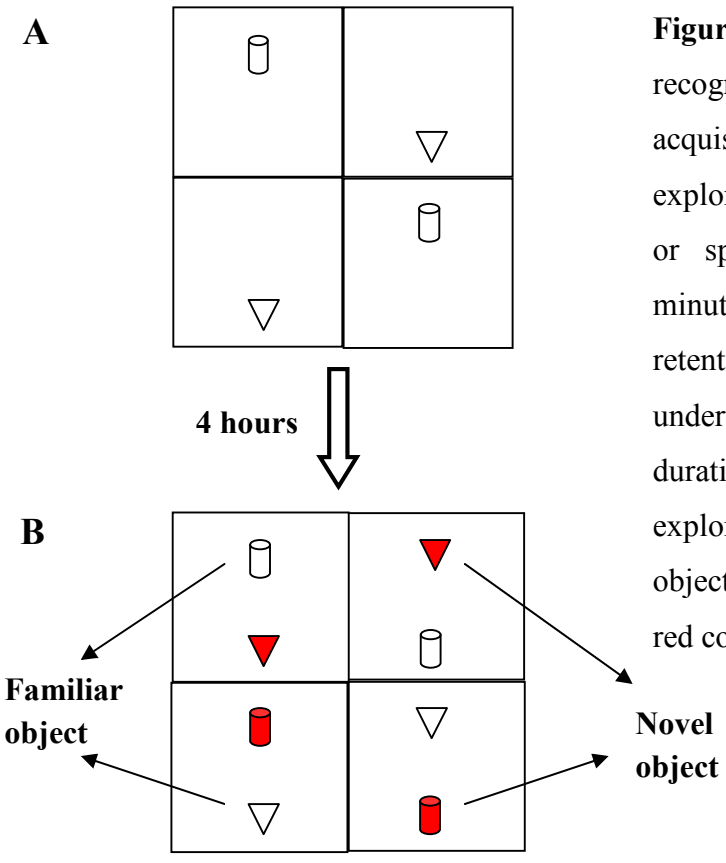

Figure 2.8 The novel object recognition test. In the acquisition trial (A), a mouse explored only one triangular or spherical object for 10 minutes. Four hours later, the retention trial (B) was undertaken for the same duration in which the mouse explored the same familiar object and the novel object, red colour.

2.8.2.3 The novel object location test (OLT)

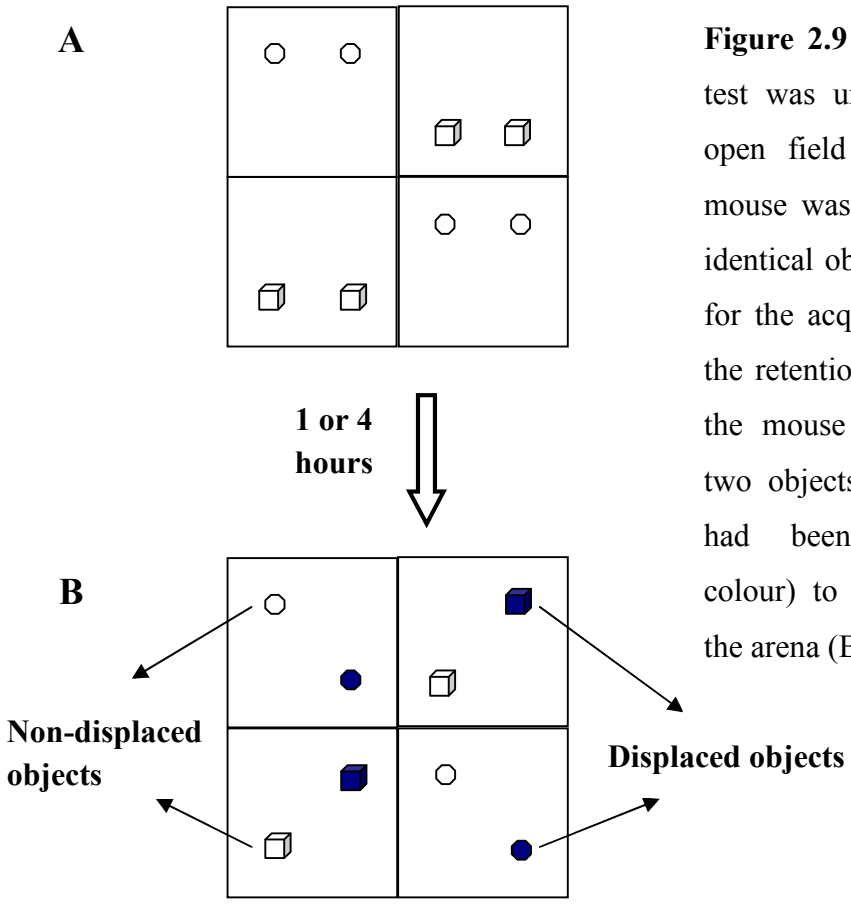

Figure 2.9 The object location test was undertaken using the open field arena in which a mouse was left to explore two identical objects for 10 minutes for the acquisition trial (A). In the retention trial (10 minutes), the mouse explored the same two objects after one of them had been displaced (blue colour) to a novel location in the arena (B)

This task aims to test spatial memory (Ennaceur *et al.*, 1997; Dix and Aggleton, 1999) through using the high tendency of rodents to explore a familiar object in the second trial for a longer time when its location has been changed rather than at a fixed location. This test was undertaken 24 hours after the novel object recognition test. Mice underwent two 10-minute trials, separated by four hours or 1 hour (for the other experiments described in Chapters 3 and 4), Fig 2.9. During the two trials, each mouse was left to explore two identical objects (hexagonal or square varnished wooden blocks), provided that one of the two objects was displaced to a different location in the arena for the second trial. Sessions were recorded by a camcorder attached to the ceiling of the testing

room which was illuminated by fluorescent lights. The mice's movement in the arena was tracked using the Ethovision software (Noldus, Netherlands). The arenas and objects were cleaned using 20 % ethanol between sessions to remove any olfactory cues. The criteria of scoring the object exploration time of each mouse was as described in the novel object recognition test. A location index (LI) was calculated using this equation:

$$\mathbf{LI} = \frac{\textbf{Displaced object exploration time X 100}}{\textbf{(Non-displaced object exploration time + displaced object exploration time)}}$$

2.8.3 Statistical analysis

Variables evaluated during the habituation session; including the total distance travelled, percent distance and percent time spent in the centre relative to total and number of defecations, were analysed using one way ANOVAs with groups as the between subject factor. Two-way ANOVAs with repeated measures were used to analyze the percentage of correct alternation and total exploration times in the novel object recognition and location tests. The between subject factor was group and repeated measures were the within subject factors: time of the alternation test (three levels) or trials (two levels). Post-hoc planned comparisons were used when appropriate. Paired sample *t*-tests were used to compare the exploration times between the familiar and novel objects and between the non-displaced and displaced objects in each group. One sample *t*-tests were used to compare the percentage of correct alternation, LI and RI to 50 % (lack of discrimination). Data are expressed as mean ± SEM. An effect was considered significant when $P \leq 0.05$.

2.9 Results

2.9.1 The spontaneous alternation test

The two-way ANOVA with repeated measures showed no significant main effects of groups ($F_{(4, 36)} = 0.55$, P = 0.70) and time ($F_{(2,72)} = 1.31$, P = 0.28) in the spontaneous alternation performance, but a significant time X group interaction effect ($F_{(8,72)} = 2.58$, p = 0.016) was detected. Post-hoc planned comparisons revealed a significant decrease in the alternation rate (P = 0.02) of mice which had a choice between water and flavoured corticosterone during withdrawal I, compared to the control-flavour group (water *versus* IMP). Also, planned comparisons showed a significant increase in the spontaneous alternation performance in all corticosterone self-administered groups during withdrawal II, compared to the control-vehicle group (water *versus* ethanol 1%, P <0.05, in all case). The percentage of correct alternations was also compared to 50% (chance alternation) using a one sample t-test for each group at each time point to test whether mice used a spatial memory strategy. All groups significantly alternated above the chance level before acquisition (baseline) and during the first withdrawal period, but mice treated with the ethanol vehicle did not significantly discriminate between the 2 arms during withdrawal II, Fig. 2.10.

2.9.2 Novel object recognition and location tests

2.9.2.1 Habituation

The one way ANOVA revealed a significant difference between groups in the total distance travelled ($F_{(4,36)} = 5.74$, P = 0.001), with all corticosterone-treated groups being less active than the control-vehicle (P = 0.001, in all case) and the control-flavour (P <0.05, in all case) groups throughout the 30-minute session, Fig. 2.11A. Also, there was a significant group effect in the distance moved in the central sector relative to total ($F_{(4, 36)} = 6.05$, P = 0.001). Planned comparisons revealed that mice which had had the choice between IMP and unflavoured corticosterone exhibited a significantly lower distance moved in the centre than all groups (P <0.01, in all case), Fig. 2.11B. There were, however, no significant group differences for the time which mice spent in the centre

relative to total ($F_{(4, 36)}$ = 0.72, P = 0.59, Fig. 2.11B) and for the number of defecations ($F_{(4, 36)}$ = 0.38, P = 0.82, Fig. 2.11C).

2.9.2.2 The novel object recognition test

The two-way ANOVA with repeated measures showed no significant main effects of groups ($F_{(4,36)}$ = 1.76, P = 0.16), trials ($F_{(1,36)}$ = 0.95, P = 0.34) or trial X group interaction ($F_{(4,36)}$ = 2.45, P = 0.064) for the total object exploration time, Fig. 2.12A. In the retention trial, the time spent exploring the familiar and novel objects differed significantly ($F_{(1,36)}$ = 39.47, P = 0.0000003), but there was no object X group interaction effect ($F_{(4,36)}$ = 2.07, P = 0.11). Post-hoc paired sample t-tests revealed that mice in the control-vehicle group and the group in which mice had a choice between IMP and unflavoured corticosterone had recognition memory deficits, as they did not spend more time exploring the novel object (t = 1.99, P = 0.09, t = 0.9, P = 0.40, respectively) than the familiar object, Fig. 2.12B. This was confirmed by one sample t-tests comparing RI of each group to 50%, although there was no significant group effect for RI ($F_{(4, 36)}$ = 1.85, P = 0.14), Fig. 2.12C.

2.9.2.3 The object location test

There were no significant main effects of groups ($F_{(4,36)}$ = 0.34, P = 0.85), trials ($F_{(1,36)}$ = 0.63, P = 0.43) and trial X group interaction ($F_{(4,36)}$ = 0.90, P = 0.48) for the total exploration times, but significant main effects of object displacement ($F_{(1,36)}$ = 13.79, P = 0.001) and trial X object interaction ($F_{(1,36)}$ = 4.60, P = 0.039) were revealed. Paired sample t-tests showed no difference in the time spent exploring the two identical objects in each group during the acquisition trial, Fig. 2.13A. In the retention trial, mice which had had a choice between water and flavoured corticosterone spent a longer time exploring the displaced object than the non-displaced object (t = 2.53, P = 0.035), Fig. 2.13B. One sample t-tests revealed that the groups in which mice had a choice between water and unflavoured corticosterone or water and flavoured corticosterone during the two-bottle choice test significantly discriminated the novel location (t

= 2.53, P = 0.035 and t = 2.54, P = 0.035, respectively compared to chance), but the other groups did not, Fig. 2.13C. However, a one way ANOVA showed no differences in LI between groups ($F_{(4, 36)}$ = 0.56, P = 0.70), Fig. 2.13C.

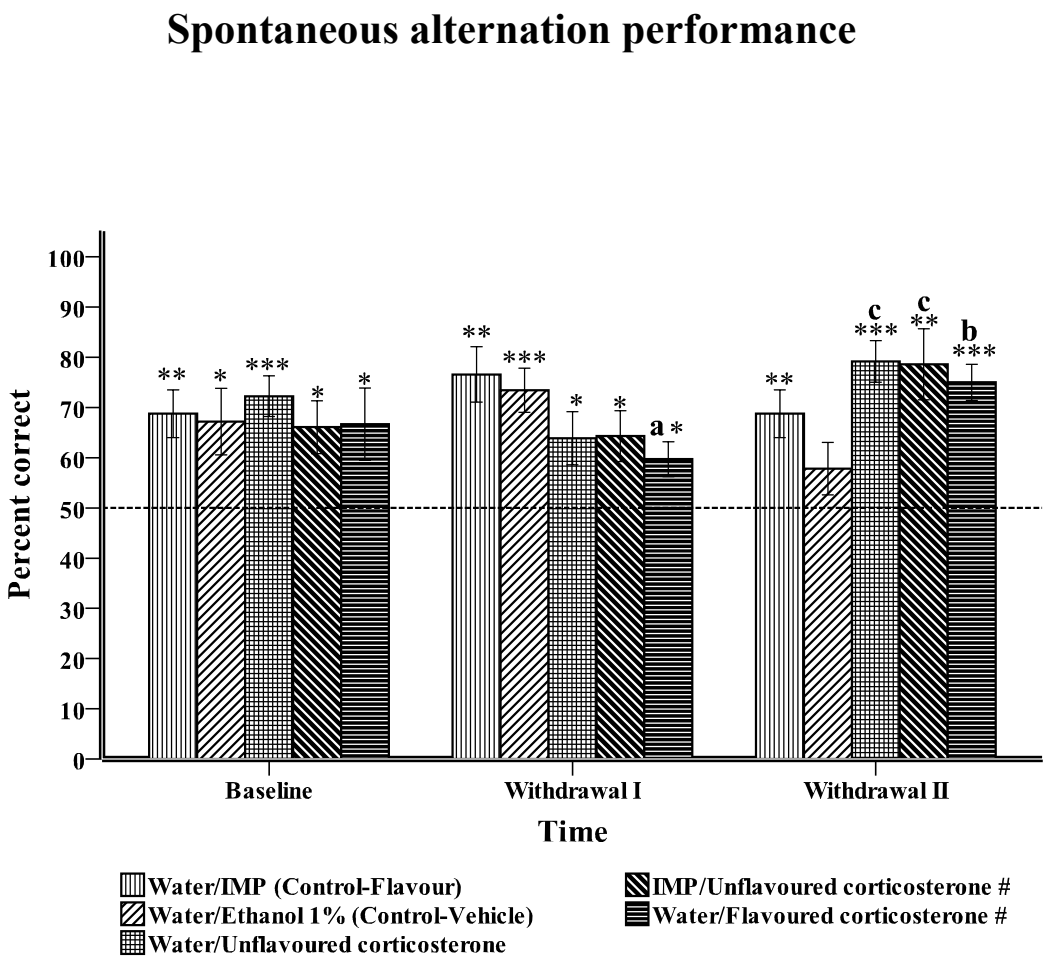

Figure 2.10 Spontaneous alternations in the T-maze. The percentage of correct alternations (percent correct) of the control and corticosterone-treated groups (n = 7-9 mice) before acquisition (baseline) and during the two withdrawal phases. Data are expressed as means ± SEM. One sample *t*-tests: *P ≤0.05, **P <0.01 and ***P <0.001 significantly different from 50% (alternation by chance). Planned comparisons: ^{a}P <0.05 significantly different from the control-flavour group (water *versus* IMP), ^{b}P <0.05 and ^{c}P <0.01 significantly different from the control-vehicle group (water *versus* ethanol 1%), # indicates groups whose solutions were exchanged during the switching phase.

Locomotion and anxiety-related behaviour in the open field

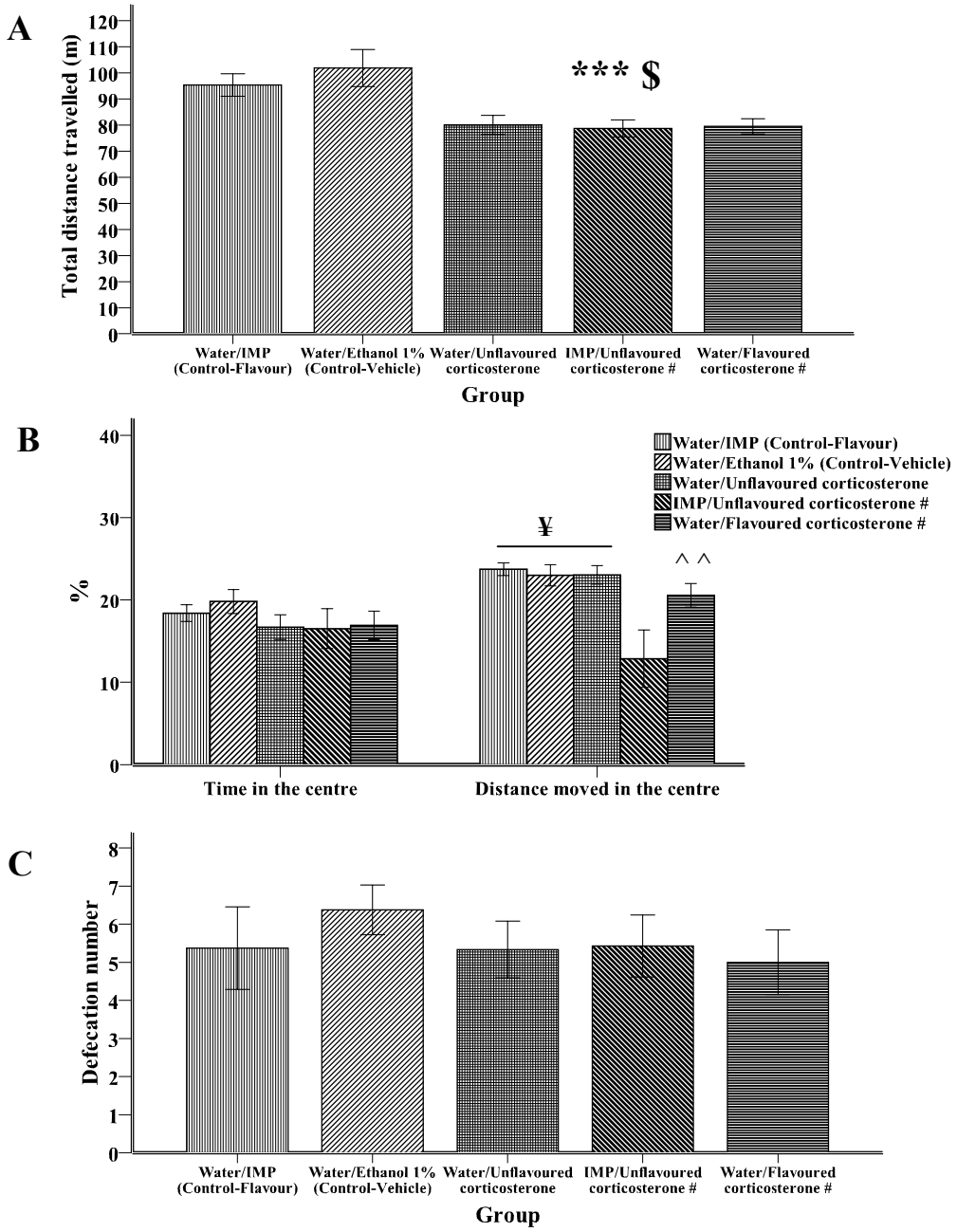

Figure 2.11 Locomotion and anxiety-like behaviour during the habituation session. (A) Total distance travelled. (B) Percent time and distance in the centre relative to total. (C) Defecation number. One week after the cessation of the two-bottle choice test, mice (n = 7-9/group) were habituated to the object test arena for 30 minutes, 24 hours before the novel object recognition test. Data are expressed as means ± SEM. Planned comparisons: ***P = 0.001 compared to the control-vehicle (water *versus* ethanol 1%) group, $P <0.05 compared to the control-flavour (water *versus* IMP) group, ^^P <0.01 and ¥P <0.001 compared to (IMP *versus* unflavoured corticosterone). # is for groups whose solutions were exchanged during the switching phase.

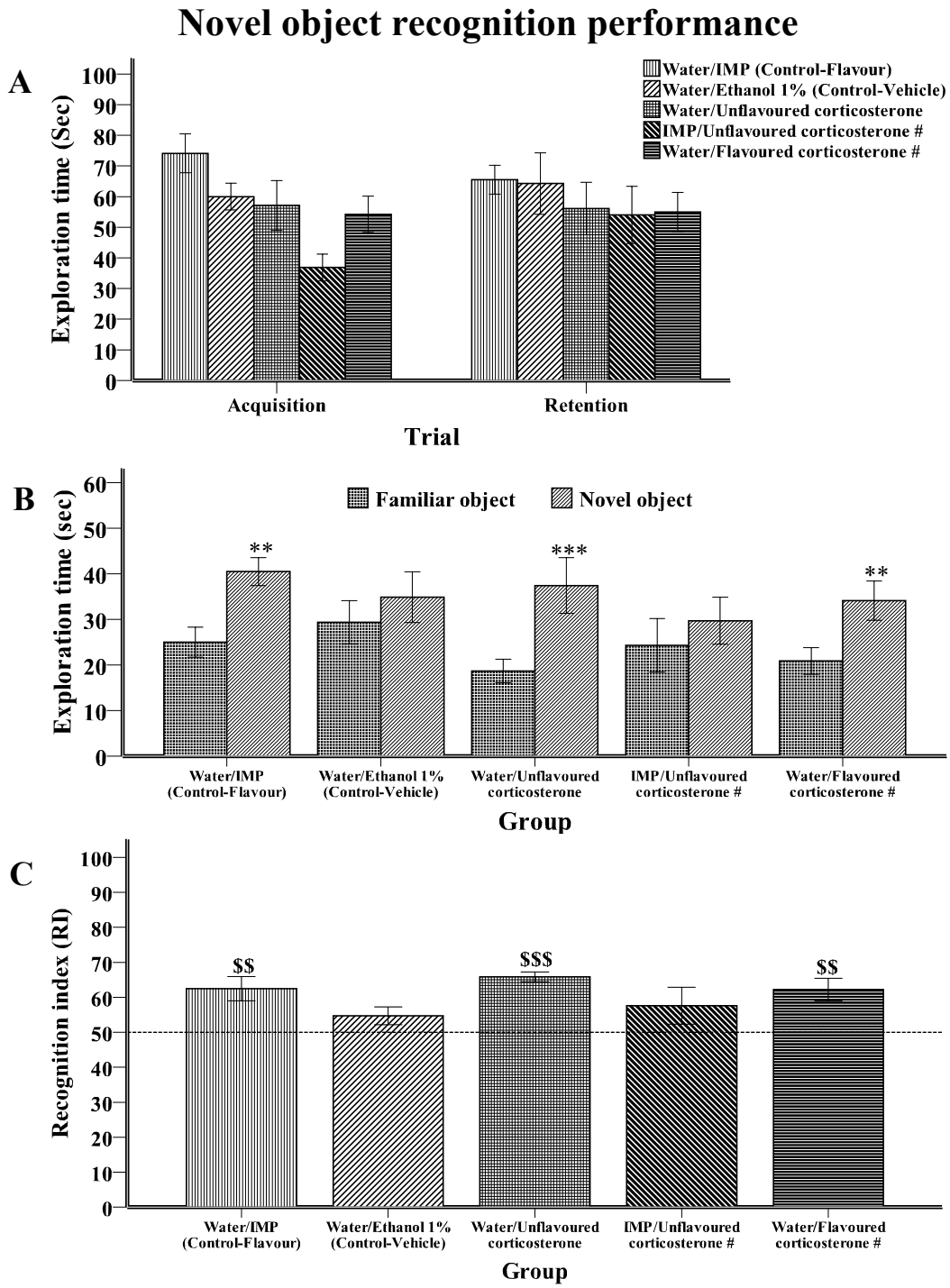

Figure 2.12 The novel object recognition test. (A) Total exploration times for each group (n = 7-9 mice) during the 10-minute acquisition and retention trials. (B) Time spent exploring the familiar and novel objects during the retention trial. (C) Recognition index (RI) was calculated as % (novel object exploration time / total exploration time). Data are expressed as means ± SEM. Paired sample *t*-tests: **P <0.01 and ***P = 0.001 compared to time spent exploring the familiar object. One sample *t*-tests: $^{\$\$}$P <0.01 and $^{\$\$\$}$P <0.001 compared to 50% (no discrimination). # indicates groups whose solutions were exchanged during the switching phase.

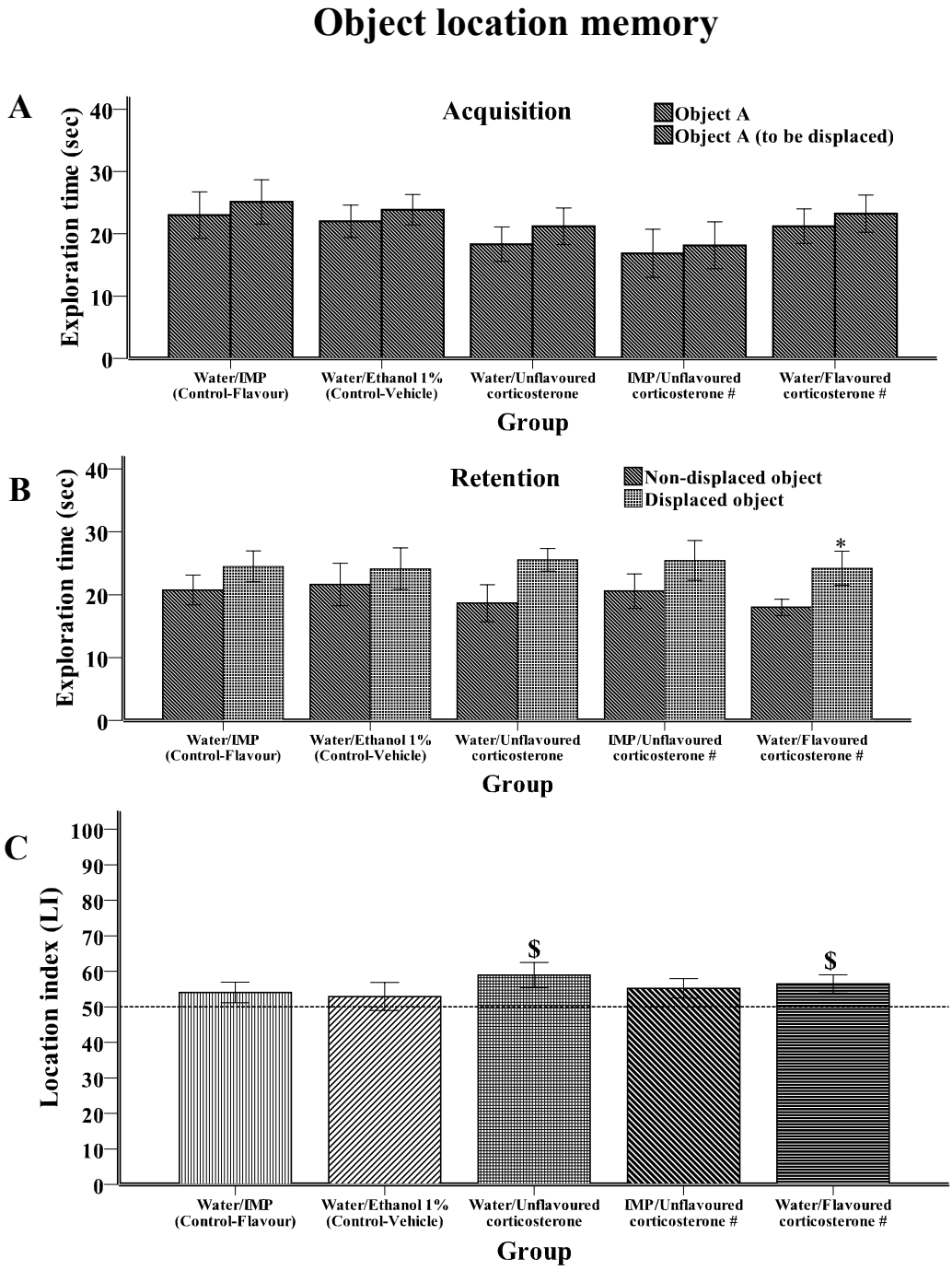

Figure 2.13 The object location test. (A) Exploration times for the two identical objects in each group (n = 7-9 mice) of corticosterone naïve and treated mice during the acquisition trial. (B) Mice's exploration times of the non-displaced and displaced objects during the retention trial. (C) Location index (LI) was calculated as % (displaced object exploration time / total exploration time). Data are expressed as means ± SEM. Paired sample *t*-tests: *P <0.05 compared to the non-displaced object. One sample *t*-tests: $P <0.05 compared to 50% (chance). # indicates groups whose solutions were exchanged during the switching phase.

2.10 Discussion

The second objective of this experiment was to test the impact of corticosterone self-administration and reinstatement on spatial and recognition memory. Chronic corticosterone self-administration did not impair spontaneous alternation performance and only a group of corticosterone-treated mice exhibited recognition memory deficits. Also, the control-vehicle group exhibited both recognition and spatial memory deterioration by the end of the experiment. It is important to emphasize that, although all corticosterone-treated mice self-administered the hormone, there were differences in the onset and magnitude of corticosterone self-administration in each group. Mice whose corticosterone solution was paired with IMP self-administered corticosterone rapidly and markedly from the first week of acquisition and the ingested doses remained at high levels during relapse and switching phases. On the other hand, when the unflavoured corticosterone solution was paired with water, mice developed corticosterone self-administration more slowly and their corticosterone doses were lower than mice which had unflavoured corticosterone *versus* IMP during acquisition. By contrast, mice whose corticosterone solution was flavoured with IMP during acquisition and relapse started to self-administer corticosterone significantly during the switching phase. Thus, the three groups chronically received different corticosterone doses, and this was accompanied with distinct effects on memory. Secondly, ethanol 1% (vehicle) possibly contributed to the effects of corticosterone on cognitive performance, since ethanol intake was dependent on the amount of corticosterone consumed in each group.

Because motor activity and anxiety-related behaviour can confound mice's performance in cognitive tasks, mice were first habituated to the open field to investigate these measures. All corticosterone-treated groups were less active in the open field arena than the control groups. Likewise, rats which were implanted with corticosterone pellets for a week exhibited a reduced motor activity but this disappeared a week after the removal of corticosterone pellets (Fernandes *et al.*, 1997). Moreover, 3 weeks of corticosterone (100 μg/ml) treatment in drinking water reduced locomotor activity of C57BL/6 mice

(Karatsoreos *et al.*, 2010). Also, corticosterone-treated groups showed no significant difference in BW during the withdrawal phases, and this excludes weight gain observed during corticosterone treatment as a reason for reduced activity. However, the decrease in motor activity could be a withdrawal symptom of the rewarding hormone, and this point needs further investigation.

Anxiety-related behaviour was generally unaltered by corticosterone except for mice which had a choice between IMP and unflavoured corticosterone, as they showed reduced activity in the central sector of the open field relative to total, compared to the other groups. However, there were no group differences for the percentage of time which mice spent in the centre and for the number of defecations, which has been used as a measure of emotionality (Candland *et al.*, 1967). A recent study showed that C57 mice exhibited enhancement of anxiety-like behaviour in the elevated plus maze test after 4 weeks of unlimited access to corticosterone at 100 μg/ml concentration in 1% ethanol, but these symptoms disappeared 4 weeks after the cessation of corticosterone treatment (Lee *et al.*, 2010). The discrepancies between all anxiety-related measures in one of the corticosterone-treated groups (IMP *versus* unflavoured corticosterone) provided insufficient evidence for changes in anxiety-like behaviour.

Spatial memory of mice was evaluated in the spontaneous alternation test at 3 time points: before acquisition and during the two withdrawal phases. The spontaneous alternation performance was preserved in all corticosterone-treated groups which alternated above the chance level at all time points, although overall performance of all three corticosterone-treated groups tended to be inferior to the two control groups after the acquisition phase but higher than control mice after switching. Interestingly, mice which had a choice between water and flavoured corticosterone had lower alternation rate than the control-flavour group after acquisition, suggesting that high corticosterone intake by the other groups which exhibited a significant preference for the hormone during acquisition did not, however, impair the spontaneous alternation behaviour. These findings are in agreement with a number of published reports, although the literature does contain contradictory findings. For instance, chronic intake of

corticosterone (400 μg/ml) in drinking water for 21 days did not impair spatial recognition memory of rats in a Y-maze task (Conrad *et al.*, 2007). Similarly, most rats receiving corticosterone in drinking fluid (400 μg/ml in 2 % ethanol, ~30 mg/kg) for 8 weeks showed no spatial memory impairment in the radial arm maze (Luine *et al.*, 1993). Under a different mode of administration, rats receiving subcutaneous injection of corticosterone at 26.8 mg/kg for 21 days showed no spatial memory impairment in the Y maze (Coburn-Litvak *et al.*, 2003). On the other hand, rats had spatial learning difficulties in the radial 8-arm maze when implanted with corticosterone pellets for 3 months (Endo *et al.*, 1996). Whereas 2 months of corticosterone injections (10 mg/kg) impaired spontaneous alternation in rats (Bardgett *et al.*, 1994), rats treated with the same dose were unimpaired for spatial memory in a platform-maze test (Bardgett *et al.*, 1996). During withdrawal II, the object location test was used to assess spatial memory of mice (Ennaceur *et al.*, 1997; Dix and Aggleton, 1999). Our results showed that control mice were not able to discriminate the displaced object to a significant degree. This is likely related to the long 4-hour delay between the two trials. Previous work showed that mice perform optimally in the object location test when the duration between the acquisition and retention trials is up to 2 hours (Murai *et al.*, 2007). The long inter-trial interval was used in the present study to detect a possible improvement in spatial memory given previous (unpublished) corticosterone self-administration work. This was confirmed in the present study as 2 of 3 groups of corticosterone-treated mice significantly discriminated the displaced object.

The novel object recognition test was used to assess non-spatial memory. Corticosterone-treated mice were generally unimpaired in this task except for the group which had a choice between IMP and unflavoured corticosterone. This could be secondary to ingesting extremely high doses of corticosterone (40 mg/kg/day on average) and/or ethanol, which peaked at 2 g/kg/day, during acquisition, since ethanol-treated control mice also showed recognition memory deficits. Although two groups showed recognition memory impairment, RIs were not significantly different between all groups. Previously, it was found that plasma levels of corticosterone had an inverted U-shaped relationship with

primed burst potentiation (PBP), a physiological model of memory (Diamond *et al.*, 1992). Thus, low plasma corticosterone levels positively correlated with PBP with the maximal correlation being with moderate levels of corticosterone (Diamond *et al.*, 1992). Also, it was reported that enhanced performance in the novel object recognition test had an inverted U-shaped relationship with corticosterone levels (Okuda *et al.*, 2004). The hippocampus is involved in the processing of spatial and recognition memory (Clark *et al.*, 2000; Gould *et al.*, 2002). However, there has been some evidence that the rhinal cortex also contributes to a great extent to recognition memory (Mumby and Pinel, 1994; Ennaceur *et al.*, 1996). The hippocampus is very vulnerable to changes in plasma corticosterone levels as GRs and MRs are highly expressed in this area (Reul and de Kloet, 1985; Diamond *et al.*, 1992; Pavlides *et al.*, 1995). As stated earlier, MRs have a 10-fold higher affinity for corticosterone than GRs which are activated by high levels of corticosterone (Reul and de Kloet, 1985). Rats showed memory improvement in the Morris water maze when they were exposed to mild stress and low doses (10 mg/kg, i.p.) of corticosterone (Akirav *et al.*, 2004). However, relatively high corticosterone doses (26.8 mg/kg) for 8 weeks caused spatial memory impairments in rats in the Y-maze task (Coburn-Litvak *et al.*, 2003). In the hippocampus, MR activation improved hippocampal LTP, while GR activation was shown to suppress it (Pavlides *et al.*, 1995). Furthermore, pre- and post-training intrahippocampal injection of a GR antagonist enhanced performance of rats in the water maze (Oitzl *et al.*, 1998), indicating that high levels of corticosterone impaired memory performance. Taken together, recognition and spatial memory deficits of one corticosterone treated group (IMP *versus* unflavoured corticosterone) could be due to elevated corticosterone consumption, compared to the other two corticosterone self-administered groups during the acquisition phase of the two-bottle choice test. To make it clear, the group whose choice was water *versus* flavoured corticosterone received the lowest corticosterone dose during acquisition and relapse (on average less than 20 mg/kg/day but 30 mg/kg during switching), while the group offered a choice between water and unflavoured corticosterone ingested average corticosterone doses of 26 mg/kg/day during acquisition but 45 mg/kg/day during relapse and switching. Mice in these groups showed a

significant preference for the object displaced in the novel location. In contrast, the corticosterone-treated group which was unable to discriminate the displaced object received much higher corticosterone doses (40 mg/kg/day on average during all testing phases). This suggests that short-term exposure to high corticosterone doses may have a positive effect on spatial memory. Also, ethanol intake is suggested to contribute to the recognition memory impairment which was seen in one of corticosterone-treated groups. In agreement with this, an acute dose of ethanol (1 g/kg) was found to impair rats' performance in a food-reinforced alternation T-maze paradigm (Rossetti *et al.*, 2002). Others found that both 0.5 g/kg and 1.5 g/kg of ethanol, i.p., impaired spatial working memory of C57BL/6J mice in a T-maze (Melchior *et al.*, 1993). It was also reported that alcoholics develop memory impairment and learning difficulties (Bowden and McCarter, 1993; Parsons and Nixon, 1993). In the present experiment, chronic small ethanol doses (0.5 g/kg) impaired the spontaneous alternation behaviour and recognition memory of the control-vehicle group. However, an acute dose of ethanol (0.5 g/kg) was found to improve working memory of mice in a delayed-alternation task (Rossetti *et al.*, 2002). Paradoxically, the two corticosterone-treated groups which consumed more than 2 g/kg/day of ethanol did not show this impairment. Overall, this suggests that corticosterone self-administration might protect, to some extent, against adverse cognitive effects of chronic alcohol intake.

It is important to point out that the corticosterone-treated group with recognition memory deficits did not exhibit memory impairment in the spontaneous alternation task. One possibility is that the two tests are controlled by different processes or brain areas which are not equally impaired by corticosterone. Another explanation is that mice had already been trained during the choice test to choose to drink from the two bottles whose position was switched daily, and this choice was controlled by the presence of flavour or corticosterone-seeking behaviour. This may have provided some forms of cognitive training to mice and might have partially contributed to keeping their spatial alternation performance unimpaired even with consuming high doses of corticosterone.

Indeed, cognitive training has been found to improve working memory and cognition (Ramsey, 2008; McNab *et al.*, 2009).

In summary, chronic oral corticosterone self-administration did not impair spatial memory of mice even at high doses. In contrast, mice's recognition memory was impaired only when mice consumed excessive amounts of corticosterone for a long time. This indicates that short-term exposure to high doses of corticosterone did not impair memory, and this provides some evidence that rewarding properties of corticosterone outweigh deteriorating effects of excessive corticosterone exposure.

Chapter 2 - Part 3

Effects of corticosterone self-administration on the expression of protein markers of neuroplasticity in the hippocampus and frontal cortex of the mouse brain

2.11 Aim

The aim of this phase of the experiment was to investigate whether oral corticosterone self-administration alters the expression of proteins involved in synaptic plasticity in the hippocampus and frontal cortex of mice. Western immunoblotting was used to measure levels of synaptophysin (SYN) and components of the brain derived neurotrophic factor (BDNF) signalling pathway including BDNF, its receptor tropomysin-related kinase B (TrkB) and phosphorylated extracellular signal regulated kinase 1 and 2 (pERK1/2) which mediates upstream BDNF/TrkB signalling.

2.12 Materials and methods

2.12.1 Tissue preparation & western immunoblotting

The hippocampus and frontal cortex were homogenised in the following lysis buffer solution (pH 7.6): 5 mM Tris, 2 mM EGTA, 80 mM β-glycerophosphate, 1 mM sodium fluoride and protease inhibitor cocktail (Roche Diagnostics, Germany). The protein concentration of homogenates was determined by the Lowry assay (Lowry *et al.*, 1951) to ensure equal loading onto gels. The protein concentration of samples was equalised by a mixture of lysis/SDS sample buffer to provide a final concentration of 1 µg/µl or 1 µg/5µl, depending on the protein. Samples were heated at 95°C for 5 min, vortex-mixed and centrifuged at 13.000 g for 1 minute or 5 minutes; to remove impurities whose bands were found to overlap with BDNF bands. Samples were loaded at a concentration of 1 µg for SYN (38 kDa) and 10 µg for all other proteins: BDNF (14 kDa), Full length TrkB (FL-TrkB, 145 kDa), truncated TrkB (t-TrkB, 95 kDa) and phosphorylated ERK1/2 (pERK1/2, 44/42 kDa) whose levels were calculated relative to total ERK1/2 (t-ERK1/2, 44/42 kDa). GAPDH (36 kDa) was also determined to correct loading errors. All proteins were separated on 10 % Tris-glycine SDS-PAGE gel except BDNF (12%). Spectra™ Multicolor Broad Range Protein Ladder (Fermentas UK, Cat SM1841) or ColorBurst™ Electrophoresis Marker (Sigma-Aldrich UK, Cat C1992) was used and loaded at 2µl. Samples were run at 200 V for 45 min for all proteins except for BDNF for which samples were run at 150V for 37 min based on prior optimisation of

the procedures. After blotting samples to a nitrocellulose membrane, a Ponceau S solution was applied to the membranes in order to ensure successful transfer and then removed by washing with TBST (Tris-buffered saline Tween-20; 150 mM NaCl, 50 mM Tris and 0.05 % v/v Tween-20, pH 7.4). The Snap ID protein detection system (Millipore UK, WBAVDBASE) was used to block and incubate the membranes with primary and secondary antibodies for all proteins except for BDNF. Using Snap ID, membranes were blocked with 1.5% cold water fish skin gelatine (Sigma-Aldrich UK, Cat G7041) dissolved in TBST. Then, for 10 minutes, separate membranes were incubated with a mixture of mouse anti-SYN (1:10.000; Millipore UK, Cat MAB5258) and rabbit anti-GAPDH (1:1000; Sigma-Aldrich UK, Cat G9545) antibodies, or with a mixture of mouse anti-ERK1/2 (1:500; Cat 9107) and rabbit anti-pERK1/2 (1:500; Cat 4377s) antibodies, both from Cell Signalling Technology UK, or with rabbit anti-TrkB antibody (1:1000; Millipore UK, Cat 07-225), or with rabbit anti-GAPDH antibody (1:10.000; Sigma-Aldrich UK, Cat G9545). Then, the membranes were washed three times with TBST followed by a 10-minute incubation period with a mixture of infrared absorbing goat anti-rabbit and goat anti-mouse antibodies. Membranes were again washed three times with TBST. For BDNF, the membranes were blocked in 3 % cold water fish skin gelatine for 1 hour and then incubated overnight at 4°C (~18 hour) with a mixture of mouse anti-BDNF (1:750; Sigma-Aldrich UK, Cat B5050) and rabbit anti-GAPDH (1:15.000; Sigma-Aldrich UK, Cat G9545) antibodies. The following day, membranes were washed with TBST three times followed by a 30-minute incubation period with a mixture of infrared absorbing goat anti-mouse and anti-rabbit antibodies at ~37ºC. Membranes again were washed three times with TBST. All antibodies were dissolved in the same blocking buffer. The Odyssey® Infrared Imaging System was used to scan all membranes and Odyssey software was used to analyse protein bands detected on all membranes according to the manufacturer's instructions.

2.12.2 Statistical analysis

Data were analysed by one way ANOVAs followed by post-hoc planned comparisons when appropriate. Pearson's correlation coefficients were calculated to estimate if the levels of the measured proteins correlate with the average corticosterone intake or with memory performance (% of correct alternation, RI and LI). Data are expressed as mean ± SEM. The level of all proteins of interest was calculated as a percent of the control-flavour group (water *versus* IMP) because mice in this group did not show memory impairment, while mice in the control-vehicle group (water *versus* ethanol 1%) showed spatial and recognition memory impairment at the end of the experiment. An effect was significant if $P \leq 0.05$.

2.13 Results

2.13.1 SYN

There were no differences between groups for SYN levels in either the hippocampus ($F_{(4, 36)} = 0.34$, $P = 0.85$), Fig. 2.14A, or the frontal cortex ($F_{(4,36)} = 2.27$, $P = 0.08$), Fig. 2.14B.

2.13.2 BDNF

There was a significant group difference in hippocampal BDNF levels ($F_{(4,36)} = 4.88$, $P = 0.003$). Post-hoc planned comparisons indicated a significant decrease in BDNF levels in the control-vehicle group ($P = 0.011$) compared to the control-flavour group, Fig. 2.15A. Also mice which had a choice between IMP and unflavoured corticosterone or water and flavoured corticosterone had significantly higher levels of hippocampal BDNF compared to the control-vehicle group ($P = 0.00039$ and $P = 0.006$, respectively), Fig. 2.15A. In the frontal cortex, no significant alterations in the levels of BDNF were observed amongst all corticosterone-treated groups compared to the control groups ($F_{(4,36)} = 0.71$, $P = 0.59$), Fig. 2.15B.

2.13.3 TrkB

For methodological reasons, one mouse was identified as a significant outlier and removed from the control-vehicle group. In the hippocampus, there were no statistical differences in the levels of FL-TrkB ($F_{(4,35)}$ = 1.36, P = 0.27) and t-TrkB ($F_{(4,35)}$ = 0.56, P = 0.69) between any of the experimental groups, Fig 2.16A. Similarly in the frontal cortex, there were no significant changes in the levels of either FL-TrkB ($F_{(4,36)}$ = 0.29, P = 0.88) or t-TrkB ($F_{(4,36)}$ = 0.67, P = 0.62) between all groups, Fig 2.16B.

2.13.4 Phospho-ERK1/2

One significant outlier in the group whose choice was water *versus* unflavoured corticosterone was removed from the analysis. There was a significant difference between groups in hippocampal pERK2 levels ($F_{(4, 35)}$ = 3.95, P = 0.01). Post-hoc planned comparisons detected a significant decrease in hippocampal pERK2 levels of mice which had a choice between IMP and unflavoured corticosterone (P = 0.035) compared to the control-flavour group, Fig. 2.17A. In addition, the two corticosterone-treated groups which had a choice between IMP and unflavoured corticosterone or water and flavoured corticosterone had low hippocampal pERK2 levels (P = 0.001 and P = 0.009, respectively) compared to the control-vehicle group, Fig. 2.17A. On the other hand, there was no significant group effect for hippocampal pERK1 levels ($F_{(4, 35)}$ = 2.44, P = 0.065), Fig. 2.17A.

In the frontal cortex, one mouse (water *versus* flavoured corticosterone) was identified as a significant outlier, and it was removed from the analysis. Significant alterations in the levels of pERK2 were found between groups ($F_{(4,35)}$ = 3.61, P = 0.015). Post-hoc planned comparisons indicated a significant decrease in pERK2 levels in the frontal cortex of mice whose choice was water *versus* flavoured corticosterone (P = 0.040) compared to the control-flavour group, Fig 2.17B. In addition, all corticosterone-treated groups showed significantly lower levels of pERK2 (P <0.05, in all case) than the control-vehicle group, Fig 2.17B. On the other hand, there were no differences in the

levels of pERK1 between groups ($F_{(4,35)}$ = 2.30, P = 0.078), Fig. 2.17B. Using GAPDH as a reference protein, no group differences in the levels of t-ERK1 and t-ERK2 were found either in the hippocampus or the frontal cortex of mice (data not shown).

2.13.5 Correlation between levels of measured proteins and memory performance using Pearson's correlation

There was a significant correlation between t-TrkB levels in the frontal cortex and location index (LI) of all corticosterone-treated mice (n = 25, r = 0.54, P = 0.005). For mice in the control-flavour group, there was an inverse relationship between recognition index (RI) and BDNF levels in the frontal cortex (n = 8, r = -0.73, P = 0.042) and between hippocampal levels of pERK2 and the percentage of correct alternation (n = 8, r = -0.75, P = 0.033). In contrast, mice in the same group showed a positive correlation between hippocampal levels of TrkB and the percentage of correct alternations (n = 8, r = 0.72, P = 0.046), and between TrkB levels in the frontal cortex and RI (n = 8, r = 0.90, P = 0.002) and between hippocampal pERK1 levels and LI (n = 8, r = 0.72, P = 0.045)

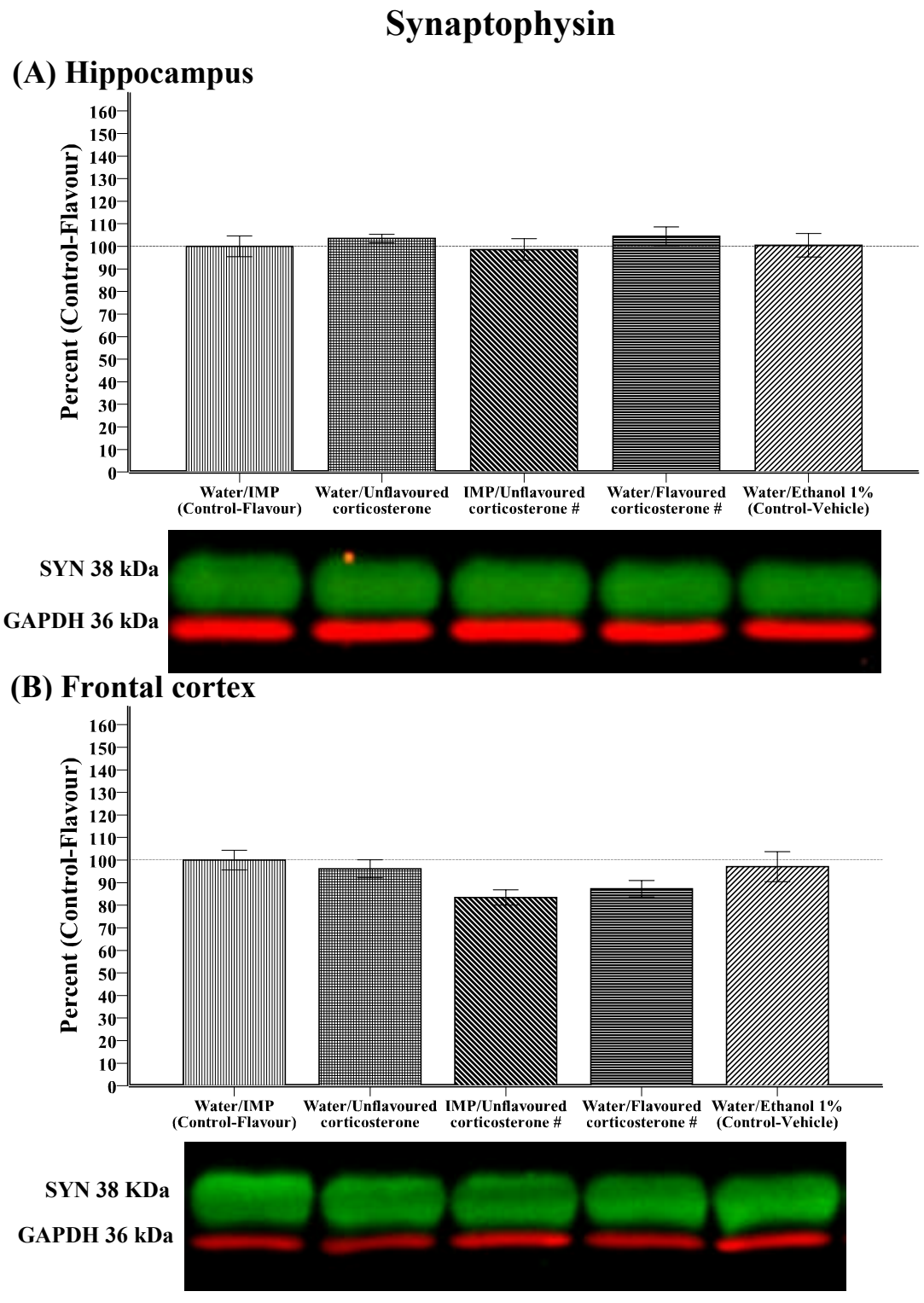

Figure 2.14 Effects of oral corticosterone self-administration on SYN levels in the hippocampus (A) and frontal cortex (B). SYN and GAPDH levels were measured using western immunoblotting. Synaptophysin/GAPDH was calculated as % of the control-flavour group (water *versus* IMP). Data are expressed as mean ± SEM (n = 7-9 mice/group). # indicates groups whose solutions were exchanged during switching.

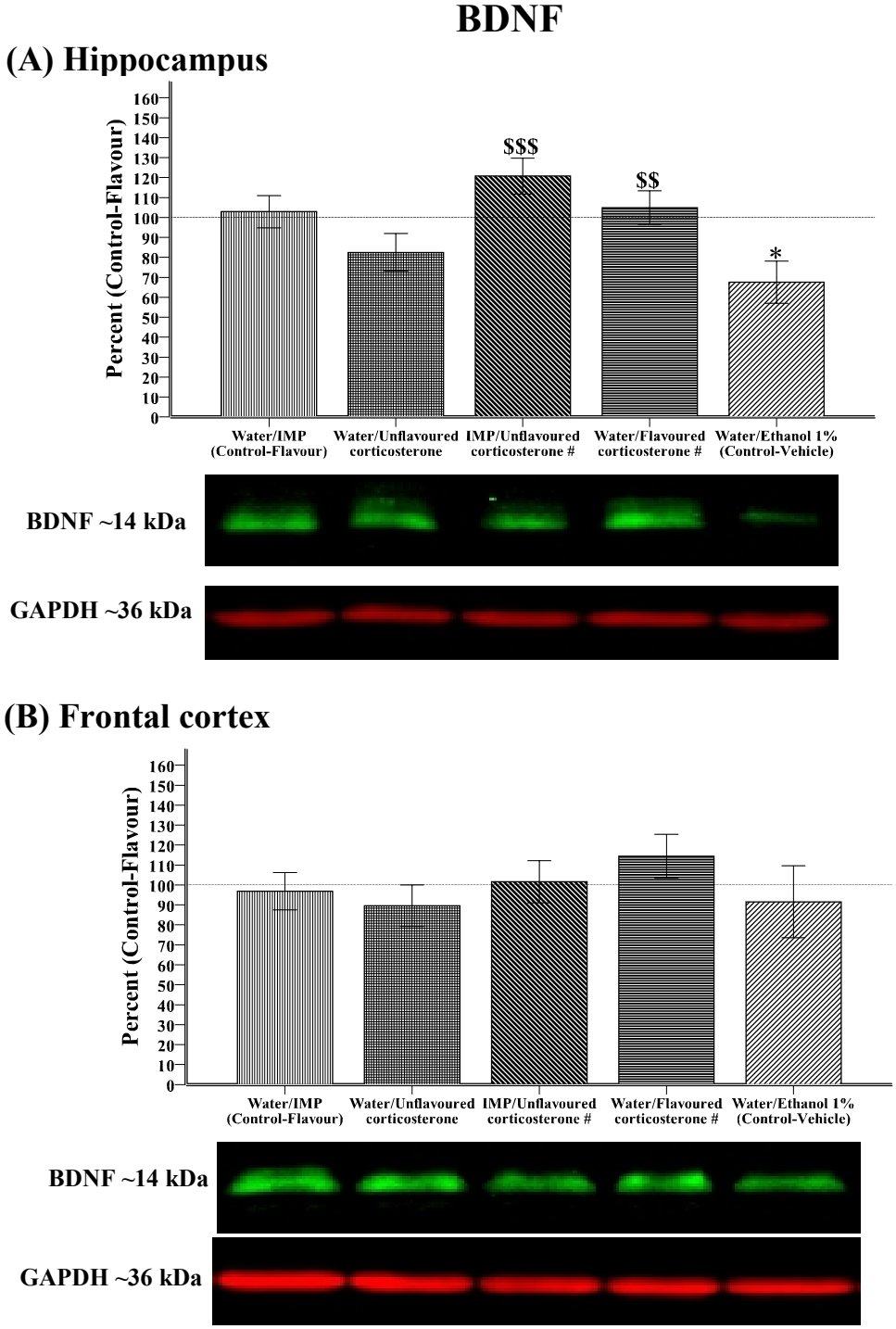

Figure 2.15 Effects of acquisition and reinstatement of oral corticosterone self-administration on BDNF levels in the hippocampus (A) and frontal cortex (B) of 5 groups (n = 7-9) of male C57BL/6J mice. BDNF/GAPDH was calculated as % of the control-flavour group (water *versus* IMP). Data are expressed as mean ± SEM. Planned comparisons: *P <0.05 *versus* the control-flavour group, $^{\$\$}$P <0.01 and $^{\$\$\$}$P <0.001 *versus* the control-vehicle group (water *versus* ethanol 1%). # indicates groups whose solutions were exchanged during the switching period.

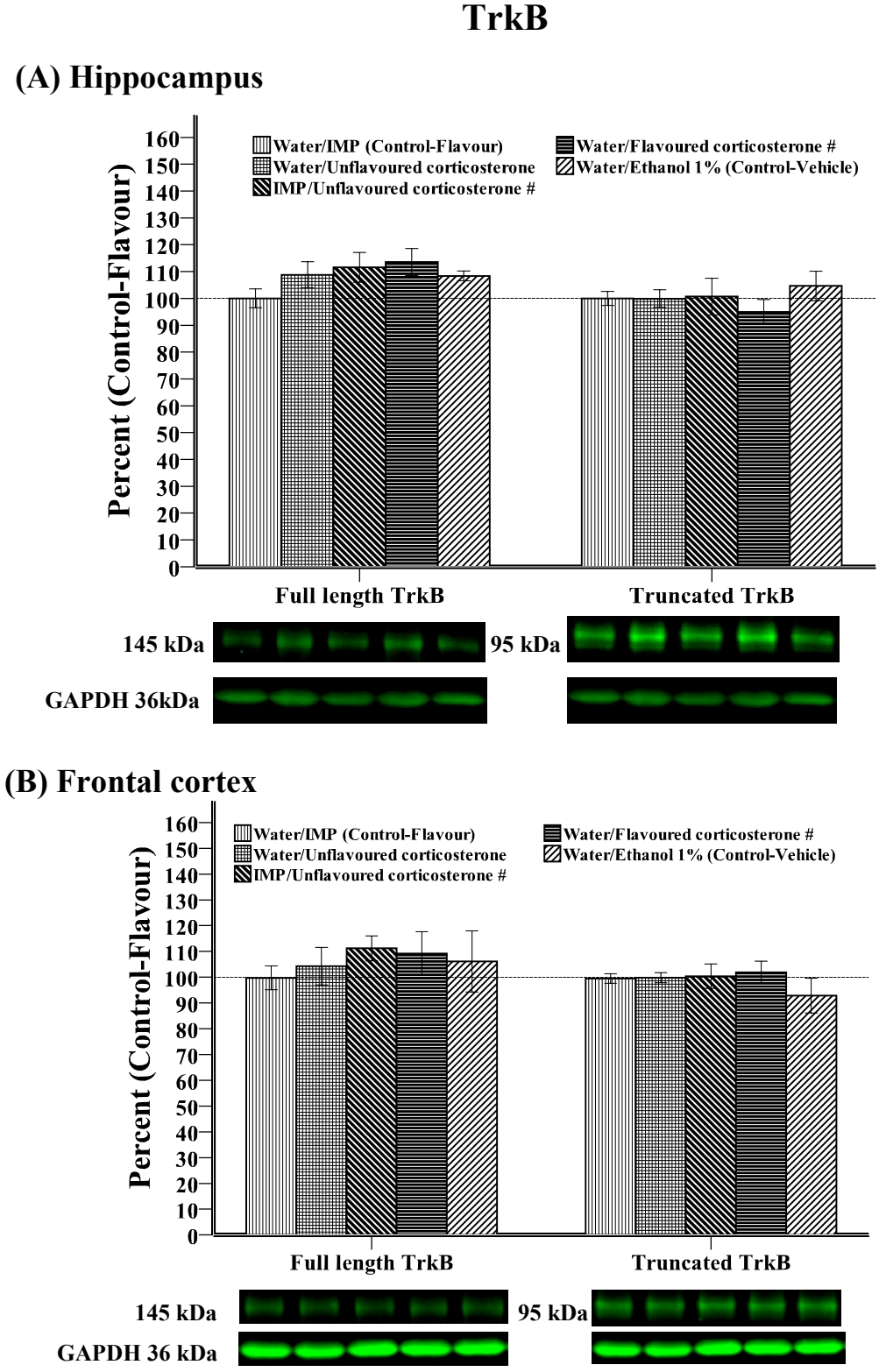

Figure 2.16 Levels of full length TrkB (145 kDa) and truncated TrkB (95 kDa) in the hippocampus (A) and frontal cortex (B) of corticosterone naïve and treated mice (n = 7-9/group). FL-TrkB/GAPDH and t-TrkB/GAPDH were calculated as % of the control-flavour group (water *versus* IMP). Data are expressed as mean ± SEM.

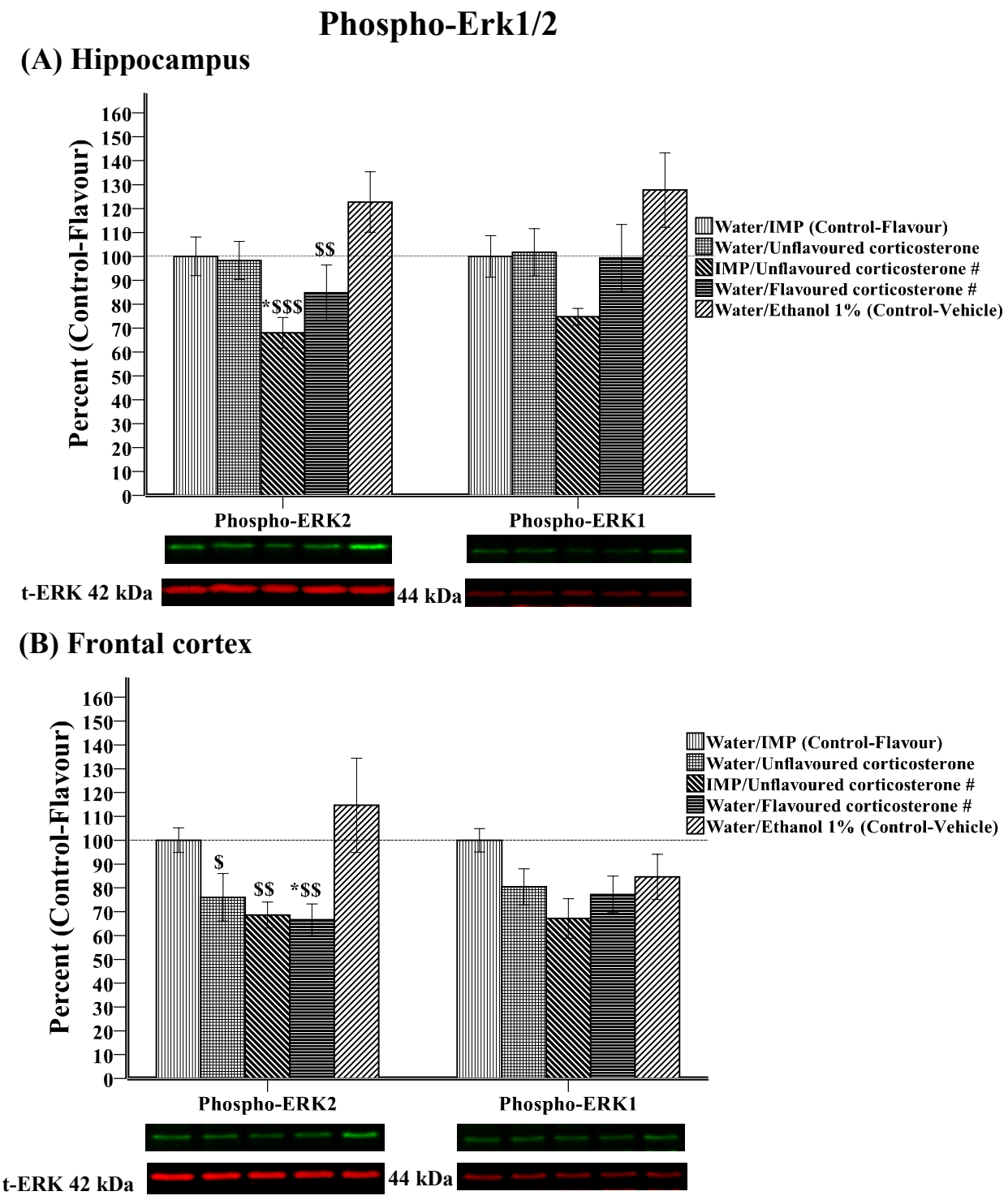

Figure 2.17 Effects of oral corticosterone self-administration on the activation of ERK1/2 in the hippocampus (A) and frontal cortex (B) of corticosterone naïve and treated mice (n = 7-9/group). pERK2/t-ERK2 and pERK1/t-ERK1 were calculated relative to the control-flavour group (water *versus* IMP). Data are expressed as mean ± SEM. Planned comparisons: *P <0.05 significantly different from control-flavour group, $^{\$}$P <0.05, $^{\$\$}$P <0.01 and $^{\$\$\$}$P ≤0.001 significantly different from the control-vehicle group (water *versus* ethanol 1%).

2.14 Discussion

The purpose of this part of the experiment was to investigate whether oral corticosterone self-administration and its effects on mice's memory are accompanied with persistent changes in markers of neuroplasticity. The present study showed that oral corticosterone self-administration reduced levels of pERK2 in the hippocampus and frontal cortex of some or all corticosterone-treated groups compared to the control groups. Also, two of the corticosterone-treated groups had higher levels of BDNF in the hippocampus than the control-vehicle group (water *versus* ethanol 1%). In addition, mice in the control-vehicle group showed low levels of BDNF in the hippocampus compared to the control-flavour group (water *versus* IMP). In contrast, the levels of SYN and TrkB in both the hippocampus and frontal cortex were not significantly different between groups.

It has been proposed that the hippocampus is critical for memory and learning (O'Keefe and Dostrovsky, 1971; Eichenbaum *et al.*, 1999) and the hippocampus greatly expresses GRs and MRs (Reul and de Kloet, 1985), and thus it was vulnerable to changes in plasma corticosterone levels according to the degree and duration of corticosterone self-administration in each group. Also, the frontal cortex is involved in cognitive performance and the development of drug addiction (Markowitsch and Pritzel, 1977; van Haaren *et al.*, 1988; Volkow *et al.*, 2011)**.**

In the hippocampus and frontal cortex, the levels of SYN were not altered by oral corticosterone self-administration. SYN has been used as a classical marker of synaptogenesis (Mazer *et al.*, 1997; Vawter *et al.*, 2002). From the literature, it is not clear whether SYN levels are affected by the development of drug addiction. However, CPP to amphetamine increased SYN levels in the hippocampus and nucleus accumbens core of rats (Rademacher *et al.*, 2006). SYN and other vesicle-associated membrane proteins are well known to influence synaptic plasticity and memory performance. For instance, SYN-knockout mice showed a significant reduction in hippocampal LTP (Janz *et al.*, 1999) and exhibited memory impairment in the Morris water maze and novel

object recognition tests (Schmitt *et al.*, 2009). Being important for associative learning performance, SYN levels were elevated in the basolateral nuclei of the amygdala of mice after they developed auditory fear conditioning (Nithianantharajah and Murphy, 2008). In contrast, environmental enrichment-induced improvement of age-related decline in mice's spatial memory was associated with low SYN levels in the hippocampus and frontal cortex, compared to control aged mice (Bennett *et al.*, 2006). Furthermore, old female C57BL/6J mice showed an increase in SYN immunoreactivity in the hippocampus, but they demonstrated spatial and passive avoidance memory impairment (Benice *et al.*, 2006). The present work indicated that chronic corticosterone intake even at high doses did not have a long-lasting effect on SYN levels in either the hippocampus or frontal cortex. However, single and repeated immobilization stress decreased SYN mRNA expression in the hippocampus and cerebral cortex of rats (Thome *et al.*, 2001), and immobilization stress was reported to substantially elevate plasma corticosterone levels (Sapolsky *et al.*, 1984). Overall, it can be concluded that chronic corticosterone self-administration did not recruit SYN, at least in the hippocampus and frontal cortex, to mediate rewarding and cognitive effects of the stress hormone.

BDNF/TrkB/ERK1/2 signalling pathway is particularly important for proper neuronal function, memory and learning capability (Minichiello *et al.*, 1999; Mazzucchelli and Brambilla, 2000; Yacoubian and Lo, 2000; Heldt *et al.*, 2007). As mentioned earlier, chronic corticosterone self-administration even at high doses did not alter levels of BDNF or its receptor (FL-TrkB or t-TrkB) in the hippocampus and frontal cortex, compared to the control-flavour group. BDNF is highly expressed in the rat hippocampus (Maisonpierre *et al.*, 1990) and is important for neuronal survival, neuroplasticity (Gooney and Lynch, 2001; West, 2008), spatial memory (Mizuno *et al.*, 2003; McGauran *et al.*, 2008) and learning (Bao *et al.*, 1998; Berchtold *et al.*, 2010). Previous work showed that chronic corticosterone treatment slightly reduced hippocampal levels of BDNF mRNA and protein in rats (Jacobsen and Mork, 2006), but adrenalectomy increased BDNF mRNA expression in the rat hippocampus

(Chao *et al.*, 1998). Also, adrenalectomized rats treated with a single dose of corticosterone which elevated plasma corticosterone levels exhibited a significant albeit small decrease in hippocampal BDNF levels 4 or 6 hours after treatment (Schaaf *et al.*, 1998). Interestingly, mice in the control-vehicle group had low BDNF levels in the hippocampus, compared to the control-flavour group and corticosterone self-administered groups which had IMP added to their test or control solutions. As seen in Chapter 2 - Part 2, mice in the control-vehicle group had spatial and recognition memory impairment. In contrast, corticosterone treatment abolished ethanol-induced memory impairment in the spontaneous alternation test, and two of corticosterone self-administered groups also had no recognition memory deficits. This suggests that memory impairment induced by 1% ethanol in the control-vehicle group was possibly secondary to the decreased hippocampal BDNF levels. In agreement with this, chronic exposure to ethanol vapour decreased the expression of BDNF mRNA but increased TrkB mRNA expression in the rat hippocampus (Tapia-Arancibia *et al.*, 2001). In contrast, expression of BDNF mRNA in the dentate gyrus was increased 12 hours after the cessation of chronic ethanol exposure (Tapia-Arancibia *et al.*, 2001).

Over the past two decades, drug self-administration studies have been used to understand the role of BDNF in drug-seeking behaviour. For instance, BDNF/TrkB signalling in the nucleus accumbens was reported to be involved in the acquisition and reinstatement of intravenous cocaine self-administration in both mice and rats (Graham *et al.*, 2007). Moreover, BDNF infusions in the ventral tegmental area or the nucleus accumbens of rats facilitated cocaine-induced behavioural sensitization (Horger *et al.*, 1999). On the other hand, BDNF was found to modulate the rewarding effects of a high fat diet *via* the enhancement of dopamine release in the nucleus accumbens shell and the dorsal striatum (Cordeira *et al.*, 2010).

A considerable body of research suggests that ERK1/2 plays a dominant role in the development of drug addiction. Effects on ERK1/2 levels vary according to the abused drug, the duration of exposure and the brain region (Zhai *et al.*,

2008). For example, acute cocaine or amphetamine administration increased ERK1/2 activity in the hippocampus and frontal cortex of mice (Valjent *et al.*, 2004; Valjent *et al.*, 2005). Further, repeated cocaine injections raised levels of pERK1/2 in the nucleus accumbens and ventral tegmental area of rats (Berhow *et al.*, 1996; Mattson *et al.*, 2005; Yoon *et al.*, 2007). Our results suggest that ERK1/2 in the frontal cortex was involved in the acquisition of corticosterone self-administration, but maintenance of corticosterone self-administration was associated with low pERK2 levels in the hippocampus. For instance, mice which were offered a new choice (IMP *versus* unflavoured corticosterone, group E) after switching started to self-administer corticosterone and showed a significant decrease in pERK2 levels in the frontal cortex, compared to the control-flavour group. Also, mice which had the same choice during acquisition and reinstatement (group D) self-administered corticosterone for a long time and showed low hippocampal pERK2 levels. Consistent with these results, chronic administration of corticosterone in drinking water reduced pERK2 levels in the hippocampus and frontal cortex of mice and this effect lasted for more than 10 days until mice were culled (Gourley *et al.*, 2008). Previous studies showed that repeated exposure to morphine was associated with low levels of pERK1/2 in the prefrontal cortex of human addicts and rats (Ferrer-Alcon *et al.*, 2004) and with high pERK1/2 levels in the nucleus accumbens of mice (Liu *et al.*, 2007).

It is interesting that the two corticosterone-treated groups which had IMP in their control or test solutions showed higher BDNF and lower pERK2 levels in the hippocampus, compared to the control-vehicle group. As mice in the control-vehicle did not show a significant preference for 1% ethanol, and corticosterone-treated groups markedly developed and reinstated corticosterone self-administration, this provides some evidence that low activity of ERK2 in the hippocampus and frontal cortex of corticosterone-dependent mice underlies the rewarding properties of corticosterone. In contrast, mice which had a choice between water and unflavoured corticosterone did not show a significant decrease in pERK2 levels in either brain area, compared to the control-flavour group. These conflicting results between corticosterone self-administered

groups suggest that IMP acted as a cue by which mice learned to choose between the two solutions of the two-bottle choice test. Clearly, mice learned an association between IMP as a cue and high doses of corticosterone as a reward. The frontal cortex was found to be implicated in associative learning tasks (Asaad *et al.*, 1998; Asaad *et al.*, 2000). For instance, C57BL/6J mice developed conditioned place preference to morphine and showed a decrease in levels of pERK2 in the frontal cortex (Li *et al.*, 2008). Also, it was found that pERK2 levels increased in the hippocampus of rats after being trained in associative learning and conditioning fear tasks (Atkins *et al.*, 1998).

On the other hand, our results showed that mice whose choice was water *versus* unflavoured corticosterone had low pERK2 levels in the frontal cortex, compared to the control-vehicle group. This indicates that the effects on pERK2 levels, in this control group, could be attributed to repeated intermittent exposure (2 withdrawals) to 1% ethanol in the control-vehicle group. It was found that continuous or intermittent moderate ethanol consumption decreased phosphorylation of ERK1/2 in different brain areas including the hippocampus and frontal cortex of rats, but pERK1/2 levels increased within 24-hour withdrawal (Sanna *et al.*, 2002). However, corticosterone probably blocked the effects of ethanol on the activity of ERK2, as the hormone decreased levels of pERK2 in the hippocampus and frontal cortex.

ERK1/2 has a key role in learning and memory formation such as recognition memory (Kelly *et al.*, 2003). Also, induction of LTP in the hippocampus slices isolated from rats was associated with the increase of pERK2 levels (English and Sweatt, 1996). It was found that stress-induced hippocampal LTP impairment was corticosterone-dependent (Korz and Frey, 2003). Taken together, our findings suggest that low ERK2 activity in the hippocampus could be involved in the recognition memory impairment of mice which ingested high doses of corticosterone during all phases of the two-bottle choice test.

It is also important to point out that the control-vehicle group showed memory impairment and low hippocampal BDNF levels in the hippocampus, but no

reduction in pERK2 levels in the hippocampus and frontal cortex, compared to the control-flavour group. This suggests that effects on ERK1/2 activity were not only linked to BDNF/TrkB signalling, but also to other upstream signalling systems, such as endogenous opioid and glutamatergic pathways which might be involved in the plasticity induced by corticosterone self-administration.

There was a positive correlation between levels of t-TrkB in the frontal cortex of all corticosterone-treated mice and LIs. This finding suggests that the improvement of LIs was dependent on t-TrkB levels in the frontal cortex, and this was accompanied with a decrease of pERK2 levels in the frontal cortex of all of corticosterone-treated mice compared to the control-vehicle group. As the control-flavour group did not develop memory deficits compared to the control-vehicle group, it was interesting to explore whether levels of the marker proteins in the hippocampus or frontal cortex might correlate with memory performance in the control-flavour group. Results revealed that RIs were negatively correlated with BDNF levels, but they were, however, positively correlated with TrkB levels in the frontal cortex. Whereas the percentage of correct alternation increased when hippocampal TrkB levels increased, it decreased when hippocampal pERK2 levels increased. Also, LIs were positively correlated with hippocampal pERK1 levels. It is difficult to understand the mechanisms behind these correlations. However, these proteins are involved in the same signalling pathway and these observations can be interpreted, as a decrease in one component of the pathway triggers a compensatory increase in the others, despite there being no correlation between the measured proteins in the same brain area. From this work, it can be concluded that the BDNF/TrKB/ERK1/2 signalling pathway is implicated in the rewarding and cognitive effects of corticosterone self-administration, but further investigation is necessary to determine the involvement of other endogenous reward modulators whose actions involve ERK1/2.

Chapter 2 - Part 4

Impact of corticosterone self-administration on levels of amine neurotransmitters in the mouse striatum

2.15 Aims

Many abused drugs mediate their rewarding effects *via* enhancing dopamine neurotransmission in the mesolimbic dopamine pathway (Hoebel *et al.*, 1983; Devine *et al.*, 1993; Glick *et al.*, 1998), and imaging studies using single-photon emission computed tomography, MRI and PET showed correlation between dopamine release in the striatum of addicts and amphetamine-induced euphoria (Laruelle *et al.*, 1995; Drevets *et al.*, 2001). Moreover, locomotor hyperactivity induced by acute morphine treatment was accompanied by increased levels of serotonin (5HT) and its major metabolite 5-hydroxyindoleacetic acid (5HIAA) in the nucleus accumbens and dorsal striatum of C57BL/6J mice (Fadda *et al.*, 2005). This suggests that corticosterone self-administration could be accompanied by alterations in the levels of amines in the striatum of corticosterone-dependent mice compared to corticosterone naïve mice. Dopamine and its metabolites 3, 4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), 5HT and 5HIAA were, therefore, measured in the striatum of mice by means of HPLC.

2.16 Materials and methods

2.16.1 HPLC determination of amine neurotransmitters in the striatum

Each mouse's striatum was homogenised in a measured volume of ice-cold perchloric acid (PCA) (0.05 M perchloric acid containing 0.02% w/v sodium metabisulfite and 0.01 % w/v EDTA dissolved in HPLC Grade water). Samples were homogenised by a sonic probe for 20-30 sec (MSE Soniprep 150 UK, power 20) and centrifuged at 14.000 g (MSE Harrier 18/80) at 4°C for 20 minutes. Then, the supernatants were removed, filtered through 0.45 μm PVDF syringe filters (Kinesis UK) and stored at -80°C until analysis. Dopamine, DOPAC, HVA, 5HT and 5HIAA were determined by HPLC with electrochemical detection. Samples (20 μl) were injected onto an electrochemical detector (Antec, the Netherlands). The mobile phase (50 mM KH_2PO_4, 0.1 mM EDTA and 0.32 mM octane sulfonic acid in HPLC Grade

water, pH 3.5 adjusted by orthophosphoric acid before addition of 13% v/v methanol) was pumped at a rate of 1 ml/min. A Phenomenex reverse phase column; C-18, 5 μm ODS-2 (150 X 4.6 μm), was used to separate and measure the previously mentioned amines and their metabolites using a glassy carbon working electrode with a potential of + 0.7 V *versus* an Ag/AgCl reference electrode. The height of peaks of each amine was measured relative to a calibration mixed standard of dopamine, DOPAC, HVA, 5HT and 5HIAA with known concentrations (5×10^{-7} M). The mixed standard solution was injected at the start and end of running samples through the HPLC system. The levels of amines or metabolites were expressed as pmol/mg tissue. An example chromatogram illustrates the separation of dopamine, 5HT and their metabolites in the standard mixture and the striatum of a corticosterone-treated mouse, Fig 2.18.

2.16.2 Statistical analysis

One way ANOVAs with groups (5 levels) as the between subject factor were used to analyse levels of dopamine, 5HT, their metabolites and their turnover ratios: DOPAC/dopamine, HVA/dopamine and 5HIAA/5HT. Post-hoc planned comparisons were used when significance ($P \leq 0.05$) was reached. Also, Pearson's correlation coefficients were calculated to investigate whether levels of the measured amines or their metabolites correlate with the average corticosterone doses consumed over the whole experiment. Data are expressed as mean ± SEM.

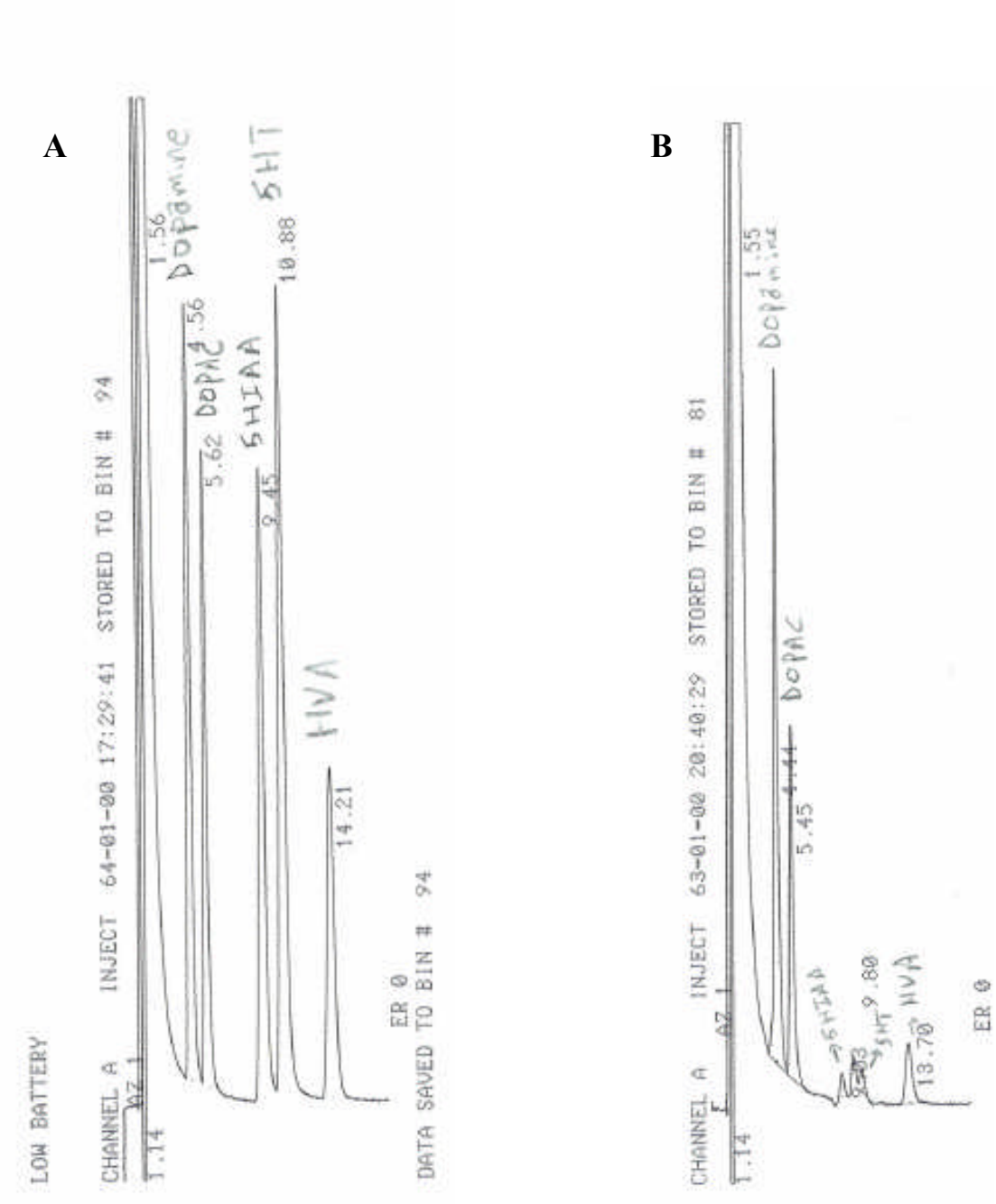

Figure 2.18 Example chromatogram produced by HPLC for the separation of dopamine, DOPAC, HVA, 5HT and 5HIAA of (A) a mixed standard at 5 x 10^{-7} M concentration and (B) mouse striatum tissue homogenate, water *versus* unflavoured corticosterone.

2.17 Results

2.17.1 Dopamine and its metabolites, DOPAC and HVA

There was no significant main group effect for the levels of dopamine in the striatum ($F_{(4,34)}$ = 1.41, P = 0.25), table 2.3. Similarly, levels of the dopamine metabolites, DOPAC and HVA, were not significantly different between groups ($F_{(4,34)}$ = 1.03, P = 0.41, $F_{(4,34)}$ = 0.65, P = 0.63, respectively), table 2.3. Nevertheless, there were significant group differences for the ratio DOPAC/dopamine ($F_{(4,33)}$ = 3.02, P = 0.032). Post-hoc planned comparisons showed a significant increase in the DOPAC/dopamine ratio in the striatum of mice which had water *versus* flavoured corticosterone compared to the control-vehicle (P = 0.006) and the control-flavour (P = 0.044) groups, table 2.3. Also, mice whose choice was IMP *versus* unflavoured corticosterone showed a significant increase in the DOPAC/dopamine ratio (P = 0.016) compared to the control-vehicle group, table 2.3. On the other hand, the HVA/dopamine ratio, another estimate of dopamine turnover, was not significantly different between groups ($F_{(4,33)}$ = 2.44, P = 0.066), table 2.3. Interestingly, there was a positive correlation between averaged corticosterone doses that mice consumed over the whole study and DOPAC levels in the striatum (n = 24, r = 0.44, P = 0.03), Fig. 2.19. In contrast, levels of dopamine and HVA or HVA/dopamine ratios did not significantly correlate with averaged corticosterone doses.

2.17.2 5HT and its metabolite 5HIAA

There were no significant group differences in the levels of 5HT and its metabolite 5HIAA in the striatum (5HT: $F_{(4,34)}$ = 1.33 , P = 0.28, 5HIAA: $F_{(4,34)}$ = 0.36, P = 0.83), table 2.3. Also, there were no alterations in the 5HIAA/5HT ratio ($F_{(4,34)}$ = 1.12, P = 0.37), table 2.3. Likewise, no correlation was found between levels of 5HT or 5HIAA and the average of corticosterone doses consumed.

Table 2.3 Striatal levels (pmol/mg tissue) of dopamine, 5HT and their metabolites. In addition, the rates of DOPAC/dopamine, HVA/dopamine, and 5HIAA/5HT were calculated in the striatum of corticosterone naïve and treated mice (n = 7-9 / group). Data are presented as mean ± SEM. There was an increase in the DOPAC/dopamine ratio which reflects dopamine utilisation, planned comparisons: *P <0.05, **P <0.01 compared to the control-vehicle group and $^{\$}$P <0.05 compared to the control-flavour group. # indicates groups whose solutions were exchanged during switching.

Variables / Groups	Dopamine	DOPAC	HVA	5HT	5HIAA	DOPAC / dopamine	HVA / dopamine	5HIAA / 5HT
Water *versus* IMP (control-flavour)	47.25 ± 5.81	28.19 ± 2.93	10.26 ± 0.98	1.41 ± 0.16	2.20 ± 0.10	0.62 ± 0.03	0.23 ± 0.01	1.76 ± 0.28
Water *versus* unflavoured corticosterone	48.49 ± 6.63	30.2 ± 4.48	10.34 ± 1.21	1.20 ± 0.14	2.39 ± 0.15	0.64 ± 0.06	0.22 ± 0.01	2.19 ± 0.28
IMP *versus* unflavoured corticosterone #	34.68 ± 6.03	32.58 ± 6.38	9.72 ± 1.43	1.70 ± 0.21	2.38 ± 0.22	0.78 ±0.09 *	0.25 ± 0.02	1.44 ± 0.08
Water *versus* flavoured corticosterone #	31.92 ± 8.03	21.55 ± 4.47	7.91 ± 1.70	1.70 ± 0.24	2.46 ± 0.19	0.80 ± 0.09**$	0.29 ± 0.03	1.77 ± 0.38
Water *versus* ethanol 1% (control-vehicle)	45.77 ± 5.35	24.03 ± 2.76	10.19 ± 0.83	1.30 ± 0.26	2.45 ± 0.20	0.53 ± 0.03	0.23 ± 0.01	2.39 ± 0.57

Correlation between average corticosterone doses and DOPAC levels in the striatum

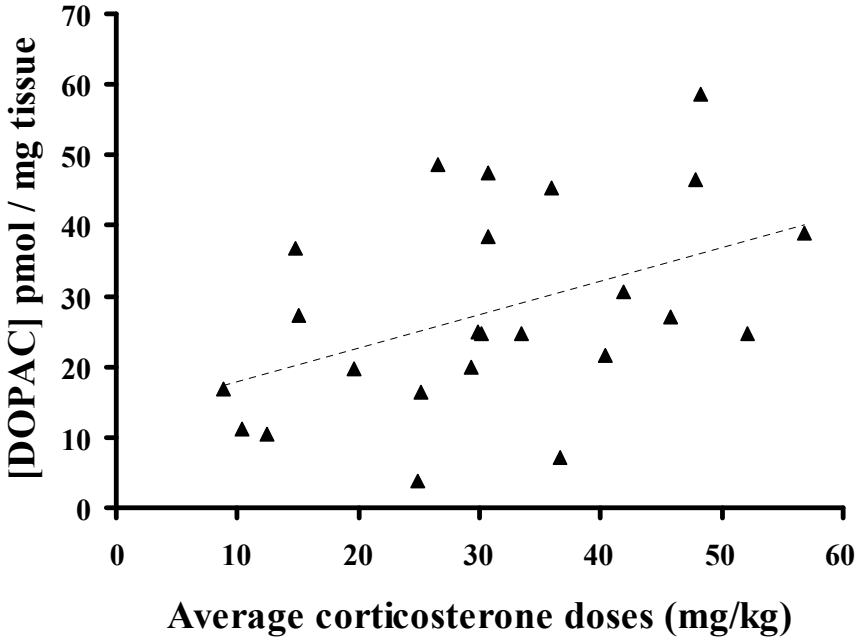

Figure 2.19 The correlation between DOPAC concentrations in the striatum of all corticosterone self-administered mice and averages of their daily corticosterone doses consumed over the whole experiment. Pearson's correlation (n = 24, r = 0.44, P = 0.03).

2.18 Discussion

The results of the present work suggest that acquisition and reinstatement of chronic oral corticosterone self-administration were accompanied by some alterations in the dopamine neurotransmission in the striatal complex. There were no significant changes in levels of dopamine, 5HT and their metabolites in the striatum of corticosterone self-administered mice compared to the two control groups. Also, the HVA/dopamine and the 5HIAA/5HT ratios were not significantly different between groups. These results are consistent with previous work which revealed that chronic exposure to high corticosterone doses (50 mg/kg, s.c.) did not significantly modify levels of these variables in the rat striatum (Inoue and Koyama, 1996), despite the fact that rats were sacrificed 24 hours after the last corticosterone injection, but in this study mice were sacrificed 10 days after the end of the corticosterone treatment. In contrast, chronic corticosterone administration increased 5HT levels and decreased 5HIAA levels in the hippocampus and frontal cortex of rats which were sacrificed 24 hours after 21 days of corticosterone (32mg/kg, s.c.) treatment (Jacobsen and Mork, 2006). Interestingly, the two groups in which mice self-administered corticosterone when it was flavoured or paired with IMP had significantly higher DOPAC/dopamine ratios, an indicator of dopamine turnover, in the striatum compared to both control groups. However, mice whose unflavoured corticosterone solution was paired with water did not show a significant change in the DOPAC/dopamine ratio. Most importantly, the average of corticosterone doses of all corticosterone-treated mice positively correlated with levels of DOPAC in the striatum.

The role of dopamine in the reward and development of drug addiction has been studied over the past few decades. There are anatomically three dopamine pathways in the brain: mesolimbic, mesocortical and nigrostriatal. Dopaminergic neurons in the mesocorticolimbic dopamine system project mainly from the ventral tegmental area to the nucleus accumbens and the frontal cortex (Wise, 1998). Nigrostriatal dopaminergic neurons originate from the substantia nigra and project to the dorsal striatum, and this pathway has been

traditionally implicated in motor function (Wise, 2009). However, there has recently been some evidence that the nigrostriatal dopamine pathway works in association with the mesocorticolimbic dopamine system in response to the rewarding substances (Wise, 2009). It is well established that the mesocorticolimbic dopamine system mediates rewarding effects of abused drugs. For example, rats self-administered amphetamine (Hoebel *et al.*, 1983) and nomifensine (Carlezon *et al.*, 1995), a selective dopamine reuptake inhibitor, into the nucleus accumbens. Additionally, rats learned to lever-press for dopamine microinjection into the nucleus accumbens (Dworkin *et al.*, 1986). Furthermore, natural rewards like palatable food (Martel and Fantino, 1996b; Martel and Fantino, 1996a) and copulation (Fiorino *et al.*, 1997) were accompanied with an increase in dopamine and their metabolites in the nucleus accumbens. Dopamine *per se* was initially given attention as a reward mediating neurotransmitter when pimozide, a selective dopamine receptor antagonist, treated rats exhibited low self-stimulation rates of the medial forebrain bundle (Liebman and Butcher, 1973). Dopamine is also believed to play a fundamental role in the habit forming actions of abused drugs. For instance, the rewarding effects of cocaine and amphetamine were diminished in rats by pimozide but not affected by phentolamine HCl, a selective noradrenergic blocker (Yokel and Wise, 1975; De Wit and Wise, 1977), or by methysergide, a 5-HT1/5-HT2 receptor blocker (Lacosta and Roberts, 1993). Moreover, other abused drugs such as morphine and nicotine increased dopamine levels, measured by microdialysis, in the nucleus accumbens of rats (Devine *et al.*, 1993; Glick *et al.*, 1998).

Whereas oral acute corticosterone treatment minimally decreased the DOPAC/dopamine ratio in the striatum, two weeks of adrenalectomy increased the DOPAC/dopamine ratio in the medial prefrontal cortex of Sprague-Dawley rats (Lindley *et al.*, 1999). In the present study, corticosterone-treated mice which had IMP in their solutions had a significantly higher DOPAC/dopamine ratio than the control groups. In contrast, the third corticosterone-treated group in which mice self-administered unflavoured corticosterone paired with water

showed no significant changes in dopamine turnover. This suggests that when mice tasted IMP, they could predict they would be rewarded. This cue probably resulted in an increase in the dopamine release when it was paired with a reward, corticosterone, as mice in the control-flavour group (water *versus* IMP) did not show a significant increase in the DOPAC/dopamine ratio, compared to the control-vehicle group. It has been thought that dopamine acts as alerting device for the receipt of a reward rather than mediating its hedonic effects (Schultz *et al.*, 1997). In agreement with this, it was observed that intracranial self-stimulation initially enhanced dopamine release, measured using cyclic voltammetry, in the rat caudate-putamen but dopamine levels began to fall as rats lever-pressed to receive more brain stimulation (Kilpatrick *et al.*, 2000). When light was used as a cue, trained monkeys showed a rise in dopamine levels which dropped when they started to consume the rewarding juice (Hollerman and Schultz, 1998; Schultz, 2010). IMP in the present experiment probably acted like the light, as the conditioned stimulus.

Corticosterone treatment increased, decreased or did not change levels of extracellular dopamine, 5HT and their metabolites in different brain regions (Rothschild *et al.*, 1985; Wolkowitz *et al.*, 1986; Dunn, 1988; Tanganelli *et al.*, 1990; Imperato *et al.*, 1991; Inoue and Koyama, 1996). It is necessary to distinguish between corticosterone self-administration and corticosterone treatment where animals had no free access to corticosterone, as the dopamine reward pathway was reported to respond to non-contingent drug injections and drug self-administration in a different way (Stefanski *et al.*, 1999). It has been found that corticosterone modulates effects of abused dugs on the dopamine reward pathway. For example, mifepristone, a glucocorticoid receptor antagonist, reduced the elevated extracellular dopamine concentrations induced by morphine in the nucleus accumbens (Marinelli *et al.*, 1998), and this could be direct effects on dopamine release, as the mesocorticolimbic and nigrostriatal dopaminergic neurons were found to be GR immunoreactive in Sprague-Dawley rats (Harfstrand *et al.*, 1986). Also, cocaine-evoked dopamine release was enhanced by stress-induced sensitization *via* corticosterone secretion

(Rouge-Pont *et al.*, 1995). Moreover, corticosterone enhanced the activity of tyrosine hydroxylase, the rate limiting step in catecholamine synthesis, in the ventral tegmental area (Nestler *et al.*, 1989). In contrast, application of mifepristone to cultured mesencephalic neurons produced a dose-dependent decrease in dopamine release (Rouge-Pont *et al.*, 1999). Our results also showed a direct relationship between DOPAC levels in the striatum and the averaged corticosterone dose consumed over the entire experiment. This suggests that dopamine turnover was altered in a dose-dependent manner during the acquisition, relapse and switching phases of the two-bottle choice test.

This work suggests possible connections between the rewarding effects of corticosterone and dopamine neurotransmission in the striatum. Further investigations using techniques such as microdialysis are required to demonstrate the immediate effects of corticosterone self-administration on the levels of dopamine and their metabolites in the nucleus accumbens and other brain regions which are involved in the reward-seeking behaviour.

General conclusion

This experiment showed that mice acquired and reinstated oral corticosterone self-administration in the two-bottle choice test. The use of IMP was extremely important, as results revealed that mice used IMP to discriminate the stress hormone, and IMP facilitated and enhanced corticosterone self-administration as a result of its initial aversive taste. The switching phase improved the interpretation of results of acquisition and relapse. Firstly, corticosterone-dependent mice (IMP *versus* unflavoured corticosterone, group D) received corticosterone doses similar to what they self-administered prior to switching. Secondly, mice in group E (water *versus* flavoured corticosterone) developed significant corticosterone self-administration after switching, provided that they did not show avoidance to flavoured corticosterone during acquisition and relapse. Altogether, switching indicated that the rewarding effects of corticosterone are flavour-independent. Apart from ethanol self-administration studies using saccharine fading procedures (the addition of a sweetener to gradually increasing concentrations of the ethanol solution to facilitate its self-administration followed by a gradual withdrawal of the sweetener after animals have self-administered the highest ethanol concentration), this is the first study in which a flavour has been used in the two-bottle free choice model for testing the rewarding effects of a substance. Short term self-administration of high corticosterone doses did not impair the spontaneous alternation performance and recognition memory but improved the object location memory. In contrast, mice whose choice was IMP *versus* unflavoured corticosterone chronically received high doses of corticosterone and consequently had recognition memory deficits but no spatial memory impairment in the alternation test. This suggests that spontaneous alternation and recognition memory are regulated by different brain circuits. Overall, rewarding effects seem to outweigh deleterious effects of excessive exposure to the stress hormone. Corticosterone self-administration in mice probably induced plasticity in the hippocampus and frontal cortex as shown by changes in pERK1/2 levels which were significantly different between corticosterone naïve and treated groups. Also, the frontal cortex appears to mediate the development of corticosterone self-administration, but the hippocampus is more likely involved in corticosterone dependence. Our

results suggest that IMP acted as a cue by which mice learned the association between the taste of flavour and the rewarding hormone, and this was accompanied by significant effects on BDNF/pERK2 levels in the hippocampus. However, mice did not show these effects when they had no IMP in the control or test solutions. Additionally, corticosterone self-administration resulted in enhancement of dopamine utilisation in the striatum as DOPAC/dopamine ratio was higher only in the corticosterone-treated groups which had IMP-flavouring solutions. This reinforces the role of IMP as a cue which triggered dopamine release in the striatum of mice when they drank from the corticosterone solution. Finally, DOPAC levels in the striatum were directly proportional to the average corticosterone doses consumed by all mice over the whole study. In summary, although this experiment has provided more evidence, in addition to previous work, that corticosterone is rewarding, its rewarding properties do not impair but are likely to improve memory. Further work is required to investigate whether corticosterone recruits certain signalling pathways in specific brain regions to mediate its rewarding effects and how all these affect cognitive performance.

Chapter 3

The rewarding and cognitive effects of voluntary wheel running: role of the stress hormone corticosterone

3.1 Introduction

It is well established that voluntary wheel running is a natural reward. Indeed, wheel running reinforces lever pressing (Iversen, 1993; Belke and Wagner, 2005), while rats develop conditioned place preference (CPP) to the after-effects of voluntary wheel running (Lett *et al.*, 2000; Lett *et al.*, 2001; Belke and Wagner, 2005). Moreover, long-term wheel running decreases dopamine D2 receptor mRNA expression in the nucleus accumbens core of rats (Greenwood *et al.*, 2011), as observed in morphine-dependent rats (Georges *et al.*, 1999). Furthermore, the mesolimbic and nigrostriatal dopamine pathways have been found to be implicated in the wheel running activity of mice bred for high running performance (Mathes *et al.*, 2010).

Voluntary wheel running has been extensively used to study the effects of exercise on cognition. Wheel running enhances spatial memory, long-term potentiation (LTP) and neurogenesis in the hippocampus of young mice (van Praag *et al.*, 1999; Vasuta *et al.*, 2007) and also improves hippocampal neurogenesis in aged mice (van Praag *et al.*, 2005). Moreover, wheel running also ameliorates aging and Alzheimer's-related cognitive decline in mice (van Praag *et al.*, 2005; Nichol *et al.*, 2007; Parachikova *et al.*, 2008).

It is also believed that voluntary wheel running activates the HPA axis and elevates plasma corticosterone levels (Girard and Garland, 2002; Droste *et al.*, 2003; Adlard and Cotman, 2004; Fediuc *et al.*, 2006). Although previous work reports increases in plasma corticosterone levels as a result of unlimited access (24 hours) to wheel running (Droste *et al.*, 2003; Fediuc *et al.*, 2006), it is not clear whether limited access (1-hour) to wheel running is sufficient to increase corticosterone levels. As mentioned in the previous chapters, corticosterone has been implicated in drug-seeking behaviour (Mantsch *et al.*, 1998; Dong *et al.*, 2006; Mantsch and Katz, 2007) and is self-administered in rats (Deroche *et al.*, 1993; Piazza *et al.*, 1993) and mice (Chapter II). Taken together, this suggests that corticosterone elevation induced by wheel running may also be involved in its rewarding and cognitive effects.

This chapter comprised two preliminary studies to investigate the involvement of corticosterone in the rewarding and/or cognitive effects of single and repeated 1-hour voluntary wheel running sessions in mice.

Chapter 3 – Part 1

Effects of repeated 1-hour voluntary wheel running sessions on HPA axis and memory in mice

3.2 Aims

In this pilot study, there were 3 questions to be addressed:

1- What is the minimum number of sessions required for mice to run effectively?
2- Do repeated 1-hour voluntary wheel running sessions increase plasma corticosterone levels after 5 weeks of exercise?
3- What is the impact of chronic intermittent wheel running on memory?

3.3 Materials and methods

3.3.1 Animals

Twenty 6-8 week old male C57BL/6J mice were bought from Charles River UK, individually caged and kept undisturbed for a week of acclimatization. Mice were kept under standard environmental conditions of temperature (20 -22 °C), humidity (55 – 60 %), ventilation and 12:12 light dark cycle (lights on at 7:00 am) throughout the experiment. Mice had unlimited access to food and water. Body weight (BW) of mice was recorded once a week. All procedures undertaken in this study were in accordance with the UK Animal Scientific Procedures Act 1986 under project licence 40/3283.

3.3.2 Running wheels

Each wheel (4.5 inches in diameter) was tightly fixed to the internal side of the cage lid facing the cage bottom. A small circular magnet was then mounted on the top of the wheel. A cycle computer was attached to the side of the cage lid with its sensor directed at a distance of < 1 cm to the magnet placed on the wheel to count the number of the revolutions. Cycle computers were calibrated according to the wheel size to calculate the running distance over the exercise sessions. The wheel running equipment is illustrated in Fig. 3.1

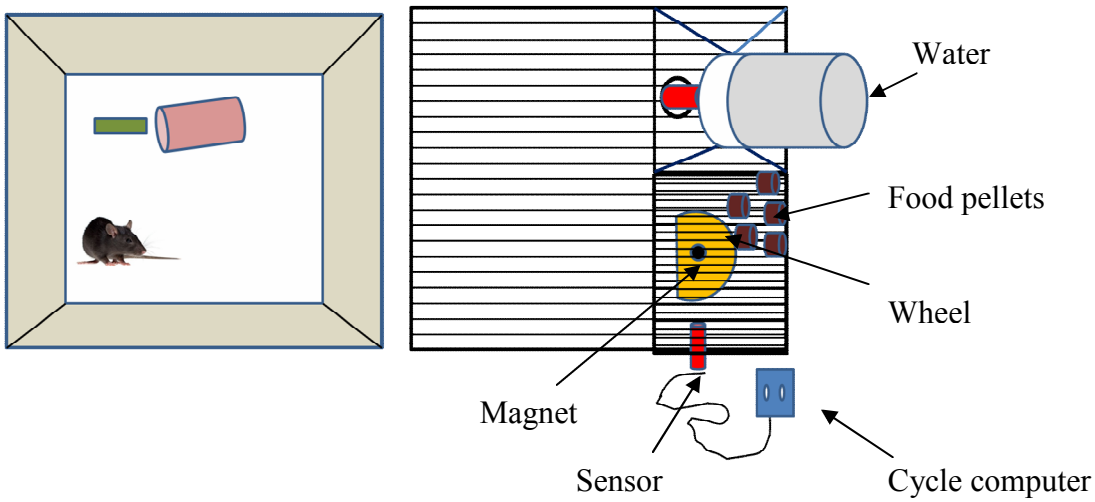

Figure 3.1 Voluntary wheel running apparatus. For each 1-hour running session, the lid of a mouse cage was replaced with another fitted with a running wheel and a cycle computer. The number of wheel revolutions was recorded by the cycle computer attached to the side of the cage lid. The distance run was calculated automatically by the cycle computer according to the wheel's diameter and number of revolutions. Each sedentary mouse had access to a static wheel during the exercise sessions. All mice had access to food and water during the exercise session.

3.3.3 Experimental design

At the end of the acclimatization period, spatial memory was examined using the spontaneous alternation test (SAT) in a T-maze. Based on performance in the alternation test, mice were assigned to one of two groups (n = 10): running and sedentary (non-exercising), to ensure equal baseline alternation performance. Mice underwent a series of daily 1-hour voluntary wheel running sessions, 5 days a week for 5 weeks, during which running distances were automatically calculated using the cycle computers. It was reported that this exercise protocol but using treadmills improved hippocampal neurogenesis and memory performance (Chang *et al.*, 2008; Liu *et al.*, 2009). Sedentary mice were given access to static wheels. After the completion of the exercise

regimen, mice were re-tested in the SAT, novel object recognition test (NORT) and object location test (OLT). Methodological details of memory tests are given in Chapter 2-Part 2. On the termination day, mice had their final running session immediately before being culled. Trunk blood was collected and plasma was kept at -80°C for measuring corticosterone levels. The timeline of the experiment is illustrated in Fig. 3.2

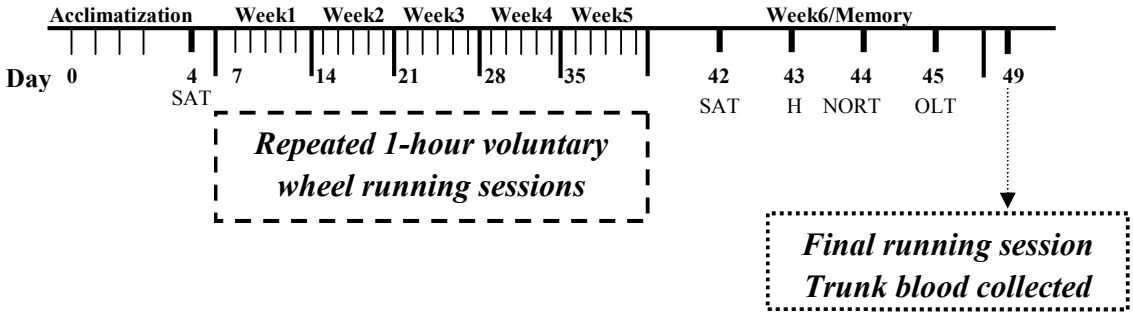

Figure 3.2 The timeline of the experiment. After acclimatization, mice underwent 5 weeks of repeated 1hour/day voluntary wheel running sessions (5 days a week). Mice were then subjected to the spontaneous alternation test (SAT), habituation (H) session in the open field arena, novel object recognition test (NORT) and object location test (OLT). On Day 49, mice were sacrificed immediately after the final running session.

3.3.4 Plasma corticosterone levels (ng/ml)

Circulating corticosterone levels were determined using a commercial ELISA kit. Details are given in Chapter 2-Part 1.

3.3.5 Statistical analysis

For the exercise group, paired sample *t*-tests were used to compare the average running distances of mice for each of the 5 weeks to the level of running activity recorded during the prior week. Two-way ANOVAs with repeated

measures were used to analyse BW, percentage of correct alternation in the SAT (time X group) and total exploration times (trial X group) in the NORT and OLT. Groups (2 levels, sedentary and running) were the between subject factor, and $time_{BW}$ (7 levels, weeks), $time_{SAT}$ (2 levels, before and after running) or $trial_{NORT,\ OLT}$ (2 levels, acquisition and retention) was the within subject factor. Paired sample *t*-tests were used to compare the exploration times between the familiar and novel objects, and between the non-displaced and displaced objects. Unpaired *t*-tests were used to analyse plasma corticosterone levels, total distance travelled and anxiety-related behaviour during the habituation session prior to the object memory tests, recognition index (RI) and location index (LI). The percentage of correct alternation, RI and LI were also compared to 50 % (lack of discrimination) using one sample *t*-tests. The Pearson's correlation coefficient was used to analyse correlations between running distances of mice over 5 weeks and memory performance or running distances of mice during the final session and their plasma corticosterone levels. Data are presented as mean ± SEM, and significance was reached when $P \leq 0.05$.

3.4 Results

3.4.1 Running distance (km)

Paired sample *t*-tests revealed a significant increase in the distance run by mice over the 5 weeks of exercise, which reflected the gradual development and maintenance of running behaviour. Mice ran only 0.143 km per 1-hour session on average during the first week, but they reached a maximum average running distance of ~ 0.88 km per 1-hour session by the end of the fifth week, Fig 3.3. Also, there was no significant difference in the running performance of mice before sacrifice (around 0.81 km) compared to their average running distance during the fifth week. Moreover, body weights of running mice were not significantly different from those of sedentary mice over the entire study ($F_{(1,18)} = 0.23$, $P = 0.64$). There was also no significant main effect of week X group interaction ($F_{(6,108)} = 1.16$, $P = 0.34$), but there was a significant time effect

($F_{(6,108)}$ = 67.07, P <0.001), which reflects the normal increase in BW of mice over time.

3.4.2 Plasma corticosterone levels (ng/ml)

There was a significant rise in circulating corticosterone levels of running mice compared to sedentary mice (the unpaired *t*-test: $t_{(18)}$ = 6.03, P = 0.000011) after the last running session, Fig 3.4. Also, there was no correlation (n = 10, r = 0.33, P = 0.35) between running distances (km) of mice during the final exercise session and their plasma corticosterone levels (ng/ml).

3.4.3 Spontaneous alternation

The two-way ANOVA with repeated measures showed no significant main effects of groups ($F_{(1,18)}$ = 4.11, P = 0.058), time ($F_{(1,18)}$ = 0.05 , P = 0.83) and time X group interaction ($F_{(1,18)}$ = 1.24, P = 0.28) on spatial alternation performance. The averaged performance of both sedentary and running groups did not differ from 50 % (chance) during acclimatization, but sedentary mice improved their spontaneous alternation performance after the second alternation test as indicated by one sample *t*-tests (t = 3.87, P = 0.004), Fig 3.5.

3.4.4 Locomotor activity and anxiety-like behaviour in the open field

Unpaired *t*-tests revealed no significant alterations in locomotor activity and measures of anxiety-related behaviour between the sedentary and running groups. Data are presented in table 3.1

Table 3.1 Locomotor activity and anxiety-related behaviour of sedentary and running groups (n = 10 mice) in the open field 24 hours before the novel object recognition test. Data are presented as mean ± SEM**.** Unpaired *t*-tests indicated that repeated 1-hour voluntary running sessions did not affect motor activity and anxiety-like behaviour.

Measures	Groups		Unpaired *t*-tests
	Sedentary	Running	
Distance travelled (m)	90.97 ± 3.22	96.17 ± 4.85	$t_{(18)}$ = 0.89, P = 0.38
% Distance in the centre relative to total	11.75 ± 0.53	10.84 ± 0.38	$t_{(18)}$ = 1.40, P = 0.18
% Time spent in the centre relative to total	9.31 ± 0.84	8.02 ± 0.71	$t_{(18)}$ = 1.17, P = 0.26
Defecation number	6.80 ± 0.61	5.90 ± 0.86	$t_{(18)}$ = 0.85, P = 0.41

3.4.5 The novel object recognition test

For the total exploration times, the two-way ANOVA with repeated measures showed no significant main effects of groups ($F_{(1,18)}$ = 2.23 , P = 0.15), trial ($F_{(1,18)}$ = 0.05 , P = 0.82) and trial X group interaction ($F_{(1,18)}$ = 0.45 , P = 0.51), but there was a significant object effect during the retention trial ($F_{(1,18)}$ = 22.56, P <0.001). Post-hoc paired sample t-tests revealed that mice significantly preferred the novel over the familiar object in both sedentary (t = 2.45, P = 0.037) and running (t = 4.17, P = 0.002) groups, table 3.2. One sample t-tests

confirmed that RIs of sedentary (t = 2.51, P = 0.033) and exercising (t = 5.95, P = 0.0002) mice were significantly different from 50% (lack of discrimination), Fig. 3.6A. There was no difference in RIs between the two groups ($t_{(18)}$ = 1.44, P = 0.17). Interestingly, there was a significant negative correlation between RIs and running distances during the last 4 weeks of exercise in the running group: week2 (r = - 0.75, P = 0.013), week3 (r = - 0.83, P = 0.003), week4 (r = - 0.72, P = 0.02) and week5 (r = - 0.79, P = 0.007).

3.4.6 The object location test

The two-way ANOVA with repeated measures revealed no differences between sedentary and running groups for the total exploration times during the acquisition and retention trials ($F_{(1,18)}$ = 0.64, P = 0.43). Also, there were no significant main effects of trials ($F_{(1,18)}$ = 0.38, P = 0.54) and trial X group interaction ($F_{(1,18)}$ = 1.42, P = 0.25). Moreover, the two-way ANOVA indicated no significant effects of object displacement ($F_{(1,18)}$ = 1.11, P = 0.31), trial X object interaction ($F_{(1,18)}$ = 3.54, P = 0.076) or object X group interaction ($F_{(1,18)}$ = 1.17, P = 0.29), table 3.2. No differences in LIs were found between groups ($t_{(18)}$ = 0.73, P = 0.47) and the LIs of both groups were not significantly different from 50 % (no discrimination), although running mice (t = 1.96, P = 0.082) tended to perform better than the sedentary (t = 1.03, P = 0.33), Fig. 3.6B.

Table 3.2 Exploration times (sec) during the novel object recognition and location memory tests**.** Data are presented as mean ± SEM (n = 10 mice/group). A1 (non-displaced object), A2 (object to be displaced), a (familiar + novel), b (object A1 + object A2), c (non-displaced + displaced). Paired sample t-tests: *P <0.05 and **P <0.01 compared to the familiar object.

Memory tests	Trials	Objects	Groups	
			Sedentary	Running
Novel object recognition	Acquisition	Single	52.20 ± 4.91	64.00 ± 9.32
	Retention	Familiar	20.25 ± 2.01	24.95 ± 3.76
		Novel	28.40* ± 3.44	40.80** ± 6.06
		Total[a]	48.65 ± 4.54	65.75 ± 9.34
Novel object location	Acquisition	Object A1	26.90 ± 3.69	27.25 ± 4.74
		Object A2	24.55 ± 2.82	27.40 ± 4.02
		Total[b]	51.45 ± 5.93	54.65 ± 8.24
	Retention	Non-displaced	21.75 ± 2.43	24.40 ± 3.63
		Displaced	24.00 ± 1.62	32.05 ± 5.17
		Total[c]	45.75 ± 2.86	56.45 ± 7.66

Development and maintenance of running behaviour in the exercising mice

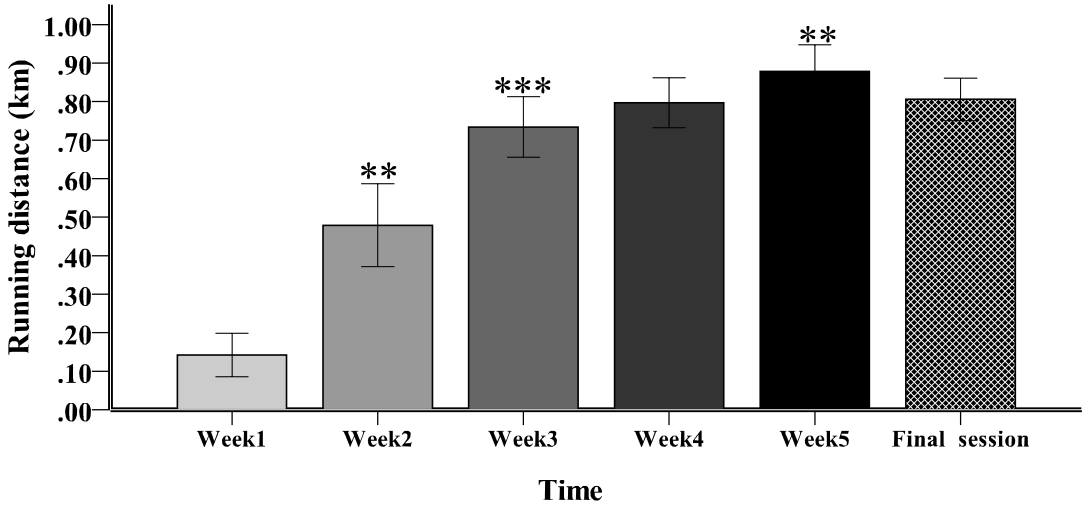

Figure 3.3 Mean running distances (km) of exercising mice (n = 10). Male C57BL/6J mice increased their running distances over the 5 weeks of repeated 1-hour/day (5 days/week) voluntary wheel running sessions. Data are expressed as mean ± SEM. Paired sample *t*-tests: **P <0.01 and ***P <0.001 compared to the prior week. The average running distances of mice was not significantly different between the fifth week and the final session.

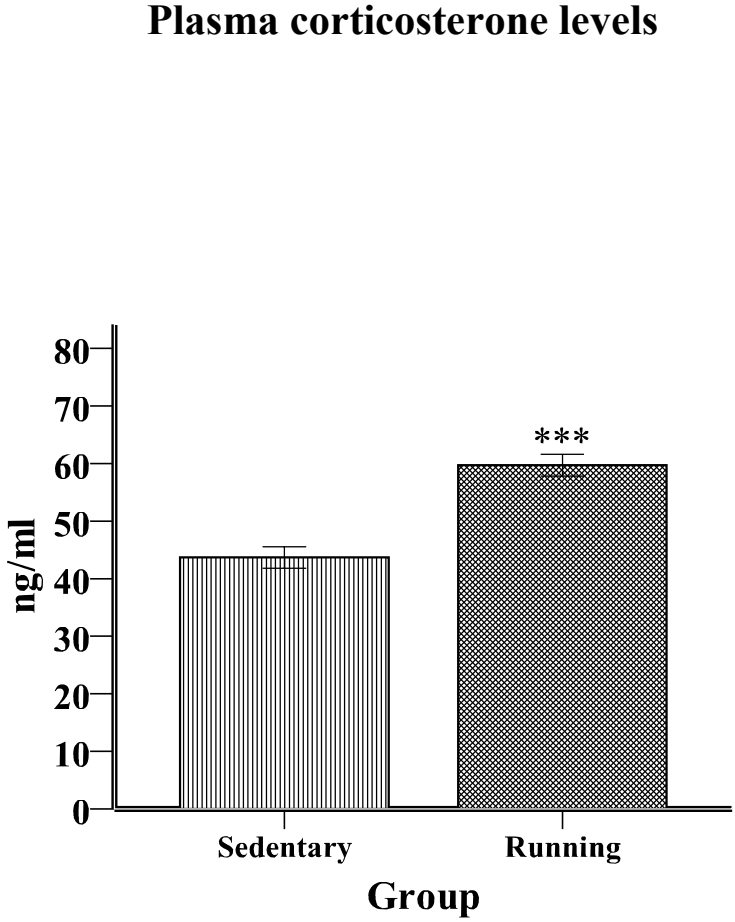

Figure 3.4 Plasma corticosterone levels (ng/ml). After the final running session, mice (n = 10 / group) were immediately sacrificed and trunk blood was collected to investigate effects of repeated 1-hour voluntary wheel running sessions on HPA axis function. Data are presented as mean ± SEM. The unpaired *t*-test revealed a significant corticosterone rise in the running group: ***P <0.001 compared to sedentary.

Percentage of correct alternations in the T-maze

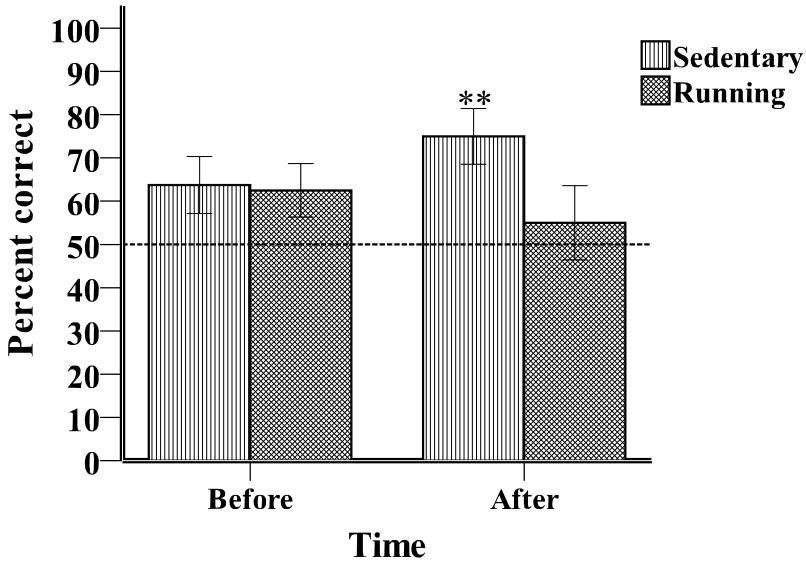

Figure 3.5 The spontaneous alternation performance in the T-maze. Sedentary and running mice (n = 10 / group) underwent the spontaneous alternation test before and after the 5 weeks of repeated 1-hour voluntary wheel running sessions. Data are expressed as mean ± SEM. One sample *t*-tests: **P <0.01 compared to 50 % (chance).

The novel object recognition and location memory performance

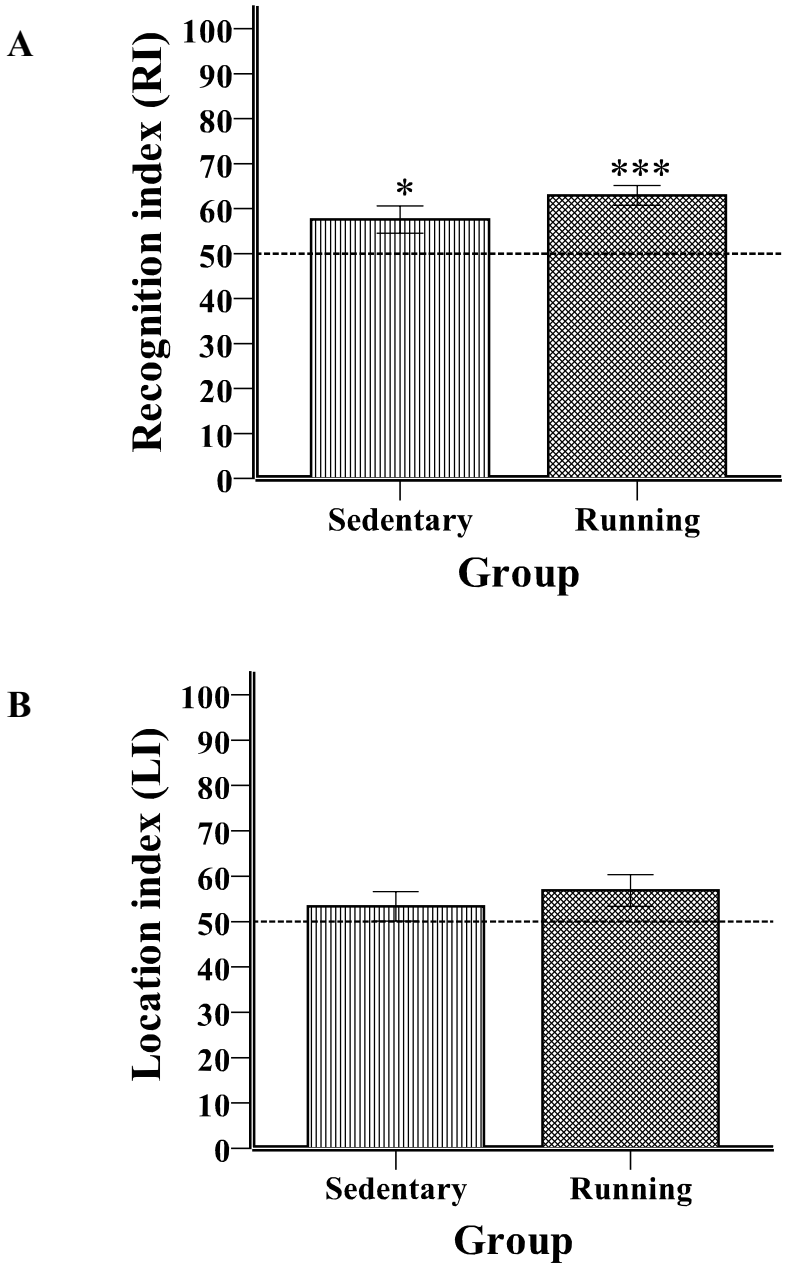

Figure 3.6 The object memory tests. (A) Recognition Index. (B) Location index. Data of sedentary and running mice (n = 10 / group) are presented as mean ± SEM. One sample *t*-tests: *P <0.05 and ***P <0.001 compared to 50 % (no discrimination).

3.5 Discussion

The present study confirmed that repeated 1-hour voluntary wheel running sessions motivated mice to run more over the course of 5 weeks and this was not accompanied with a habituation of exercise-induced elevation of corticosterone levels. Whereas sedentary mice exhibited an improvement in the spontaneous alternation performance by the end of the experiment, both sedentary and running groups did not effectively acquire the object location task. In contrast, recognition memory was intact in both groups.

These results emphasize that wheel running is a motivated behaviour, as mice kept increasing their running performance over consecutive limited (1-hour) running sessions, consistent with previous work showing that wheel running is reinforcing and rewarding (Iversen, 1993; Belke and Wagner, 2005). Furthermore, it is believed that wheel running is a spontaneous behaviour which increases with experience and, therefore, animals do not habituate to wheel running (Lett *et al.*, 2001; Cosgrove *et al.*, 2002).

These results also showed that exercising mice exhibited a significant increase in plasma corticosterone levels after the final running session, which was undertaken 10 days after a 5-week exercise regimen. This is in agreement with previous work which demonstrated that C57 mice had high corticosterone levels after 4 weeks of continuous wheel running (Droste *et al.*, 2003). Likewise, chronic running increased the weight of adrenal glands and decreased thymus weight of exercising mice (Droste *et al.*, 2003), indicating corticosterone elevation. In contrast, rats exercising for 5 weeks in the wheel exhibited enhancement of circadian corticosterone levels after the first and second week, but there was a gradual decline in the elevated corticosterone levels so that further running no longer increased corticosterone levels compared to the sedentary group (Fediuc *et al.*, 2006). Both sedentary and exercising rats had similar GR levels in the hippocampus and anterior pituitary (Fediuc *et al.*, 2006), reflecting HPA axis adaptation. Nevertheless, in running animals, HPA axis responses to novel stressors such as restraint stress, new environments and

forced swimming were maintained (Droste *et al.*, 2003; Fediuc *et al.*, 2006). In fact, diminished HPA axis responsiveness with chronic exercise was indicated by the increment of corticosterone levels but low levels of ACTH in exercising animals in response to the previously mentioned stressors compared to sedentary animals (Droste *et al.*, 2003; Fediuc *et al.*, 2006). Also, C57BL/6J mice exercising for 2 weeks exhibited increased plasma corticosterone levels without a comparable increase in ACTH levels. This was, however, accompanied by a significant rise in mRNA expression for adrenal steroidogenic acute regulatory protein and tyrosine hydroxylase, both of which are directly or indirectly involved in corticosterone synthesis (Otawa *et al.*, 2007). In the present study, running mice did not develop HPA axis adaptation in response to long-term intermittent exercise-induced corticosterone elevation, suggesting that running mice were exposed to high corticosterone levels until the last session.

Mice did not show differences in their locomotor activity and anxiety-like behaviour during the 30-minute habituation session which was carried out 24 hours prior to the novel object recognition test. This eliminates the possibility that effects on memory performance were secondary to motor or emotional variability between groups. Interestingly, only sedentary mice showed an improvement in spontaneous alternation performance. As stated earlier, sedentary mice were left to explore the static wheels during the exercise sessions, but running mice had the opportunity to run in the wheels and explore them during the running session. This suggests that exploration behaviour without running enhanced spontaneous alternation performance, reflecting spatial memory improvement. In the object location test, however, neither sedentary nor running groups were able to discriminate the displaced object during the retention trial, which was carried out 1 hour after the acquisition trial. Although the LI of the running group was slightly higher than the LI of the sedentary group, the LIs of both groups did not significantly differ from 50 % (no discrimination). It was found that mice demonstrated a significant preference for the displaced object, when the inter-trial interval between the

acquisition and retention trials was not more than 2 hours (Murai *et al.*, 2007). The inability of mice in this study to spend more time exploring the displaced objects could be method-related. For example, the lack of significant extra-field cues was found to disrupt the ability of mice to discriminate the displaced object (Murai *et al.*, 2007). In contrast, the arrangement of objects in the arena and the entry position of a mouse into the arena were not critical for the optimum performance (Murai *et al.*, 2007). Mice were also examined in the novel object recognition test, in which both groups spent more time exploring the novel object than the familiar object. The RIs of both sedentary and running mice were significantly higher than 50%, and there was no significant difference in performance between the two groups, although running mice tended to outperform sedentary mice. In agreement with these results, it was found that short-term (13 days) unlimited wheel running improved recognition memory and enhanced neurogenesis and BDNF levels in the hippocampus of mice (Lafenetre *et al.*, 2010). A recent study indicated that metyrapone-treated or adrenalectomized rats did not exhibit spatial memory enhancements induced by wheel running (10 days) in saline-treated and sham-operated rats (Hajisoltani *et al.*, 2011), suggesting the involvement of exercise-induced corticosterone rise in the cognition-enhancing effects of short-term exercise.

The lack of statistical differences in the cognitive performance between running and non-running mice could be explained as follows. First, sedentary mice had access to a static wheel during the exercise sessions, and this probably provided mice with some forms of environmental enrichment which was reported to improve memory and hippocampal neurogenesis (Kempermann *et al.*, 1997; Bennett *et al.*, 2006). Previously, it was reported that mice housed with static wheels exhibited enhancement of their cognitive performance (Pietropaolo *et al.*, 2006; Nichol *et al.*, 2007). This suggests that the cognitive effects generated by wheel exploration were almost equal to those produced by the limited duration of wheel running. Therefore in the experiment described in Chapter 4, a no-wheel group was added to investigate the cognitive effects of wheel exploration. Second, repeated 1-hour running sessions could be insufficient to

show enhancements of cognitive performance compared to sedentary mice, despite the elevated plasma corticosterone levels of the running group. Thus, it can be suggested that short 1-hour wheel running sessions were sufficient to increase corticosterone levels, but they were insufficient for other exercise-related effects on cognition to take place. For instance, exercise was found to increase BDNF mRNA and protein levels in the hippocampus of rats and mice after having unlimited access to wheel running or during the dark phase (Neeper *et al.*, 1995; Oliff *et al.*, 1998; Lafenetre *et al.*, 2010). In agreement with this, rats having unlimited access (24 hours) to wheel running for only 5 days showed enhanced spatial memory performance (Ebrahimi *et al.*, 2010), and also running for 4 days at night ameliorated spatial memory impairment in rats (Shaw *et al.*, 2003). Thirdly, rats running as short a distance as 0.1 km per night reversed spatial memory deficits (Shaw *et al.*, 2003), suggesting that little exercise but in the dark/active phase is more beneficial for cognitive performance than in the light/inactive phase despite corticosterone elevation. However in the present study, 1-hour access to wheel running in the light phase was a priority in this work to avoid confounding effects of circadian corticosterone peaks in the dark phase and to prevent chronic exercise-induced HPA axis adaptation. Also, it was necessary to keep corticosterone levels at high values in the running group to study whether corticosterone is implicated in the rewarding/cognitive effects of chronic wheel running. As described in Chapter 4, pharmacological interventions were used to test this hypothesis and short running sessions were necessary for the optimum effects of the drugs used, and to avoid injecting animals more than once daily. In addition, it was suggested that short exercise sessions would allow clear accurate assessment of drugs' effects on running performance of mice. As stated earlier, it has been reported that 1-2 hours of voluntary wheel running is rewarding and reinforcing (Belke and Wagner, 2005), and, therefore, 1-hour wheel running per day is believed to be rewarding.

Finally, running mice showed extensive running performance over the last three weeks of exercise as a result of limited access to wheel running. Subsequently,

running mice showed no improvement in their spontaneous alternation performance, and their RIs were inversely proportional to average running distances on most weeks of exercise, suggesting that exercise-induced corticosterone release was indeed linked to cognitive performance. In agreement with this, high-running rats showed recognition memory deficits but low-running rats exhibited improvement in memory performance, as compared to the sedentary group (Garcia-Capdevila *et al.*, 2009).

To summarize, mice showed a great tendency to increase their running performance over repeated 1-hour exercise sessions. Also, five weeks of repeated 1-hour voluntary wheel running sessions induced plasma corticosterone elevation after the final running session. Although chronic intermittent wheel running did not improve spontaneous alternation performance, it did not impair recognition memory despite the negative correlation between running distances and memory performance. Lastly, a no-wheel group was added to the study described in Chapter 4 to investigate whether exploration of the wheel without running improves learning and memory of sedentary mice.

Chapter 3 – Part 2

The corticosterone synthesis inhibitor metyrapone attenuates rewarding effects of voluntary wheel running through decreasing the exercise-induced elevation in corticosterone levels in trained mice

3.6 Aims

Metyrapone is a corticosterone synthesis inhibitor which suppresses the conversion of 11-desoxycorticosterone to corticosterone *via* the inhibition of 11 β-steroid hydroxylase (Jenkins *et al.*, 1958; Akirav *et al.*, 2004), and it has been used to investigate the role of corticosterone in the development of drug dependence (Piazza *et al.*, 1994). It is, therefore, suitable for the investigation of the role of corticosterone in the motivation of mice for wheel running. The aim of this pilot study was to demonstrate that a single 1-hour voluntary wheel running session is sufficient to elevate plasma corticosterone levels in trained mice and to determine whether rewarding effects of wheel running are dependent on exercise-induced elevation in plasma corticosterone. We hypothesised that metyrapone would attenuate the running performance of mice, if corticosterone is involved in the rewarding effects of wheel running.

3.7 Materials and methods

3.7.1 Animals

Thirty two 6-8 week old male C57BL/6J mice were purchased from Charles River UK. They were individually housed and kept undisturbed for a week to acclimatize to the animal facility. Mice were kept under standard environmental conditions of temperature (20 – 22 °C), humidity (55 – 60 %), ventilation and 12:12 light dark cycle (lights on at 7:00 am) throughout the experiment. Food and water were available *ad libitum*. Body weight (BW) of mice was recorded weekly. All procedures undertaken in this experiment were in compliance with the UK Animal Scientific Procedures Act 1986 under project licence 40/3283.

3.7.2 Metyrapone

Metyrapone, 2-Methyl-1,2-di-3-pyridyl-1-propanone, (Sigma-Aldrich UK) was dissolved in saline immediately before use. Mice were injected with metyrapone (35 mg/kg, i.p.) 30 minutes before the challenge running session. This dose was previously found to significantly block corticosterone elevation induced by electric footshocks in mice (Chauveau *et al.*, 2010).

3.7.3 Experimental design

Mice underwent a daily 1 hour-running session (5 days a week) until they met running criteria; showing motivation to run for 3 consecutive sessions with a total running distance over 0.3 km, to be assigned to running groups. On the challenge day, mice were divided into 3 groups: saline-treated sedentary (Sed-Sal, n = 11), and running (Run-Sal, n = 9) and metyrapone-treated running (Run-Met, n = 12). Metyrapone (35 mg/kg) or saline was intraperitoneally injected 30 minutes before the challenge session. Sedentary mice were offered a static wheel during the challenge session. Immediately after the challenge session, mice were sacrificed by cervical dislocation and trunk blood was collected and kept at -80 °C for further analysis of plasma corticosterone levels. The experimental timeline is described in Fig. 3.7

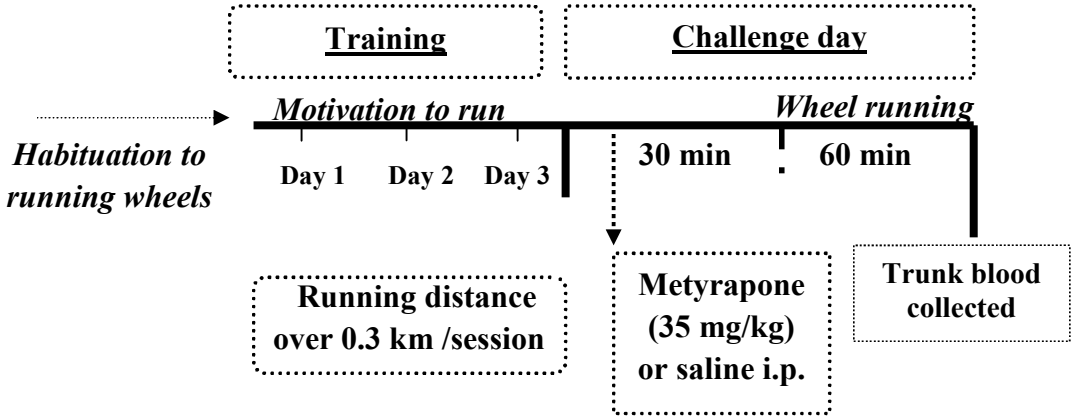

Figure 3.7 The experimental design. Mice were trained to meet the running criteria. On the challenge day, mice had a 1-hour voluntary wheel running session 30 minutes after saline or metyrapone treatment. Simultaneously, each sedentary mouse had access to a static wheel.

3.7.4 Plasma corticosterone levels (ng/ml)

A commercial ELISA kit was used to measure plasma corticosterone levels; details are provided in Chapter 2-Part 1.

3.7.5 Statistical analysis

Running distances were analysed using a two-way ANOVA with repeated measures with treatment (2 levels, saline or metyrapone) as the between subject factor, and training time (3 levels, days) as the within subject factor. Post-hoc paired sample *t*-tests were used to analyse the motivation of mice to increase their running distances over the 3 day training period *versus* the first training session after reaching the criterion of over 0.3 km per session. Also, paired sample *t*-tests were used to analyse differences in the running distances between the last training day and the treatment day. Running distances during the challenge session, plasma corticosterone levels and BW were analysed using unpaired *t*-tests. The Pearson's correlation coefficient was used to analyse the relationship between plasma corticosterone levels of exercising mice and their running distances on the challenge day. An effect was considered significant when the P value was ≤ 0.05

3.8 Results

3.8.1 Running activity (km)

Data showed that saline-treated mice ran ~ 0.6 km during the 1-hour challenge session on average and this level of exercise increased plasma corticosterone levels (83.98 ng/ml ± 6.46) compared to sedentary mice (58.77 ng/ml ± 5.72). In contrast, the mean running distance of metyrapone-treated mice was 0.37 km during the acute session and this was accompanied by a lack of elevation in corticosterone levels (65.41 ng/ml ± 4.87) compared to saline-treated exercising mice. The two-way ANOVA with repeated measures showed neither significant main effects of groups ($F_{(1,19)} = 0.85$, P = 0.37) nor time X group interaction ($F_{(2,38)} = 0.72$, P = 0.49), but there was a significant time effect ($F_{(2,38)} = 6.91$, P = 0.003) which indicates the increase in running performance over training days. Post-hoc paired sample *t*-tests showed a significant increase in the distances run by saline-treated mice during the last training session compared to the first one (t = 3.26, P = 0.012), Fig. 3.8. During the challenge session, there was a decrease in the distances run by metyrapone-treated mice compared to saline-treated mice ($t_{(19)} = 2.44$, P = 0.025), Fig. 3.8. Moreover, paired sample *t*-

tests indicated that metyrapone-treated mice ran significantly less during the challenge session than during the last training session ($t = 2.98$, $P = 0.012$), Fig 3.8. Finally, unpaired *t*-tests revealed no differences in BW between saline-treated sedentary and running mice ($t_{(18)} = 0.12$, $P = 0.91$) and between saline and metyrapone treated running mice ($t_{(19)} = 1.47$, $P = 0.16$).

3.8.2 Plasma corticosterone levels (ng/ml)

Results revealed that a 1-hour voluntary wheel running session significantly increased plasma corticosterone levels in saline-treated exercising mice compared to sedentary mice ($t_{(18)} = 2.92$, $P = 0.009$), Fig 3.9. By contrast, metyrapone treatment attenuated the exercise-induced corticosterone rise compared to saline-treated running mice ($t_{(19)} = 2.34$, $P = 0.030$), Fig 3.9.

3.8.3 Correlations

There was a positive correlation between the running distances of all exercising mice during the challenge session and their plasma levels of corticosterone ($n = 21$, $r = 0.375$, $P = 0.047$), Fig. 3.10

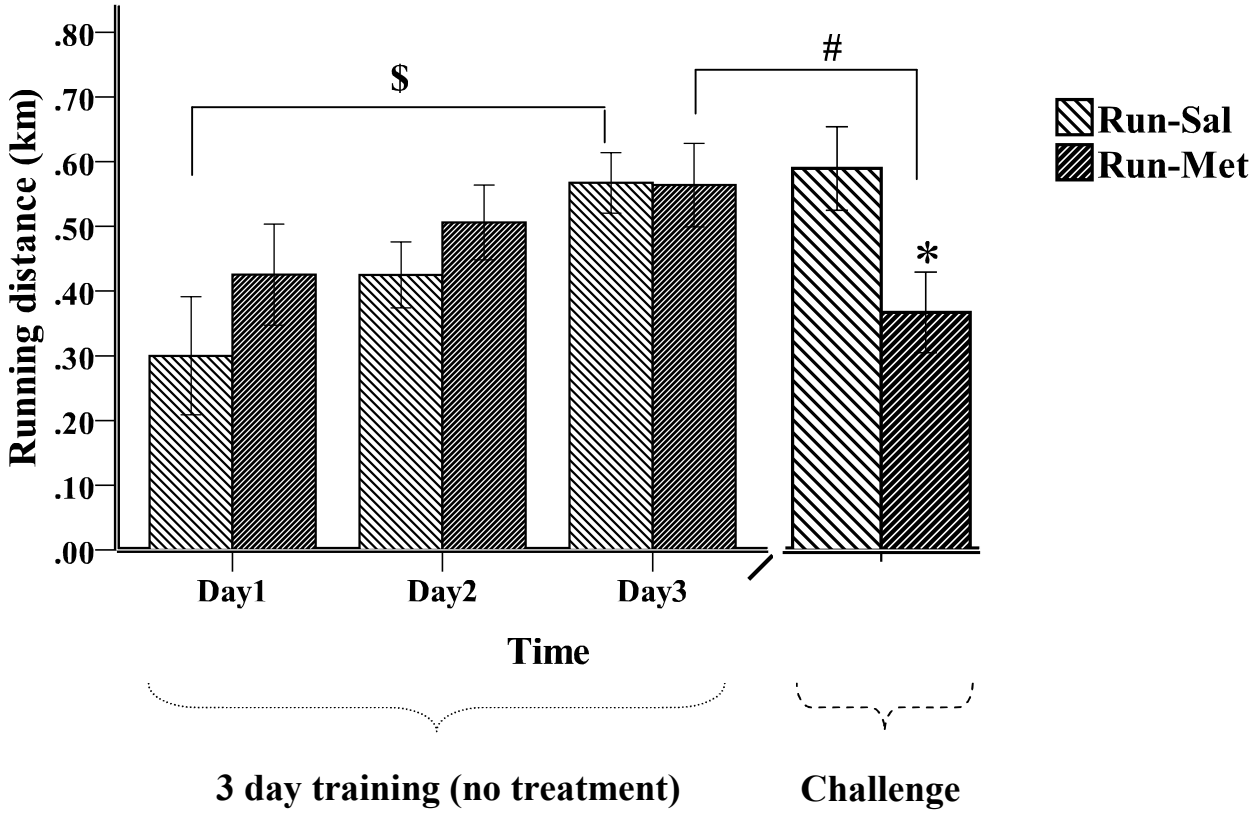

Figure 3.8 Running distances (km) in the wheel during 1-hour voluntary exercise sessions: training (Day1 to Day3) and challenge. Development of running performance in male C57BL/6J mice over the three days of training and effects of treatment with saline (n = 9) or metyrapone (n = 12) on running activity during the challenge session. Data are expressed as mean ± SEM. Paired sample *t*-tests: $^{\$}P < 0.05$ compared to Run-Sal (Day1) and $^{\#}P < 0.05$ compared to Run-Met (Day3). Unpaired *t*-tests: $^{*}P < 0.05$ compared to Run-Sal.

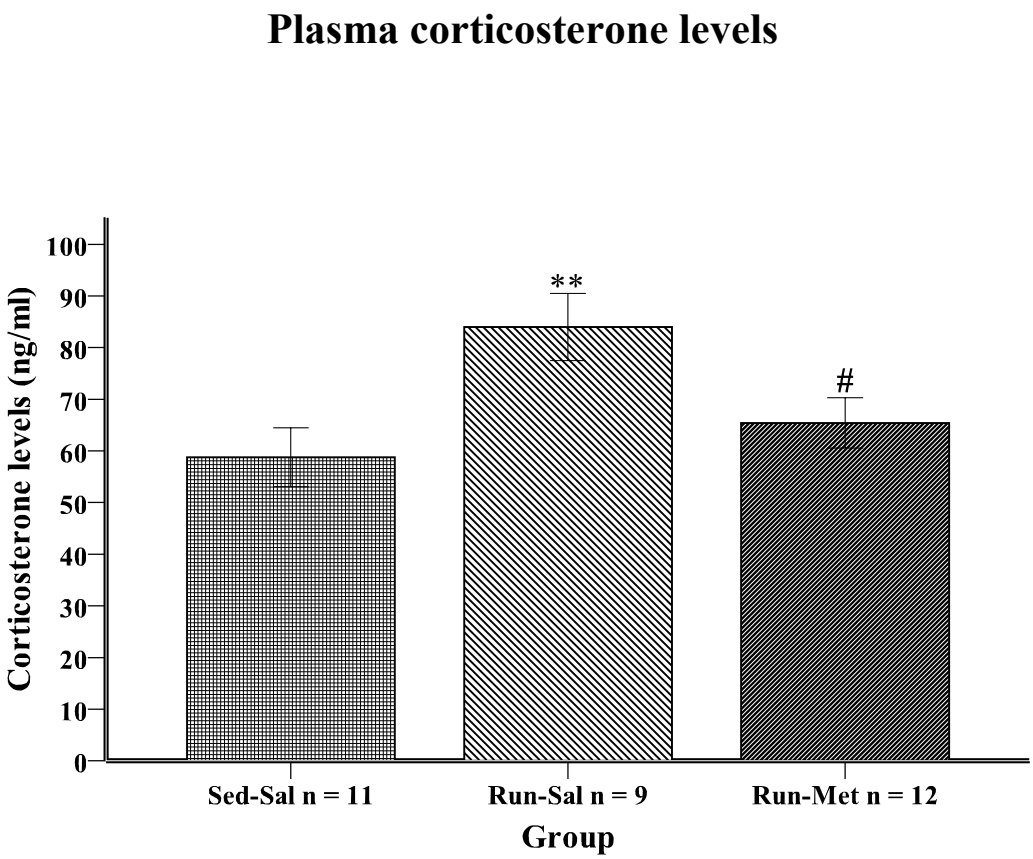

Figure 3.9 Plasma corticosterone levels (ng/ml). Data are expressed as mean ± SEM (n = 9-12 mice /group). Unpaired *t*-tests: **P <0.01 compared to Sed-Sal, $^{\#}$P <0.05 compared to Run-Sal.

Correlation between plasma corticosterone levels and running distances during the challenge session

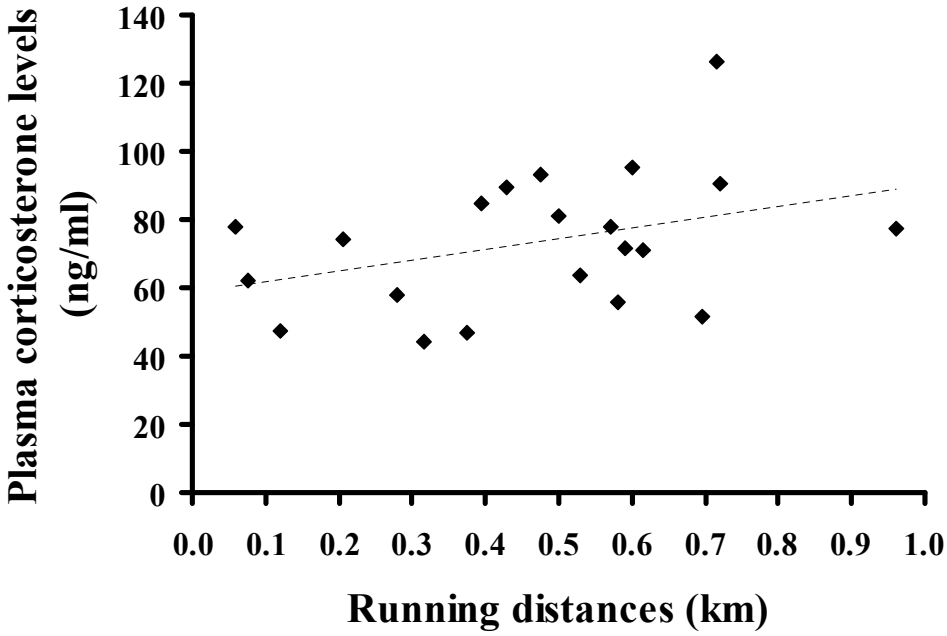

Figure 3.10 The positive correlation (n = 21, Pearson's r = 0.375, P = 0.047) between running distances (km) of mice during the challenge session and their plasma corticosterone levels (ng/ml) at the end of the session.

3.9 Discussion

The present experiment demonstrates that a single 1-hour voluntary wheel running session is sufficient to increase plasma corticosterone levels of trained mice compared to sedentary mice. In contrast, metyrapone prevented the exercise-induced increase in corticosterone levels, and attenuated the motivation of mice to run during the challenge session. Consistent with our results, one or two weeks of unlimited voluntary wheel running resulted in an increase in circadian corticosterone peaks in rats (Fediuc *et al.*, 2006) and C57 mice (Otawa *et al.*, 2007), respectively. These findings suggest that corticosterone is involved in the rewarding effects of voluntary wheel running. It is well established that metyrapone prevents rise in circulating corticosterone levels *via* inhibition of 11 β-steroid hydroxylase in the adrenal cortex which converts 11-desoxycorticosterone to corticosterone (Jenkins *et al.*, 1958).

Mice underwent a series of training sessions until they met the suggested criteria: running distance over 0.3 km per 1-hour session for 3 consecutive sessions. These criteria were derived from the chronic pilot study, Chapter 3-Part I, which showed that once mice ran over 0.3 km per 1-hour session, they kept increasing their running performance over the following sessions. Although most mice in the chronic pilot study required 1-2 weeks to achieve the training criteria, a few mice in this study were absolutely unwilling to exercise even after weeks of access to wheel running equipment and were allocated to the sedentary group. In contrast, mice and rats were successfully bred to exhibit great tendency to exercise spontaneously in the running wheels (Swallow *et al.*, 1998; Naylor *et al.*, 2005). For example, female Hsd:ICR mice demonstrated an increased tendency for wheel running and a greater exercise-induced rise in corticosterone levels than male counterparts (Coleman *et al.*, 1998). However, male mice were used in the present study to avoid confounding effects of the oestrous cycle which was previously found to influence running activity (Girard and Garland, 2002). Nevertheless, C57BL/6J mice, which were used in the present study, possess a natural tendency to run in the wheel (Brene *et al.*, 2007). In the present study, training was, however, necessary to ensure that mice in the exercising groups were sufficiently motivated to run during the

short challenge session in order to elevate plasma corticosterone levels in the control group, and to exclude the possibility that metyrapone-treated mice did not run because of a lack of motivation. Interestingly, rats having unlimited access to a running wheel for 5 weeks demonstrated the highest running distances during the fifth week of exercise, but this exercise level no longer increased corticosterone levels (Fediuc *et al.*, 2006). This suggests that corticosterone is involved in the acquisition of wheel running but its role in maintaining running levels is unclear. In contrast, other work including the present study showed that running mice had high corticosterone levels even after 4 weeks of wheel running (Droste *et al.*, 2003).

Our results also showed that there was a correlation between the running distances of all exercising mice and their plasma corticosterone levels after the challenge session, suggesting that the increase in corticosterone levels of running mice was dependent on their running distances during the challenge session, or that mice increased their running distances to enhance the secretion of the rewarding hormone. In agreement with this, it was previously found that plasma corticosterone levels were directly proportional to the number of wheel revolutions during the 20 minute wheel running prior to blood sampling (Girard and Garland, 2002).

It has been reported that corticosterone elevation increases motor activity. For example, adrenalectomy was found to attenuate amphetamine-induced locomotor hyperactivity in rats (Cador *et al.*, 1993), and to decrease the exploratory activity of rats in the open field (Veldhuis *et al.*, 1982). Similarly, metyrapone-treated and adrenalectomized rats having unlimited access to wheel running for 10 days tended to run less and exhibited a significant decrease in their running distances during the last two days of wheel running compared with vehicle-treated and sham-operated running rats, respectively (Hajisoltani *et al.*, 2011). On the other hand, some work suggests that metyrapone has a direct non glucocorticoid-dependent central effect. In a study using metyrapone at a high dose (150 mg/kg), rats showed a rapid (1-3 min) decrease of their motor activity in the actimeter (Canini *et al.*, 2009), and it was found that the fastest

glucocorticoid- mediated genomic effects required at least 15 minutes (Hallahan *et al.*, 1973). On the contrary, metyrapone treatment and adrenalectomy had similar influences on the wheel running performance of rats (Hajisoltani *et al.*, 2011), and this provides some evidence that metyrapone decreased running distances of mice as a result of lowering corticosterone synthesis, rather than acting centrally.

In summary, metyrapone attenuated the rewarding effects of a single 1-hour voluntary wheel running session in trained mice in conjunction with a decrease in plasma corticosterone levels. However, more work is required to determine whether corticosterone mediated these effects through non transcriptional actions.

General conclusion

Results of this chapter suggest that single and repeated 1-hour voluntary wheel running sessions activated HPA axis and increased plasma corticosterone levels. Moreover, metyrapone attenuated running performance of mice compared to saline-treated group *via* the inhibition of exercise-induced corticosterone rise, and there was a positive correlation between running distances of mice and their plasma corticosterone levels during the challenge session. Overall, this indicates that corticosterone is involved in the rewarding effects of voluntary wheel running. Furthermore, the exercise regimen used in this study did not improve spontaneous alternation performance and spatial memory of running mice in the novel object location test, compared to sedentary mice.

It is, however, interesting to understand how corticosterone could mediate rewarding and/or cognitive effects of wheel running. As a result of repeated voluntary wheel running, corticosterone levels were repeatedly and transiently elevated, and this probably contributed to rewarding/cognitive effects of exercise. As mentioned previously, MRs and GRs are highly expressed in the brain particularly the hippocampus (Reul and de Kloet, 1985), and therefore, the hippocampus is vulnerable to fluctuation in corticosterone levels (Diamond *et al.*, 1992). Thus, it is suggested that repeated fluctuations of corticosterone levels in response to intermittent running probably contribute to its rewarding effects, and expectedly imposes long-term effects on learning and memory of running mice, in particular hippocampus-dependent memory such as spatial and recognition memory (O'Keefe and Dostrovsky, 1971; Clark *et al.*, 2000; Broadbent *et al.*, 2004). Most importantly, a number of studies showed that GRs are essential for drug seeking behaviour (Fiancette *et al.*, 2010) and corticosterone was only self-administered at high doses which produced high plasma corticosterone levels, required to activate GRs (Reul and de Kloet, 1985; Piazza *et al.*, 1993), and Chapter II. This suggests that GRs are a potential target to study mechanisms underlying corticosterone involvement in the rewarding and cognitive effects of voluntary wheel running. It is also important to investigate whether dopamine, which is the major reward neurotransmitter in the brain (Wise and Rompre, 1989; Wise, 2009), is involved in the rewarding/cognitive effects of voluntary wheel running. Chronic wheel running

has been reported to produce plasticity changes in the mesolimbic reward pathway, such as reduced mRNA expression for dopamine D2 receptors in the nucleus accumbens core of rats (Greenwood *et al.*, 2011). Altogether, this suggests that dopamine and/or corticosterone *via* GRs play a role in exercise-related reward and memory enhancing effects, in particular if cognitive effects of exercise depend on its rewarding properties.

As described in Chapter 4, a study was therefore carried out to investigate whether GRs and/or D2 receptors mediate the cognitive effects of voluntary wheel running *via* its rewarding effects. In an attempt to make effects of this exercise protocol on memory stand out, one more control group was added to the study in which mice had no access to running wheels in order to examine the influence of wheel exploration on memory.

Chapter 4

Glucocorticoid and dopamine D2 receptors: Are they involved in the rewarding and cognitive effects of the repeated 1-hour voluntary wheel running sessions?

4.1 Introduction

Voluntary wheel running is rewarding to mice (Brene *et al.*, 2007), elevates plasma corticosterone levels (Droste *et al.*, 2003) and is reported to enhance learning and memory performance (van Praag *et al.*, 1999; Lafenetre *et al.*, 2010; Hajisoltani *et al.*, 2011). In agreement with this, the two pilot studies described in Chapter 3 demonstrated that single and repeated 1-hour voluntary wheel running sessions evoked corticosterone elevation. Also, mice were motivated to run and continued to increase their running performance over 5 weeks of the repeated 1-hour wheel running sessions, Chapter 3-Part 1. On the other hand, repeated exercise sessions did not improve alternation rates or recognition memory performance of exercising mice compared to sedentary mice. Instead, there was a negative correlation between running distances of mice and their recognition indices. Also, results of the pilot study, Chapter 3-Part 2, showed that metyrapone, a corticosterone synthesis inhibitor, decreased the running activity of mice. Similarly, metyrapone attenuated the running

performance of rats which had unlimited access to the wheel running for 10 days (Hajisoltani *et al.*, 2011). Altogether, corticosterone is suggested to modulate the rewarding and cognitive effects of repeated 1-hour voluntary wheel running sessions.

Glucocorticoid receptors (GRs) are thought to mediate rewarding effects of corticosterone. First, GRs have a 10-fold lower affinity for corticosterone than mineralocorticoid receptors (MRs) (Reul and de Kloet, 1985), thus GRs are mainly activated by high corticosterone levels of circadian corticosterone peaks or elevations induced by stress. It has been thought that GRs are involved in the rewarding effects of corticosterone, since corticosterone is self-administered at levels comparable to those induced by stress (Piazza *et al.*, 1993).

A considerable body of research suggests that corticosterone is critical for cognitive performance (Belanoff *et al.*, 2001b; Akirav *et al.*, 2004), since GRs are widely expressed in the brain particularly the hippocampus, suggesting a possible role of GRs in the cognitive effects of repeated exercise-induced corticosterone elevation. Thus, GRs represent a potential target to examine whether corticosterone elevation mediates the rewarding and/or cognitive effects of voluntary wheel running. Mifepristone (RU-38486) was the first synthetic GR antagonist (Bertagna *et al.*, 1984), which also has antiprogestin activity (Cadepond *et al.*, 1997; Niinimaki *et al.*, 2009). Mifepristone has been shown to attenuate self-administration of cocaine in rats and mice (Deroche-Gamonet *et al.*, 2003; Fiancette *et al.*, 2010) and to impair the expression of CPP to morphine in rats (Dong *et al.*, 2006), reinforcing the hypothesis that GRs mediate the rewarding effects of corticosterone.

It has been reported that dopamine levels in the nucleus accumbens increase in response to natural reward (Hernandez and Hoebel, 1988; Damsma *et al.*, 1992) and abused drugs (Di Chiara and Imperato, 1988; Koob and Le Moal, 2008; Volkow *et al.*, 2011). Voluntary wheel running is a natural reward (Brene *et al.*, 2007) and is found to influence the mesolimbic dopamine pathway in a way similar to addictive drugs (Werme *et al.*, 2000; Werme *et al.*, 2002b). For

instance, long-term voluntary wheel running is accompanied by plasticity changes in the mesolimbic reward pathway such as reduction of dopamine D2 receptors in the nucleus accumbens core (Greenwood *et al.*, 2011), possibly *via* exercise-enhanced dopamine release. Similarly, morphine-dependent rats exhibited a low level of D2 receptors in the caudate-putamen and the nucleus accumbens core (Georges *et al.*, 1999). Dopamine D2 receptors have been shown to have a significant involvement in the development of drug addiction (Volkow *et al.*, 2002). Moreover, D2 receptor blockade by sulpiride (a D2/D3 receptor antagonist) attenuates self-administration of muscimol (a GABA A receptor agonist) and morphine (David *et al.*, 2002; Gavello-Baudy *et al.*, 2008). In the present study, sulpiride was used to block dopamine D2 receptors because it showed more selectivity on mesolimbic than nigrostriatal dopamine pathways (White and Wang, 1983) and, therefore, the decrease in the running performance would reflect the lack of motivation to run. Also, sulpiride in the doses used in this study showed no inhibitory effects on spontaneous motor activity (Simon *et al.*, 2000; Hidaka *et al.*, 2010).

4.2 Hypothesis

The present experiment used pharmacological approaches to test the hypothesis that the rewarding and cognitive effects of repeated 1-hour/day voluntary wheel running sessions are mediated *via* the activation of GRs and/or D2 receptors**.**

4.3 Materials and methods

4.3.1 Animals

Eighty three 6-8-week-old male C57BL/6J mice were purchased from Charles River UK. Mice were individually housed and left undisturbed for a week to acclimatize to the animal holding room. Standard environmental conditions of temperature (20 – 22 °C), humidity (55 – 60 %), ventilation and 12:12 light/dark cycle (lights on at 7:00 am) were maintained throughout the experiment. Mice had unlimited access to food and water. Body weights (BW) of mice were recorded weekly at the time of cage cleaning. All experimental

procedures and animal care were performed in accordance with the UK Animal Scientific Procedures Act 1986 under project licence 40/3283.

4.3.2 Drugs

4.3.2.1 Mifepristone

Mifepristone (MIF, RU-38486) was purchased from Sigma-Aldrich UK and kept at 2-8 °C. Immediately before use, MIF was freshly suspended in vehicle A, 1% Tween 80 in saline (Mesripour *et al.*, 2008), and sonicated for ~30 sec on ice. MIF (30 mg/kg, i.p.) was injected 2 hours before the running sessions. The chosen dose was reported to attenuate cocaine self-administration in C57BL/6J mice (Fiancette *et al.*, 2010). For the vehicle-treated group, vehicle A was also sonicated for ~30 sec on ice.

4.3.2.2 (±)-Sulpiride

(±)-Sulpiride was bought from Sigma-Aldrich UK and freshly dissolved in vehicle B (1 % acetic acid/saline) and the pH of the solution was then neutralised to 7 by 1M NaOH. Sulpiride (25 or 50 mg/kg, i.p.) was injected 30 minutes before the running sessions. The sulpiride 50 mg/kg (Sul50) was reported to reduce reinforcing properties of abused drugs such as morphine and muscimol (a GABA A receptor agonist) in mice (David *et al.*, 2002; Le Merrer *et al.*, 2007; Gavello-Baudy *et al.*, 2008). The sulpiride 25 mg/kg (Sul25) was previously used to examine the involvement of dopamine D2 receptors in the rewarding food-induced locomotor hyperactivity (Le Merrer and Stephens, 2006).

4.3.3 Experimental design

The experimental timeline is described in Fig 4.1. After acclimatization, all mice were subjected to a 2-week training phase of 1-hour/day (5 days/week) voluntary wheel running sessions to ensure that they were motivated to use wheels, and it was critical to verify that effects of drugs were not due to a lack

of motivation to run. Based on the pilot studies described in Chapter 3, mice were assigned to running groups if they ran over 0.3 km per 1-hour session for three consecutive running sessions during the training period. After mice met these criteria, their baseline memory performance was evaluated on Day 18 in the spontaneous alternation test (SAT) using a T-maze. Mice were assigned to two main groups, sedentary and running, and mice which demonstrated no motivation to run during the training period were excluded from the running groups. Mice were subdivided into 4 groups (n = 8-9): vehicle (A, n = 4-5 and B, n = 4, i.p.), MIF (30 mg/kg, i.p), Sul25 (25 mg/kg, i.p.) and Sul50 (50 mg/kg, i.p.). Each sedentary mouse had access to a static wheel during the running session. One more running group was added in which mice were not injected to assess injection-related stress on the running performance. A vehicle-treated no-wheel group was added in which mice were not offered access to a static wheel to evaluate the impact of this environmental enrichment on cognition. Mice underwent 4 weeks of pharmacological treatment and running sessions. On Days 49-52, mice were examined in the SAT, novel object recognition test (NORT) and object location test (OLT); all methods are described in detail in Chapter 2-Part2. On Day 56, mice were treated exactly as before the memory tests and had their final running session. Immediately after, mice were sacrificed, trunk blood was collected and plasma was kept at -80 °C for the determination of plasma corticosterone levels.

4.3.4 Plasma corticosterone levels (ng/ml)

Circulating corticosterone levels were determined using a commercial ELISA kit. Details are given in Chapter 2-Part 1.

4.3.5 Statistical analysis

Data are presented as mean ± SEM. Averaged running distances of the 4 treated running groups during the last 3 days of the training phase (during which mice ran over 0.3 km per 1-hour session) were used to analyse differences between the pre-treatment running performance of mice and their running activity during

each of the following 4 weeks of the treatment X exercise phase. Mice were assigned to running groups according to their running and spontaneous alternation performances during the training phase, to control for possible confounding effects of variability in performance between the experimental groups. Two-way ANOVAs with repeated measures; with group as the between subject factor (4 levels) or (2 levels, non-injected and vehicle-treated groups) and time as the within subject factor (5 levels, training and 4 weeks of treatment), was used to analyse the running distances (km) of mice during the repeated 1-hour voluntary wheel running sessions. The unpaired *t*-test was used to analyse running distances of the non-injected and vehicle-treated running groups during the final session. In addition, separate one way ANOVAs with repeated measures were performed to demonstrate time effects of Sul25 or Sul50 on repeated running sessions. A one way ANOVA was used to analyse the running distances of mice during the final session prior to sacrifice, with groups (4 levels) as the between subject factor.

Two-way ANOVAs were used to analyse plasma corticosterone levels, total distance travelled, measures of anxiety-related behaviour during the habituation session in the open field, recognition index (RI) and location index (LI), with exercise (2 levels, sedentary and running) and groups (4 levels) as the between subject factors. Three-way ANOVAs with repeated measures were used to analyse the percentage of correct alternation, BW, and the total exploration times during the novel object recognition and location memory tests, with exercise (2 levels) and groups (4 levels) as the between subject factors, and $time_{SAT}$ (2 levels, before and after), $time_{BW}$ (8 levels, weeks) or trials (2 levels, acquisition and retention) as the within subject factor. Two-way ANOVAs with repeated measures were used to analyse the total exploration times during NORT and OLT, the percentage of correct alternation and BW, and unpaired *t*-tests were used to analyse plasma corticosterone levels, RI and LI between the vehicle-treated sedentary and no-wheel groups and between the non-injected and vehicle-treated running groups. Post-hoc planned comparisons and paired sample *t*-tests were used as required. Paired sample *t*-tests were also used to compare the exploration times between the familiar and the novel objects and

between the non-displaced and the displaced objects in each group. One sample *t*-tests were used to compare the percentage of correct alternation, RI and LI to 50%. Pearson's correlations were calculated when required. An effect was considered significant when P was ≤0.05.

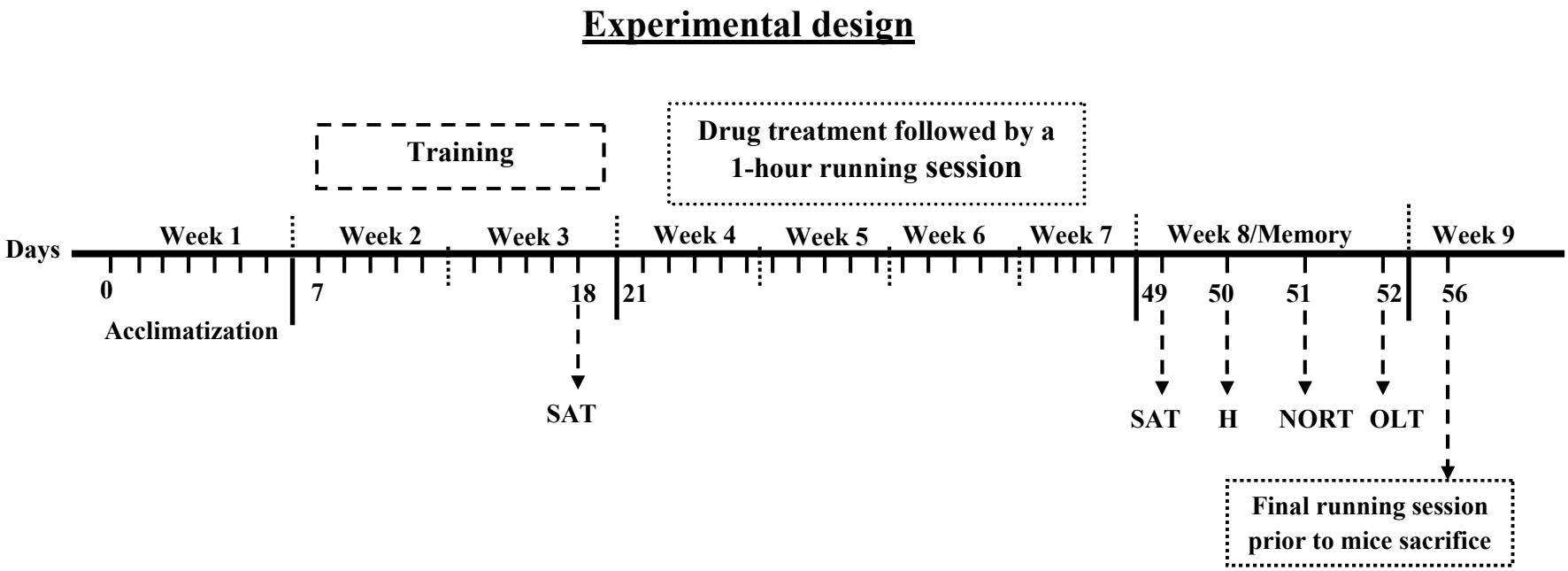

Figure 4.1 The experimental design. After one week of acclimatization, mice were trained over two weeks to meet the running criteria (over 0.3 km per 1-hour session for 3 consecutive sessions). Then, mice (n = 8-9 / group) underwent 4 weeks (5 days/week) of treatment with vehicle (A and B, i.p.), MIF (30 mg/kg, i.p.) or sulpiride (25 or 50 mg/kg, i.p.) followed by a 1-hour voluntary wheel running session. Corresponding sedentary groups were treated exactly as running groups except that they had access to static wheels during the exercise sessions. In week 8, mice were examined in the spontaneous alternation test (SAT), a 30-minute habituation (H) session in the open field, the novel object recognition test (NORT) and the object location test (OLT). Prior to sacrificing mice, on Day 56, mice were treated in the same way as before and had their final running session.

4.4 Results

4.4.1 Running distances (km)

The two-way ANOVA with repeated measures revealed significant main effects of groups ($F_{(3,31)} = 8.54$, $P < 0.001$), time ($F_{(4,124)} = 11.56$, $P < 0.001$) and time X group interaction ($F_{(12,124)} = 5.61$, $P < 0.001$). Planned comparisons showed no differences in the running performance of MIF or sulpiride-treated groups compared to vehicle-treated groups ($P_{MIF} = 0.70$, $P_{Sul25} = 0.95$, $P_{Sul50} = 0.92$) during training, indicating equal distribution of mice performance amongst all groups. Moreover, paired sample *t*-tests showed that the running performance of vehicle-treated mice was unaltered over 4 weeks of treatment (week1: $t = 0.45$, $P = 0.67$; week2: $t = 0.64$, $P = 0.54$; week3: $t = 0.28$, $P = 0.79$; week4: $t = 0.23$, $P = 0.82$) compared to the training performance, Fig. 4.2. This suggests that running mice were motivated to maintain their running performance over all weeks. Although there were no significant differences in the running performance of MIF-treated mice compared to the vehicle-treated mice (planned comparisons: $P > 0.05$, over all weeks), they ran less than their training level during the first week of treatment (week1: $t = 2.67$, $P = 0.028$; week2: $t = 0.002$, $P = 0.99$; week3: $t = 0.54$, $P = 0.61$; week4: $t = 0.08$, $P = 0.94$), Fig. 4.2. Sul25 reduced running distances of mice over most weeks of treatment compared to their training performance (week1: $t = 2.67$, $P = 0.028$; week2: $t = 1.70$, $P = 0.13$; week3: $t = 2.94$, $P = 0.019$; week4: $t = 3.08$, $P = 0.015$), and planned comparisons showed also a decrease in the mice's running distances during the third ($P = 0.037$) and fourth ($P = 0.024$) weeks of treatment compared to the vehicle-treated mice, Fig. 4.2. In contrast, Sul50 markedly lowered running distances of mice compared to the vehicle-treated group ($P < 0.001$, over all weeks) and to the mean distance which they ran during the training phase (week1: $t = 4.85$, $P = 0.002$; week2: $t = 4.24$, $P = 0.004$; week3: $t = 6.13$, $P = 0.00048$; week4: $t = 6.95$, $P = 0.00022$), Fig. 4.2. Moreover, the one way ANOVA detected a significant group effect during the final exercise session ($F_{(3,31)} = 3.27$, $P = 0.034$), and planned comparisons revealed that Sul50-treated mice exhibited decreased running distances ($P = 0.005$) compared to vehicle-treated mice, Fig 4.2. To show time effects of sulpiride on the running performance of mice over repeated sessions, separate ANOVAs with repeated

measures revealed significant day effects by Sul25 ($F_{(21, 168)} = 1.86$, P = 0.017) and Sul50 ($F_{(21, 147)} = 3.24$, P <0.001), and paired sample *t*-tests were used to show significant effects on separate days, Fig. 4.3. Averaged running distances were not significantly different between the non-injected and vehicle-treated groups ($F_{(1,15)} = 0.07$, P = 0.79), and the unpaired *t*-test also showed no significant difference in the running activity of the two groups during the final running session ($t_{(15)} = 1.06$, P = 0.31), table 4.1.

Table 4.1 Distances (km) run by mice in the non-injected group (n = 8) compared to the vehicle-treated group (n = 9) during the 3-day training phase, 4 weeks of treatment and final running session. Data are presented as mean ± SEM

Time	Groups	
	Non-injected	**Vehicle**
Training	0.603 ± 0.025	0.735 ± 0.052
Week1	0.674 ± 0.082	0.699 ± 0.080
Week2	0.778 ± 0.126	0.784 ± 0.060
Week3	0.771 ± 0.103	0.759 ± 0.081
Week4	0.773 ± 0.102	0.754 ± 0.076
Final session	0.654 ± 0.083	0.759 ± 0.058

4.4.2 Plasma corticosterone levels (ng/ml)

The two-way ANOVA revealed significant main effects of exercise ($F_{(1,58)} = 26.85$, P <0.001) and groups ($F_{(3,58)} = 2.88$, P = 0.043), but there was no significant exercise X group interaction effect ($F_{(3,58)} = 2.24$, P = 0.09). Post-hoc planned comparisons indicated that vehicle- and MIF-treated running mice had higher corticosterone levels than the corresponding sedentary group (P <0.001,

in all case), Fig. 4.4. In contrast, Sul25 and Sul50 attenuated exercise-induced corticosterone rise in the running mice (planned comparisons: P = 0.11 and P = 0.33, respectively) compared to their respective sedentary controls. Also, Sul25 (P = 0.027) and Sul50 (P <0.001) reduced corticosterone levels in exercising mice compared to vehicle-treated exercising mice, Fig. 4.4. However, there were no alterations in plasma corticosterone levels between any of the sedentary groups (P >0.05, in all case). Also, there were no differences in plasma corticosterone levels between the vehicle-treated sedentary and no-wheel groups ($t_{(14)}$ = 0.48, P = 0.64) and between the non-injected and vehicle-treated exercising groups ($t_{(15)}$ = 0.55, P = 0.59), see table 4.2.

4.4.3 Spontaneous alternations

The three-way ANOVA with repeated measures showed no significant main effects of exercise ($F_{(1,59)}$ = 0.05, P = 0.82) and groups ($F_{(3,59)}$ = 0.56, P = 0.64), but there was a significant time effect ($F_{(1,59)}$ = 16.78, P <0.001). There were also no significant main effects of exercise X group interaction ($F_{(3,59)}$ = 0.25, P = 0.86), time X exercise interaction ($F_{(1,59)}$ = 0.18, P = 0.67), time X group interaction ($F_{(3,59)}$ = 1.36, P = 0.27) and time X exercise X group interaction ($F_{(3,59)}$ = 0.56, P = 0.65). Paired sample *t*-tests revealed improvement of the spontaneous alternation performance only in Sul50-treated sedentary mice (t = 2.97, P = 0.021) and Sul25-treated running mice (t = 3.54, P = 0.008) compared to prior treatment performance, Fig. 4.5. Similarly, one sample *t*-tests comparing the percentage of correct alternation to 50% (chance) showed enhancements of the spontaneous alternation performance after treatment (Sul25: t = 3.16, P = 0.013; Sul50: t = 2.76, P = 0.028), Fig. 4.5. There were no differences between the vehicle-treated sedentary and no-wheel groups ($F_{(1,14)}$ = 0.02, P = 0.88) or between the non-injected and vehicle-treated running mice ($F_{(1,15)}$ = 0.02, P = 0.90), table 4.2.

Table 4.2 Plasma corticosterone levels (ng/ml), after the final running session, and % of correct alternation (before and after treatment) of mice (n = 8-9) in the no-wheel and non-injected groups compared to the vehicle-treated sedentary and running groups, respectively. Data are presented as mean ± SEM. ***P <0.001 compared to vehicle-sedentary mice.

Variable		Group			
		Vehicle-Sedentary	No-wheel	Vehicle-Running	Non-injected running
Plasma corticosterone levels (ng/ml)		63.80 ± 5.66	60.24 ± 4.94	106.05*** ± 7.52	100.01 ± 8.08
Percentage of correct alternation	Before	45.31 ± 7.45	43.75 ± 9.45	45.83 ± 8.84	45.31 ± 11.56
	After	62.50 ± 11.08	60.94 ± 7.26	62.50 ± 6.59	60.94 ± 6.44

4.4.4 Locomotor activity and anxiety-related behaviour during the habituation session

For the total distances travelled in the arena, there was a significant main effect of groups ($F_{(3,59)}$ = 3.53 P = 0.02), but no significant main effects of exercise ($F_{(1,59)}$ = 3.35, P = 0.07) or exercise X group interaction ($F_{(3,59)}$ = 0.12, P = 0.95). Post-hoc planned comparisons showed that Sul25-treated running mice moved shorter distances (P = 0.031) during the habituation session than vehicle-treated running mice, table 4.3. On the other hand, measures of anxiety-like behaviour were not significantly altered between groups. Firstly, percent distance moved in the central zone relative to total was not significantly altered by exercise ($F_{(1,59)}$ = 0.90, P = 0.35), groups ($F_{(3,59)}$ = 0.66, P = 0.58) and exercise X group interaction ($F_{(3,59)}$ = 0.01, P = 0.999), table 4.3. Also, there were no significant main effects of exercise ($F_{(1,59)}$ = 0.57, P = 0.45), groups

($F_{(3,59)}$ = 0.80, P = 0.50) and exercise X group interaction ($F_{(3,59)}$ = 0.09, P = 0.97) on percent time spent in the central sector relative to total, table 4.3. Similarly, the number of defecations were not affected by exercise ($F_{(1,59)}$ = 0.11, P = 0.74), groups ($F_{(3,59)}$ = 0.53, P = 0.67) and exercise X group interaction ($F_{(3,59)}$ = 1.21, P = 0.31), table 4.3. Moreover, locomotor activity and anxiety-like behaviour did not differ between the vehicle-treated sedentary and no-wheel groups (total distances: $t_{(14)}$ = 0.001, P = 0.999; % distance: $t_{(14)}$ = 1.13, P = 0.28; % time: $t_{(14)}$ = 0.84, P = 0.42; number of defecations: $t_{(14)}$ = 0.44, P = 0.66) and between the non-injected and vehicle-treated running mice (total distances: $t_{(15)}$ = 0.03, P = 0.97; % distance: $t_{(15)}$ = 0.15, P = 0.86; % time: $t_{(15)}$ = 0.41, P = 0.69; number of defecations: $t_{(15)}$ = 0.71, P = 0.49), see table 4.3

Table 4.3 Locomotor activity and variables of anxiety-like behaviour of mice (n = 8-9 / group) during the 30-minute habituation session in the open field, which was undertaken 24 hours before the novel object recognition test Data are expressed as mean ± SEM. Planned comparisons: *P <0.05, compared to vehicle-treated running mice. MIF = mifepristone (30 mg/kg, i.p.), Sul25 = sulpiride (25 mg/kg, i.p.), Sul50 = sulpiride (50 mg/kg, i.p.)

Group	Total distance travelled (km)	Percent distance	Percent time	Defecation number
No-wheel	82.06 ± 5.33	13.39 ± 0.97	10.70 ± 1.21	5.00 ± 0.46
Non-injected	88.73 ± 4.65	15.36 ± 0.72	11.88 ± 0.85	5.63 ± 1.10
Vehicle-Sedentary	82.07 ± 4.20	14.88 ± 0.89	11.96 ± 0.89	4.63 ± 0.71
MIF-Sedentary	83.82 ± 2.55	14.66 ± 0.97	11.38 ± 1.42	4.88 ± 1.11
Sul25-Sedentary	72.69 ± 2.83	14.60 + 1.12	12.24 ± 1.35	4.00 ± 0.89
Sul50-Sedentary	75.59 ± 1.65	15.82 ± 0.77	14.09 ±1.48	5.13 ± 0.72
Vehicle-Running	88.50 ± 4.94	15.55 ± 0.98	12.57 ± 1.40	4.22 ± 0.72
MIF-Running	88.23 ± 4.09	15.17 ± 1.07	12.69 ± 1.18	3.33 ± 0.71
Sul25-Running	75.87* ± 5.74	15.40 ± 0.99	13.37 ± 1.69	5.44 ± 0.87
Sul50-Running	83.41 ± 4.93	16.51 ± 1.04	14.06 ± 1.70	4.88 ± 0.52

4.4.5 The novel object recognition test

The three-way ANOVA with repeated measures showed no significant main effects of exercise ($F_{(1,59)}$ = 0.05, P = 0.82) and trials ($F_{(1,59)}$ = 0.60, P = 0.44), but there was a significant group effect ($F_{(3,59)}$ = 10.73, P <0.001). Also, there were no significant main effects of exercise X group interaction ($F_{(3,59)}$ = 1.97, P = 0.13), trial X exercise interaction ($F_{(1,59)}$ = 0.11, P = 0.74), trial X group interaction ($F_{(3,59)}$ = 2.02, P = 0.12) and trial X exercise X group interaction ($F_{(3,59)}$ = 1.09, P = 0.36). Post-hoc planned comparisons revealed that running mice treated with Sul25 (P = 0.002) or with Sul50 (P <0.001) spent a longer time exploring the single object during the acquisition trial compared to vehicle-treated exercising mice, table 4.4. Sul50 also increased the total object exploration times of exercising mice (P = 0.008) during the retention trial compared to vehicle-treated exercising mice, table 4.4. Moreover, there were significant object effects during the retention trial ($F_{(1,59)}$ = 104.58, P <0.001). Post-hoc paired sample *t*-tests showed that all exercising and sedentary groups preferred to explore the novel object to the familiar one (P <0.05, in all case), table 4.4. Furthermore, the two-way ANOVA analysing RIs revealed significant main effects of exercise ($F_{(1,59)}$ = 4.42, P = 0.04) and groups ($F_{(3,59)}$ = 3.13, P = 0.032), but there was no exercise X group interaction effect ($F_{(3,59)}$ =1.75, P = 0.17). Planned comparisons indicated that MIF (P = 0.044) and Sul50 (P = 0.05) reduced RIs of sedentary mice compared to vehicle-treated non-exercising mice, Fig. 4.6. Also, Sul25-treated running mice had a lower RI than vehicle-treated running mice (P = 0.01) and their respective sedentary mice (P = 0.005), Fig. 4.6. One sample *t*-tests comparing RIs of all groups to 50% (no discrimination) indicated that all groups had a higher RI than 50% (P <0.05, in all case), Fig. 4.6. Similarly, the no-wheel and non-injected groups explored the novel object for a longer time than the familiar object (P = 0.026, P = 0.025, respectively), and their recognition indices were higher than 50% (P = 0.022, P = 0.023, respectively), table 4.4.

Also, the two-way ANOVA with repeated measures indicated a significant main group effect on the total exploration times between the vehicle-treated sedentary and no-wheel groups ($F_{(1,14)}$ = 20.43, P <0.001). Mice in the no-wheel group

spent a shorter time exploring objects during the acquisition and retention trials (planned comparisons: $P<0.001$ and $P = 0.008$, respectively) than vehicle-treated sedentary mice, table 4.4. Nevertheless, the unpaired *t*-test ($t_{(14)} = 0.72$, $P = 0.48$) showed no significant difference between RIs of the vehicle-treated sedentary (65.54 ± 1.99) and no-wheel (62.21 ± 4.17) groups. On the other hand, there were no significant differences in the total exploration times between the non-injected and vehicle-treated running mice ($F_{(1,15)} = 2.05$, $P = 0.17$), table 4.4. Also, RIs of the non-injected (59.54 ± 3.31) and vehicle-treated exercising mice (62.98 ±1.82) did not significantly differ ($t_{(15)} = 0.94$, $P = 0.36$).

Table 4.4 Exploration times (sec) of the familiar and novel objects during the retention trial and total exploration times (TET) during the 10-minute acquisition and retention trials of the novel object recognition test which was carried out 5 days after the last week of treatment. Data are expressed as mean ± SEM (n = 8-9 mice/group). Planned comparisons: **P <0.01 and ***P <0.001 compared to the vehicle-treated running group, ^{xx}P <0.01 and ^{xxx}P <0.001 compared to the vehicle-treated sedentary group; paired sample *t*-tests: ^{a}P <0.05, ^{b}P <0.01 and ^{c}P = 0.001 compared to the familiar object. MIF = mifepristone (30 mg/kg, i.p.), Sul25 = sulpiride (25 mg/kg, i.p.), Sul50 = sulpiride (50 mg/kg, i.p.).

Group		TET (acquisition)	Familiar object	Novel object	TET (retention)
No-wheel		34.94 ^{xxx} ± 7.13	16.38 ± 3.20	26.38 ^{a} ± 4.01	42.75 ^{xx} ± 6.34
Non-injected		84.25 ± 7.30	28.94 ± 4.33	40.56 ^{a} ± 4.24	69.50 ± 7.54
Sedentary	**Vehicle**	73.25 ± 5.83	23.44 ± 1.60	45.19 ^{c} ± 3.60	68.63 ± 3.93
	MIF	65.69 ± 5.26	26.94 ± 2.56	39.69 ^{a} ± 5.27	66.63 ± 6.79
	Sul25	78.75 ± 8.56	25.75 ± 1.82	46.88 ^{b} ± 4.20	72.63 ± 4.89
	Sul50	86.06 ± 5.31	34.69 ± 2.72	50.00 ^{a} ± 4.29	84.69 ± 5.84
Running	**Vehicle**	59.89 ± 9.09	23.17 ± 3.45	39.50 ^{c} ± 5.53	62.67 ± 8.59
	MIF	48.00 ± 6.27	24.50 ± 2.38	36.83 ^{a} ± 4.83	61.33 ± 6.34
	Sul25	90.06** ± 8.89	36.39 ± 3.62	44.00 ^{a} ± 4.60	80.39 ± 7.70
	Sul50	101.75***± 5.97	38.38 ± 3.26	51.13 ^{a} ± 5.17	89.50** ± 7.08

4.4.6 The novel object location test

The three-way ANOVA with repeated measures revealed significant main effects of exercise ($F_{(1,59)} = 20.24$, $P < 0.001$), groups ($F_{(3,59)} = 4.09$, $P = 0.011$) and trial X exercise interaction ($F_{(1,59)} = 5.89$, $P = 0.018$), but there were no significant main effects of trials ($F_{(1,59)} = 0.16$, $P = 0.69$) or exercise X group interaction ($F_{(3,59)} = 0.25$, $P = 0.86$). Also, there was no significant object displacement effect ($F_{(1,59)} = 2.92$, $P = 0.093$). Planned comparisons showed a significant increase in the total exploration times of Sul25 ($P < 0.001$) or Sul50 ($P = 0.003$) treated running groups during the acquisition trial compared to their corresponding sedentary mice, table 4.5. Also, Sul50 increased the total exploration times of exercising mice ($P = 0.038$) during the acquisition trial compared to vehicle-treated exercising mice, table 4.5. On the other hand, paired sample *t*-tests indicated that mice in all groups exhibited no preference for any of the two identical objects during the acquisition trial ($P > 0.05$, in all case). Similarly, mice did not spend more time exploring the displaced object compared to the non-displaced object during the retention trial ($P > 0.05$, in all case), table 4.5. Also, the two-way ANOVA revealed no significant main effects of exercise ($F_{(1,59)} = 3.00$, $P = 0.089$), groups ($F_{(3,59)} = 1.78$, $P = 0.16$) and exercise X group interaction ($F_{(3,59)} = 0.77$, $P = 0.52$) on the LIs of sedentary and running groups. Likewise, LI of each group was not significantly higher than 50% (no discrimination), as indicated by one sample *t*-tests ($P > 0.05$, in all case), Fig. 4.7. Moreover, the total exploration times did not differ significantly between the vehicle-treated sedentary and no-wheel groups ($F_{(1,14)} = 2.60$, $P = 0.13$) and between the non-injected and vehicle-treated running groups ($F_{(1,15)} = 0.06$, $P = 0.81$), table 4.5. However, mice in the no-wheel and non-injected groups demonstrated a significant preference for the displaced object to the non-displaced one ($P = 0.019$, $P = 0.024$, respectively), table 4.5. Likewise, LIs of mice in the no-wheel (65.83 ± 4.00) and non-injected (59.89 ± 3.44) groups were higher than 50% ($P = 0.005$ and $P = 0.024$, respectively). Nevertheless, unpaired *t*-tests showed no differences in LIs between the vehicle-treated sedentary and no-wheel groups ($t_{(14)} = 1.80$, $P = 0.094$) and between the non-injected and vehicle-treated running groups ($t_{(15)} = 0.52$, $P = 0.61$).

Table 4.5 Exploration times (sec) of the non-displaced and displaced objects during the retention trial and total exploration times (TET) during the 10 minute acquisition and retention trials of the novel object location test which was carried out 24 hours after the novel object recognition test. Data are expressed as mean ± SEM (n = 8-9 mice/group). Planned comparisons: *P <0.05 compared to vehicle-treated running mice, $^{\#\#}$P <0.01 and $^{\#\#\#}$P <0.001 compared to the corresponding sedentary group; paired sample *t*-tests: ^{x}P <0.05 compared to the non-displaced object. MIF = mifepristone (30 mg/kg, i.p.), Sul25 = sulpiride (25 mg/kg, i.p.), Sul50 = sulpiride (50 mg/kg, i.p.).

Group		TET acquisition	Non-displaced object	Displaced object	TET retention
No-wheel		39.00 ± 7.22	15.44 ± 2.70	30.13^{x} ± 4.91	45.56 ± 6.26
Non-injected		74.75 ± 10.05	25.63 ± 2.83	38.19^{x} ± 4.28	63.81 ± 5.80
Sedentary	**Vehicle**	51.75 ± 2.91	24.94 ± 2.81	30.63 ± 3.50	55.56 ± 3.92
	MIF	45.50 ± 1.90	22.75 ± 2.39	26.69 ± 2.11	49.44 ± 2.07
	Sul25	52.13 ± 4.08	26.00 ± 6.49	29.06 ± 3.56	55.06 ± 5.82
	Sul50	58.50 ± 5.05	33.75 ± 4.45	32.00 ± 1.40	65.75 ± 4.42
Running	**Vehicle**	66.89 ± 10.11	28.56 ± 3.06	37.89 ± 4.26	66.44 ± 5.97
	MIF	59.89 ± 4.70	31.06 ± 2.51	29.39 ± 2.37	60.44 ± 2.20
	Sul25	79.67 $^{\#\#\#}$ ± 5.57	37.50 ± 4.91	29.67 ± 3.19	67.17 ± 6.40
	Sul50	83.63* $^{\#\#}$ ± 9.46	43.94 ± 7.33	28.69 ± 4.01	72.63 ± 5.73

4.4.7 Body weight (gram)

The three-way ANOVA with repeated measures showed no significant main effects of exercise ($F_{(1,59)} = 1.76$, $P = 0.19$) and groups ($F_{(3,59)} = 1.86$, $P = 0.15$), but there was a significant time effect ($F_{(7,413)} = 175.51$, $P < 0.001$). Also, there were significant main effects of time X group interaction ($F_{(21,413)} = 8.89$, $P < 0.001$) and time X exercise X group interaction ($F_{(21,413)} = 1.95$, $P = 0.007$), but no significant exercise X group interaction effect ($F_{(3,59)} = 0.56$, $P = 0.64$) or time X exercise interaction effect ($F_{(7,413)} = 0.26$, $P < 0.97$) were detected. Planned comparisons indicated that there was significant weight gain in MIF-treated running group compared to MIF-treated sedentary group during week 1 ($P = 0.019$) and week 2 ($P = 0.047$), table 4.6, but this was MIF-independent as the treatment phase began from week 3 (Day 21). In contrast, there was significant weight loss in MIF-treated running mice during week 8 ($P = 0.036$) compared to vehicle-treated running mice, table 4.6. Also, MIF-treated sedentary mice showed significant weight loss during week 7 ($P = 0.037$) compared to vehicle-treated sedentary mice, table 4.6. On the other hand, BW of the no-wheel group did not differ from vehicle-treated sedentary mice ($F_{(1,14)} = 0$, $P = 0.95$), but there was a significant time effect ($F_{(7,98)} = 22.57$, $P < 0.001$), reflecting the normal weight gain of mice over time. Similarly, there was no group effect ($F_{(1,15)} = 0.83$, $P = 0.38$), but a significant time effect ($F_{(7,105)} = 59.42$, $P < 0.001$) was detected between the non-injected and vehicle-treated running mice.

4.4.8 Correlations

Interestingly, corticosterone levels of all exercising mice ($n = 42$) after the final session positively correlated with their running distances during the 4 weeks of treatment (week1: $r = 0.42$, $P = 0.005$; week2: $r = 0.46$, $P = 0.002$; week3: $r = 0.51$, $P = 0.001$; week4: $r = 0.55$, $P < 0.001$), Fig. 4.8. However, there was no correlation between corticosterone levels and distances run during the final session ($r = 0.25$, $P = 0.12$). Also, there was a positive correlation between running distances of Sul50-treated running mice and their LI during week1 ($n = 8$, $r = 0.93$, $P = 0.001$) and week 3 ($n = 8$, $r = 0.76$, $P = 0.027$)

Table 4.6 Body weight (gram) of mice in all groups (n = 8-9) from D 4 to D53. Data are expressed as mean ± SEM. Planned comparisons: $^{¥}P < 0.05$ compared to the MIF-treated sedentary group, *P <0.05 compared to the vehicle-treated sedentary group, $^{\#}P < 0.05$ compared to the vehicle-treated running group. Underlined weeks indicate the treatment phase

Group		Body weight (gram)							
		Week 1 (D 4)	Week 2 (D 11)	Week 3 (D 18)	Week 4 (D 25)	Week 5 (D 32)	Week 6 (D 39)	Week 7 (D 46)	Week 8 (D 53)
No-wheel		23.56 ± 0.58	24.49 ± 0.62	24.51 ± 0.61	24.75 ± 0.46	25.06 ± 0.45	25.39 ± 0.48	25.85 ± 0.48	25.30 ± 0.32
Non-injected		22.13 ± 0.63	23.60 ± 0.49	23.75 ± 0.42	24.26 ± 0.51	24.85 ± 0.52	25.46 ± 0.54	25.74 ± 0.51	25.51 ± 0.51
Sedentary	**Vehicle**	23.50 ± 0.88	23.75 ± 0.79	24.18 ± 0.76	24.80 ± 0.72	25.16 ± 0.56	25.69 ± 0.59	25.91 ± 0.68	25.50 ± 0.64
	MIF	22.65 ± 0.54	22.98 ± 0.65	23.81 ± 0.66	23.69 ± 0.65	23.66 ± 0.65	24.20 ± 0.56	24.23* ± 0.59	24.00 ± 0.45
	Sul25	23.63 ± 0.47	24.09 ± 0.36	24.46 ± 0.42	25.13 ± 0.42	26.04 ± 0.41	26.58 ± 0.42	26.88 ± 0.44	26.34 ± 0.52
	Sul50	22.91 ± 0.88	23.26 ± 0.80	24.25 ± 0.85	24.66 ± 0.86	25.60 ± 0.93	26.36 ± 0.94	26.89 ± 0.96	25.96 ± 0.81
Running	**Vehicle**	23.46 ± 0.56	23.67 ± 0.35	24.23 ± 0.23	24.60 ± 0.41	25.23 ± 0.34	25.76 ± 0.34	26.26 ± 0.38	26.18 ± 0.23
	MIF	24.49$^{¥}$ ± 0.38	24.53$^{¥}$ ± 0.35	24.72 ± 0.28	24.88 ± 0.34	25.14 ± 0.32	25.30 ± 0.37	25.59 ± 0.37	24.58$^{\#}$ ± 0.45
	Sul25	23.04 ± 0.84	23.68 ± 0.52	24.59 ± 0.55	25.58 ± 0.37	26.40 ± 0.37	26.88 ± 0.42	27.02 ± 0.41	26.56 ± 0.29
	Sul50	23.83 ± 0.54	24.68 ± 0.48	24.84 ± 0.50	25.23 ± 0.48	26.06 ± 0.45	26.58 ± 0.50	26.93 ± 0.51	26.18 ± 0.59

Running performance during repeated 1-hour voluntary wheel running sessions

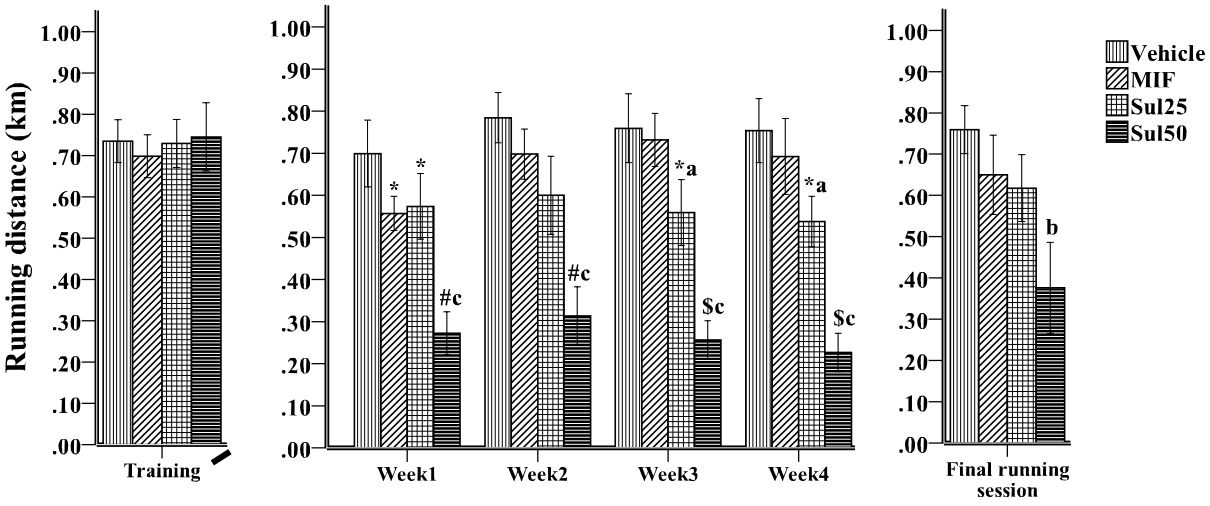

Figure 4.2 The distances (km) run by mice during the pre-treatment training phase, the 4-week exercise X treatment phase and the final running session. Male C57BL/6J mice (n = 8-9 / group) were treated with vehicle, MIF (mifepristone, a GR antagonist, 30 mg/kg, i.p.), Sul25 (sulpiride 25 mg/kg, a D2/3 antagonist) or Sul50 (sulpiride 50 mg/kg). Mice underwent repeated 1-hour/day (5 days/week) voluntary wheel running sessions during the training (3 days) and treatment phase (4 weeks) prior to memory tests. Before termination, mice had the final running session; 10 days after week4, after being treated exactly as during the treatment X exercise phase. Data are presented as mean ± SEM. Planned comparisons: $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.001$ compared to vehicle-treated mice. Paired sample *t*-tests: $*P < 0.05$, $^{\#}P < 0.01$ and $^{\$}P < 0.001$ compared to training.

Daily effects of sulpiride on running performance

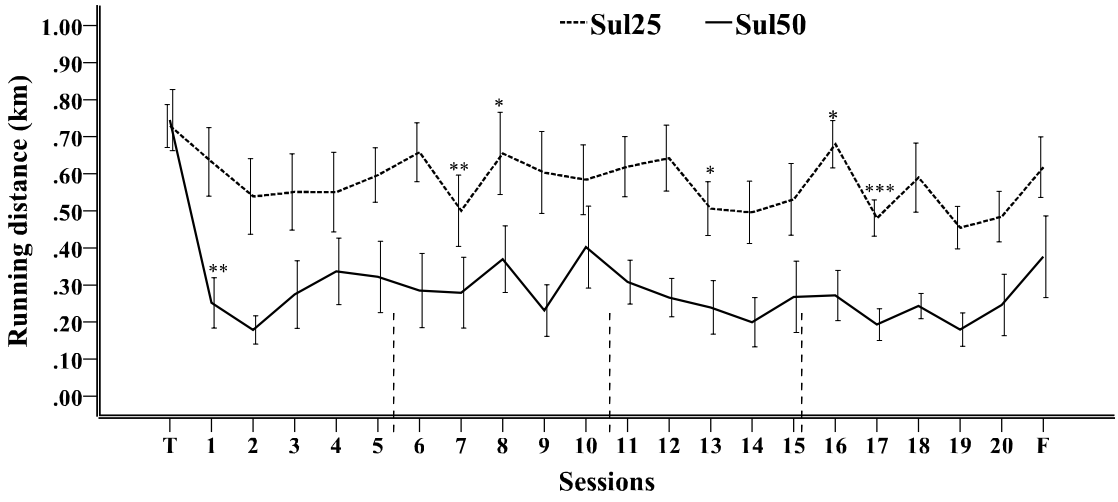

Figure 4.3 Effects of sulpiride (a D2/3 receptor antagonist) at two dose levels: Sul25 (25 mg/kg, i.p.) and Sul50 (50 mg/kg, i.p.), on the running distances of mice (n = 8-9 / group) during daily 1-hour sessions. T is for training and F is for final running session which was carried out 10 days after session 20. After training, mice had two exercise-free days (dotted lines) every 5 days of 1-hour/day running sessions. From session 13 on, running distances of Sul25-treated mice gradually decreased until session 15, then increased on session 16 (the start of the following week) and again slowly decreased until session 20, suggesting a withdrawal effects of Sul25 on D2 receptor expression in running mice. In, contrast, Sul50 decreased running distances from the first day and there were no significant differences between repeated sessions of the following weeks. Data are expressed as mean ± SEM. Paired sample *t*-tests: *P <0.05, **P <0.01 and ***P <0.001 compared to the prior session.

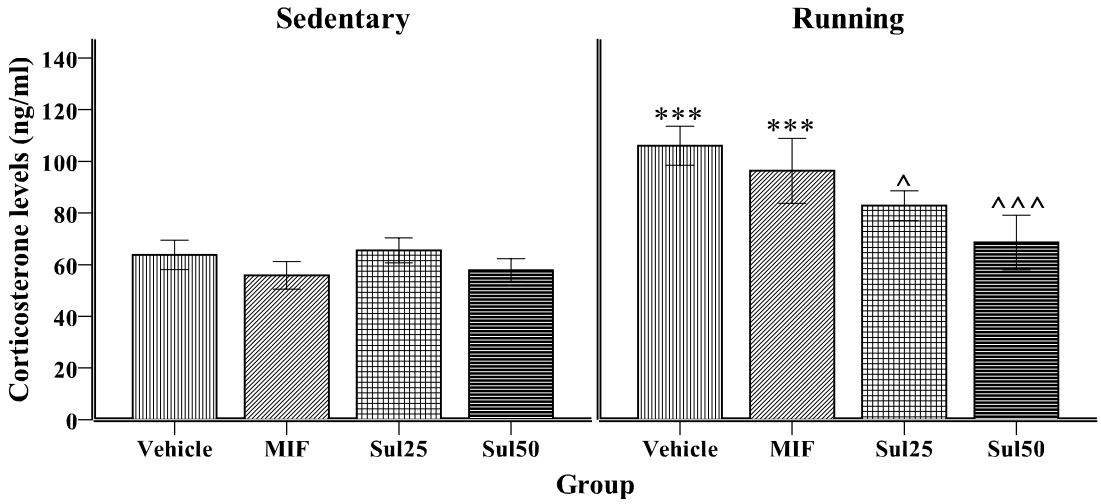

Figure 4.4 Plasma corticosterone levels (ng/ml). Immediately after the final running session, circulating corticosterone levels of sedentary and running mice (n = 8-9 / group) treated with vehicle, MIF (mifepristone, a GR antagonist, 30 mg/kg, i.p.), Sul25 (sulpiride 25 mg/kg, a D2/3 antagonist) or Sul50 (sulpiride 50 mg/kg) were measured in duplicate using a commercial ELISA kit. The exercise session was carried out 10 days after 4 weeks of (1-hour running session/day (5days/week) X treatment). Data are expressed as mean ± SEM. Planned comparisons: ***P <0.001compared to corresponding sedentary groups, ^P <0.05 and ^^^P <0.001 compared to the vehicle-treated running group.

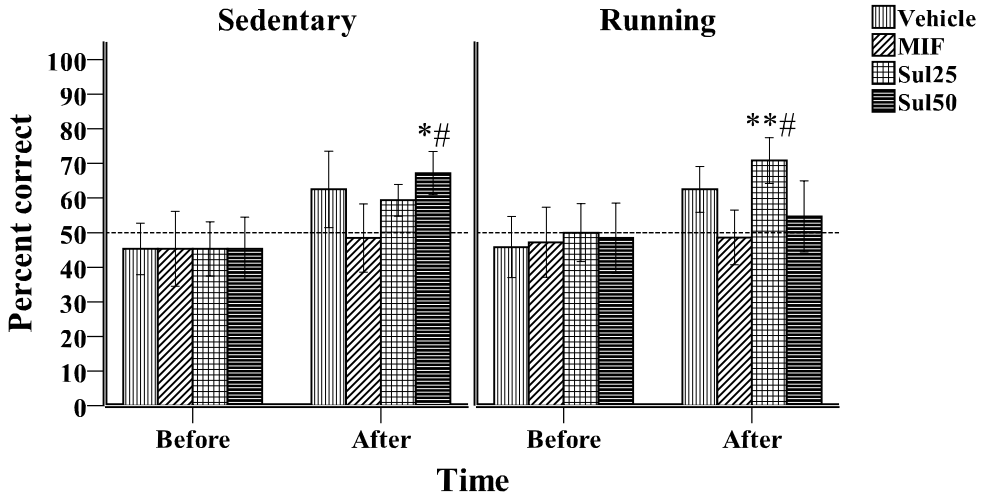

Figure 4.5 The percentage of correct alternations in a T-maze. Vehicle, MIF (mifepristone, a GR antagonist, 30 mg/kg, i.p.), Sul25 (sulpiride 25 mg/kg, a D2/3 receptor antagonist) and Sul50 (sulpiride 50 mg/kg) treated mice (n = 8-9 / group) were examined in a T-maze before and 3 days after the 4 week (exercise X treatment) phase. After 1 minute habituation, each mouse underwent a series of 9 consecutive trials testing its spontaneous alternation between the two arms of the T-maze. Data are expressed as mean ± SEM. Paired sample *t*-tests: *P <0.05 and **P <0.01 compared to before. One sample *t*-tests: $^{\#}$P <0.05 compared to 50% (chance).

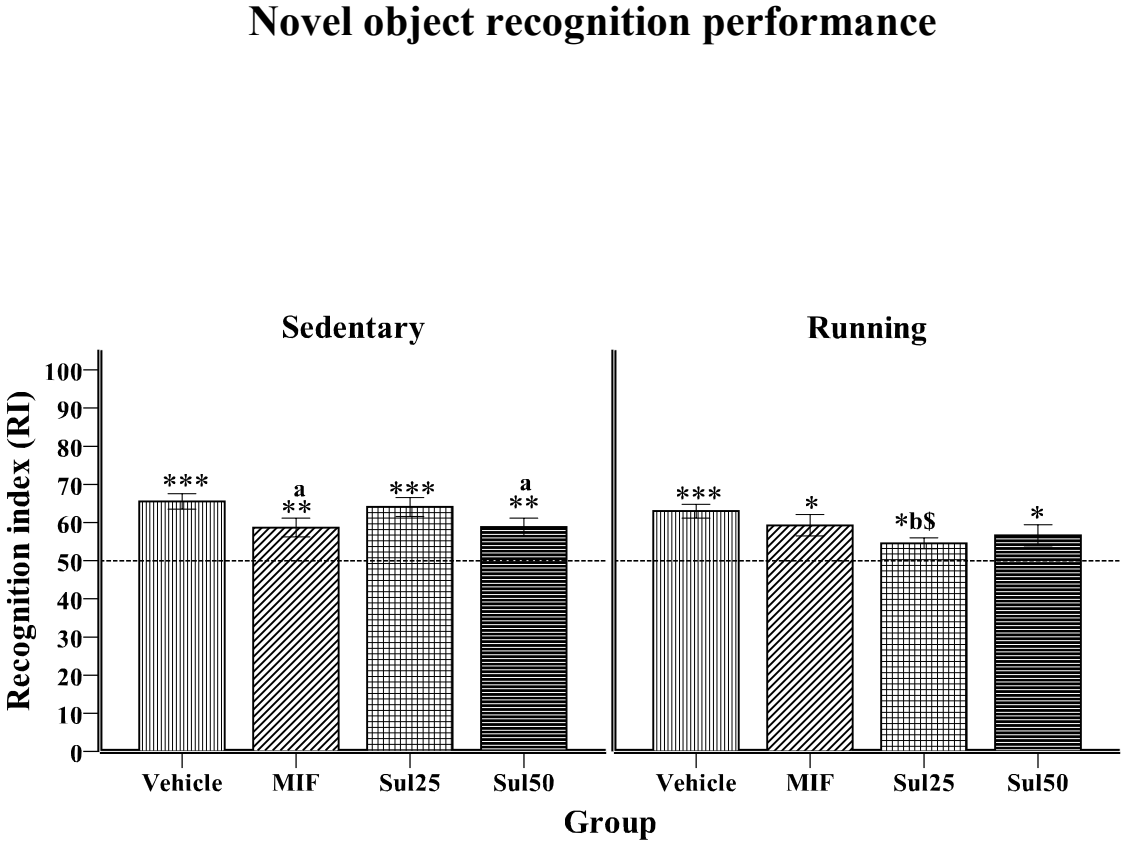

Figure 4.6 The recognition index. Vehicle, MIF (mifepristone, a GR antagonist, 30 mg/kg, i.p.), Sul25 (sulpiride 25 mg/kg, a D2/3 receptor antagonist) and Sul50 (sulpiride 50 mg/kg) treated mice (n = 8-9 / group) were subjected to two 10-minute trials (acquisition and retention) of the novel object recognition test with 4 hour inter-trial interval. Recognition index was calculated as % (novel object exploration time / total exploration time). Data are expressed as mean ± SEM. One sample *t*-tests: *P <0.05, **P ≤0.01 and ***P ≤0.001 compared to 50% (no discrimination), planned comparisons: ^{a}P ≤0.05 compared to vehicle-treated sedentary mice, ^{b}P = 0.01 compared to vehicle-treated running mice, $^{\$}$P <0.01 compared to Sul25-treated sedentary mice.

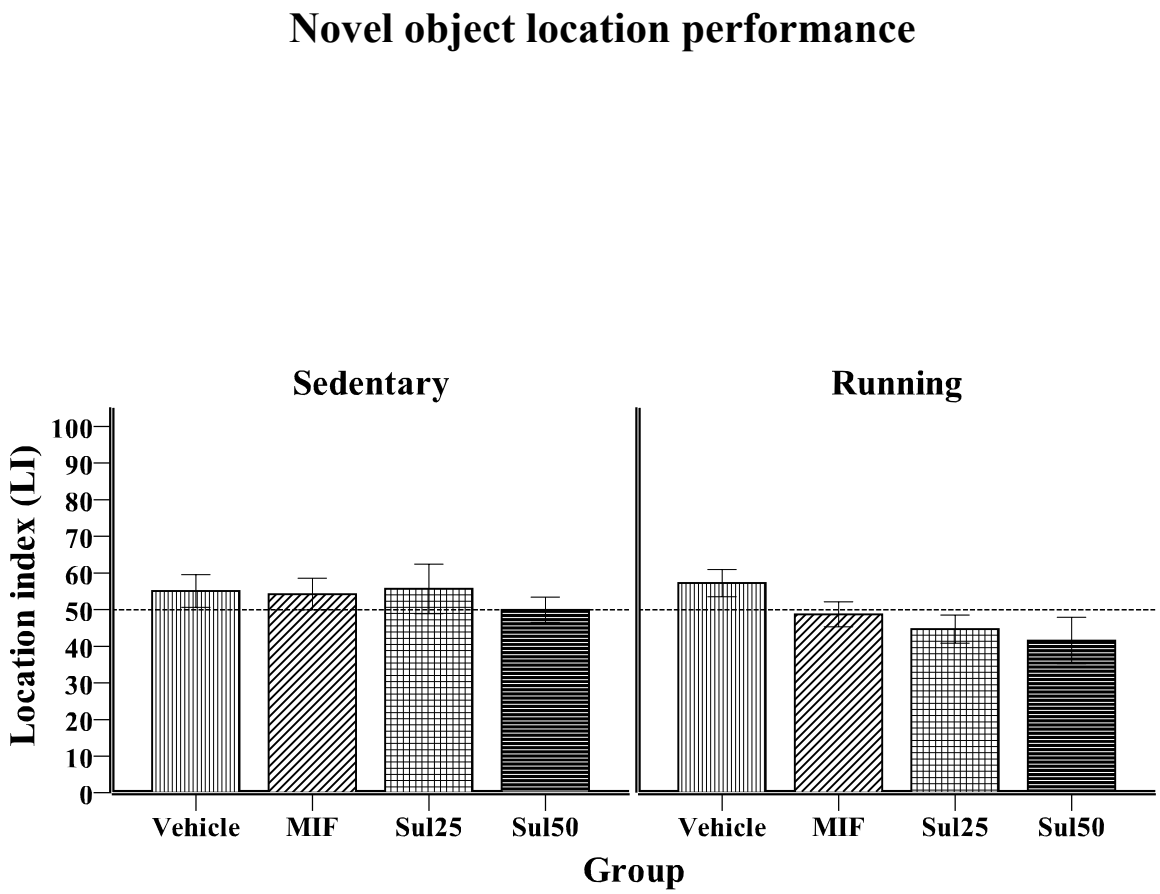

Figure 4.7 The location index. 24 hours after the novel object recognition test, sedentary and running mice (n = 8-9/group) treated with vehicle, MIF (mifepristone, a GR antagonist, 30 mg/kg, i.p.), Sul25 (sulpiride 25 mg/kg, a D2/3 receptor antagonist) or Sul50 (sulpiride 50 mg/kg) underwent acquisition and retention trials (10 minutes each) of the novel object location test with a 1-hour inter-trial interval. Location index was calculated as % (displaced object exploration time / total exploration time). Data are expressed as mean ± SEM.

Correlations between plasma corticosterone levels and running distances

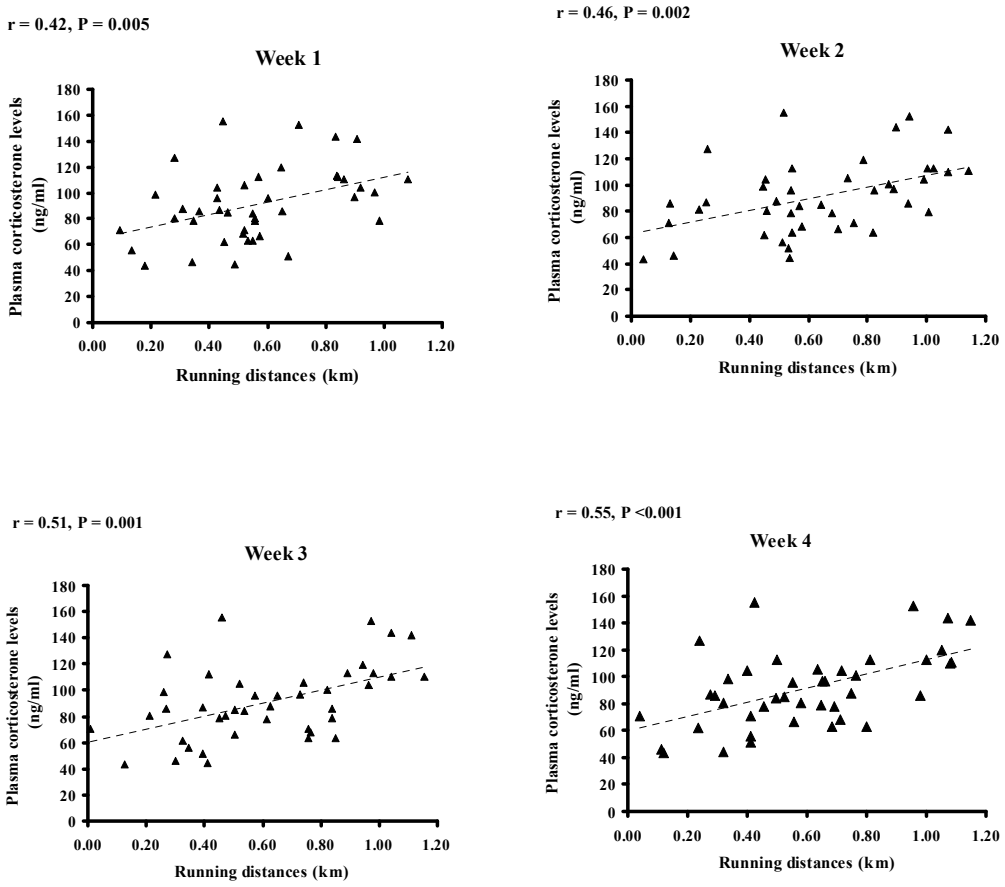

Figure 4.8 Pearson's correlations between plasma corticosterone levels (ng/ml), after the final exercise session, and mean running distances of all exercising mice (n = 42) during week 1, week 2, week 3 and week 4 of the treatment X exercise phase.

4.5 Discussion

The results of the present experiment demonstrate that although MIF did not significantly influence the running activity and/or corticosterone levels of exercising and sedentary mice compared to corresponding vehicle-treated mice, it only impaired recognition memory in sedentary mice, suggesting that repeated voluntary wheel running sessions protected against MIF-induced memory deficits. In contrast, D2 receptors did appear to be involved in the rewarding and cognitive effects of voluntary wheel running. Whereas the two sulpiride doses lowered running distances and corticosterone levels of mice, they had exercise-dependent opposing effects on mice's memory performance.

4.5.1 Running performance and corticosterone levels

First of all, there were no differences in corticosterone levels between any of the sedentary groups, indicating that none of the treatment conditions affected HPA axis activity of the sedentary mice. Also, the lack of significant differences in corticosterone levels between the vehicle-treated sedentary and no-wheel mice suggests that exploring the static wheel did not increase corticosterone levels. Moreover, it is important to point out that there were no differences in the running distances and corticosterone levels between the non-injected and vehicle-treated exercising mice, indicating negligible stressful effects of injection on the running performance and HPA axis activity.

The vehicle-treated exercising mice sustained high running activity which was not significantly different from their training level (0.73 km ± 0.05, per session on average) over all weeks, and displayed high plasma corticosterone levels (106.05 ng/ml ± 7.52) after the final session (10 days after the last week of treatment X exercise phase) compared to the vehicle-treated sedentary mice (63.80 ng/ml ± 5.66). In agreement with this, after 5 weeks of treadmill exercise (1hour/day, 5 days a week), male C57BL/6J mice showed a significant elevation of corticosterone levels (Chang *et al.*, 2008). Also, mice which ran continuously for 4 weeks exhibited higher circadian corticosterone peaks than sedentary mice, after they had no access to

running wheels (Droste *et al.*, 2003). Additionally, results of the chronic pilot study, Chapter 3-Part 1, showed that corticosterone levels of exercising mice, which were subjected to 5 weeks of the same exercise protocol used in the present experiment, increased after the final 1-hour wheel running session. This indicates that both continuous and intermittent wheel running effectively elevates corticosterone levels.

Results also revealed that MIF minimally decreased running distances of mice only during the first week of treatment compared to the training level. This suggests that the activation of GRs is not crucial for mice to maintain high running performance. MIF-treated mice ran almost as long distances as vehicle-treated mice during the pre-sacrifice session and subsequently exhibited higher plasma corticosterone levels than the corresponding sedentary mice. It is also important to emphasize that chronic exposure to MIF did not apparently influence negative feedback imposed by corticosterone on HPA axis, since there were no differences in corticosterone levels between the non-exercising MIF and vehicle treated mice, suggesting that the increase in corticosterone levels in MIF-treated exercising mice was secondary to their running activity and was not produced by disruption of HPA axis tone. In agreement with this, chronic voluntary exercise in the wheel was reported to induce HPA axis adaptation, as GR expression in the hippocampus of running rats or mice was not significantly different from sedentary groups (Droste *et al.*, 2003; Fediuc *et al.*, 2006). On the contrary, MIF treatment has been reported to disrupt HPA axis activity. For example, MIF blocked dexamethasone-, a GR agonist, induced cortisol suppression in healthy volunteers (Bertagna *et al.*, 1984).

It has been suggested that GRs mediate the rewarding effects of corticosterone. In agreement with this, rats self-administered corticosterone resulting in high levels comparable to those induced by stress (Piazza *et al.*, 1993), and it has been found that GRs are mainly activated by high corticosterone levels (Reul and de Kloet, 1985), suggesting a potential role of GRs in the rewarding effects of the stress hormone. A number of studies have also shown that GRs are important for drug-seeking behaviour. For instance, MIF attenuated cocaine-self administration in rats and mice (Deroche-Gamonet *et al.*, 2003; Fiancette *et al.*, 2010). Furthermore, MIF impaired morphine-induced CPP in rats (Dong *et al.*, 2006). Thus, although GRs have been

implicated in the rewarding effects of certain abused drugs, MIF did not attenuate running performance of mice.

In the present experiment, it is also important to distinguish between the acquisition and maintenance of the voluntary running behaviour. MIF did not have a significant influence on the running performance of mice which had already acquired the running behaviour during the training phase, but it is not known whether GR blockade could attenuate the initiation of this behaviour in running naïve mice. This may explain why MIF slightly decreased the running activity of mice during the first week of the treatment phase compared to the training level. In support of this, corticosterone treatment enhanced the acquisition of cocaine self-administration (Goeders and Guerin, 1996; Mantsch *et al.*, 1998), but stress-induced corticosterone elevation was not required for reinstatement of cocaine-seeking behaviour (Erb *et al.*, 1998). However in the results presented in Chapter 3-Part 2, metyrapone decreased the running distances of trained mice after a single session, and metyrapone slowly attenuated the running performance of Wistar rats over 10 days of unlimited access to running wheels (Hajisoltani *et al.*, 2011), suggesting that corticosterone regulates the long-term motivation to run in C57BL/6J mice probably *via* a GR-independent mechanism.

Our results also showed that both sulpiride doses attenuated the running performance of mice during some or all weeks of treatment, compared to the training levels and vehicle-treated running mice. Also, Sul50 reduced the running distances of mice, and, subsequently, attenuated exercise-evoked corticosterone increase during the final session, compared to vehicle-treated exercising mice. In contrast, Sul25 lowered corticosterone levels of running mice during the final session without a significant decrease in their running activity, compared to vehicle-treated running mice. It was reported that the D2 receptor agonist , quinpirole, activated the HPA axis and sulpiride abolished this stimulatory effect in Sprague-Dawley rats (Borowsky and Kuhn, 1992), suggesting that Sul25 had direct effects on HPA axis activity. However, it is unclear why Sul25-treated sedentary mice did not show a decrease in corticosterone levels compared to vehicle-treated sedentary mice. One explanation could be that Sul25 blocked mainly D2 autoreceptors (Hahn *et al.*, 2006), resulting in

enhancement of dopamine release (Jaworski *et al.*, 2001) and, subsequently, the running activity of mice increased (Cador *et al.*, 1993). Overall, results indicate that D2 receptor activation is needed for mice to sustain high running performance.

Sulpiride preferentially blocks D2-like receptors in particular D2 and D3 receptors (Vallone *et al.*, 2000). Sulpiride was selected because it was found to be more selective on dopamine receptors located in the mesolimbic dopamine pathway than those located in the nigrostriatal dopamine pathway (White and Wang, 1983), so that the decrease in the running performance of mice would reflect the motivation to run, not the inability to run. It has been established that nigrostriatal dopaminergic A9 neurons; which project from the substantia nigra to the dorsal striatum (caudate-putamen), regulate the motor function (Marsden, 2006). Also, sulpiride at the two selected doses attenuated locomotor hyperactivity induced by abused drugs or electric shock (Manzanedo *et al.*, 1999; Hidaka *et al.*, 2010), but they did not influence spontaneous motor activity (Manzanedo *et al.*, 1999; Simon *et al.*, 2000). Considerable research work has shown a pivotal role of D2 receptors in the development of drug addiction (Volkow *et al.*, 2002; Le Merrer *et al.*, 2007; Gavello-Baudy *et al.*, 2008). For example, morphine reduced mRNA expression for D2 receptors in the nucleus accumbens and caudate-putamen of rats (Georges *et al.*, 1999). Previous work also showed that long-term wheel running reduced the mRNA expression for D2 receptors in the nucleus accumbens core (Greenwood *et al.*, 2011), indicating both wheel running and abused drugs share common plasticity changes in the mesolimbic reward pathway. This also suggests that wheel running enhances dopamine release, resulting in D2 receptor downregulation. In line with this, dopamine release increased in the nucleus accumbens of rats running on treadmills (Freed and Yamamoto, 1985; Wilson and Marsden, 1995). Altogether, results strongly implicated D2 receptors in the rewarding effects of voluntary wheel running, which could be attenuated by blocking D2 receptors in the mesolimbic reward system or *via* direct effects on HPA axis activity.

D2 receptors are located at presynaptic and postsynaptic sites in the striatum, nucleus accumbens and olfactory tubercle (Marsden, 2006). Presynaptic D2 receptors are inhibitory autoreceptors so that dopamine release is eventually increased when these

receptors are blocked (Jaworski *et al.*, 2001; Hahn *et al.*, 2006). Small sulpiride doses were reported to act mainly at these receptors and enhance dopamine transmission in the striatum (Jaworski *et al.*, 2001) and amphetamine-induced stereotypy (Ljungberg and Ungerstedt, 1985), and these autoreceptors have been thought to be involved in tardive dyskinesia, involuntary movement of the orofacial region and other parts of the body (Marsden, 2006). Also, it has been reported that (-) sulpiride acts mainly on D2 autoreceptors (Rossi and Forgione, 1995) which regulate dopamine release (Marsden, 2006). Additionally, small doses (20 mg/kg) of sulpiride increased locomotor activity of rats, but high sulpiride doses (40 mg/kg) blocked morphine-induced locomotor hyperactivity (Manzanedo *et al.*, 1999), suggesting that small sulpiride doses enhance dopamine release which increases locomotor activity.

It is also important to point out that running distances of Sul25-treated mice significantly decreased only during the last two weeks of treatment but not during the final session which was carried out 10 days after the fourth week of treatment, compared to vehicle-treated exercising mice. As described in Fig. 4.3, repeated 2-day withdrawals of Sul25 indicated that mice ran longer distances at the start of the third and fourth weeks of treatment than the prior session and then their running distances decreased over the rest of these weeks' sessions. In contrast, Sul25-withdrawn running mice moved shorter distances in the open field during the habituation session than vehicle-treated exercising mice. This suggests that chronic Sul25 upregulated D2 receptors at presynaptic sites, resulting in reduced activation of D1 receptors *via* a decrease in dopamine release. On the other hand, Sul50 markedly decreased running distances of mice from the first day of treatment, as described in Fig. 4.3, and during the final session compared to vehicle-treated exercising mice, suggesting that Sul50 did not only block presynaptic D2 receptors, but also postsynaptic D2 receptors, and, therefore, upregulation of D2 receptors at both sites probably neutralised their inhibitory effects on postsynaptic D1 activation. In support of this, sulpiride-treated groups tended to move shorter distances during the habituation session and during the acquisition and/or retention trials of the object memory tests, data not shown.

It was reported that chronic sulpiride treatment resulted in upregulation of D2 receptors in the striatum (Motohashi *et al.*, 1992; Hurley *et al.*, 1996). Also, chronic

(-) sulpiride injection enhanced D2 autoreceptors expression in the substantia nigra of rats (Stefanini *et al.*, 1991). Moreover, chronic treatment with a high dose of sulpiride (100 mg/kg) was found to upregulate D2 receptors in the caudate-putamen and nucleus accumbens of rats (McGonigle *et al.*, 1989). Furthermore, imaging studies using pharmacological MRI and PET showed enhancement of D2 receptor sensitivity in the rat striatum subjected to 6-hydroxy dopamine (Nguyen *et al.*, 2000b), which is toxic to dopaminergic neurons. In fact, it was found that upregulation of D2 receptors was maintained after cessation of chronic D2 receptor blockade (Hurley *et al.*, 1996). Overall, it is suggested that repeated sulpiride injections upregulated D2 receptors and in particular Sul25 might have primarily affected presynaptic D2 receptors, which did not only influence the running activity of mice in the wheels, but it also reduced locomotor activity in the habituation/memory sessions.

Interestingly, the increase in corticosterone levels due to exercise in all groups was dependent on the running activity, since there was a direct relationship between corticosterone levels of exercising mice during the final session and their running distances over the 4 weeks of treatment, suggesting that running mice retained active HPA axis activity in response to wheel running. This also suggests that the observed individual variability in the motivation of mice to run may be related to their corticosterone response to wheel running. However, it is yet to be investigated whether corticosterone elevation was just a result of running or also promotes the motivation to run.

4.5.2 Memory performance

After the training phase, mice in all groups displayed no significant differences from chance in the spontaneous alternation test. The spontaneous alternation behaviour was found to reflect spatial short-term working memory performance (Sarter *et al.*, 1988), and rodents were found to alternate spontaneously above 50% (Dember and Fowler, 1958). It is important to point out that poor spatial alternation performance prior to treatment was unlikely to be related to exercise-induced corticosterone elevation during the short training phase, since sedentary mice which had no access to running wheels exhibited no significant alternation above the chance level prior to

accessing the static wheels, Chapter 3-Part 1. Nevertheless, the same mice strain C57BL/6J prior to corticosterone self-administration, Chapter 2-Part2, had a higher percentage of correct alternation than chance, suggesting that the performance level could be different between mice batches although Charles River UK was the source of mice used in all experiments. On the other hand, this suggests that the protocol used is sensitive enough to detect any possible improvement of the alternation performance induced by drug treatment.

In the second alternation test, there were no significant differences in the percentage of correct alternation between the vehicle-treated sedentary and running mice, suggesting no role of chronic intermittent exercise-evoked corticosterone elevation on the spontaneous alternation behaviour. Among all groups, sedentary Sul50 and running Sul25 treated mice demonstrated significant improvement in their spatial alternation rates compared to chance and the pre-treatment performance, although other groups excluding MIF-treated sedentary and running groups displayed slightly better performance during the post-treatment than the pre-treatment alternation tests. It was reported that acute sulpiride treatment did not rescue alternation impairment induced by repeated electric shocks (Hidaka *et al.*, 2010). However, a small sulpiride dose (10 mg/kg) abolished scopolamine-induced alternation impairment of mice in the Y-maze (Ukai *et al.*, 1998). In addition, a single methylphenidate (a dopamine reuptake inhibitor) injection dose-dependently ameliorated the spontaneous alternation impairment in juvenile stroke-prone spontaneously hypertensive rats, which were used as a model of attention deficit hyperactivity disorder (Ueno *et al.*, 2002), suggesting that dopamine neurotransmission is involved in this behaviour. Whereas Sul50 improved the spontaneous alternation performance of sedentary mice, running enhanced Sul25-induced improvement in the percentage of correct alternation. As mentioned earlier, it is suggested that D2 receptor upregulation accompanied chronic sulpiride treatment and this effect hypothetically controlled the degree of dopamine release and activation of postsynaptic D1 and D2 receptors. Also, results suggest that wheel running modified sulpiride effects on the alternation behaviour. It was found that activation of D1 receptors had an inverted U-shaped relationship with the cognitive performance (Goldman-Rakic *et al.*, 2000; Williams and Castner, 2006), suggesting that moderate activation of D1 receptors is required

for unimpaired memory. Overall, results indicate that running modified dopamine neurotransmission and this resulted in enhancement or impairment of moderate stimulation of D1 receptors.

Mice were also tested for their spatial memory performance in the novel object location test and all mice, except those in the no-wheel and non-injected groups, failed to show a significant preference for the object displaced in the novel location. It is unclear why mice in the previous two groups acquired the object location, since they exhibited no significant differences in the corticosterone levels and/or running performance compared to the vehicle-treated sedentary mice and to the vehicle-treated running mice, respectively. Interestingly, there was a positive correlation between LIs of Sul50-treated exercising mice and their running distances during some weeks of exercise, suggesting that the increase in the running performance at low levels, induced by Sul50, enhances spatial memory (van Praag *et al.*, 1999; van Praag *et al.*, 2005). It was reported that running at low levels improved memory performance, but extensive exercise induced memory deficits (Garcia-Capdevila *et al.*, 2009). A standard protocol was used to carry out the novel object location test (Murai *et al.*, 2007), and the inter-trial interval was made shorter (1 hour) than previously tested in order to help mice to acquire the task (Hikichi *et al.*, 2010). Moreover, it was reported that the inter-trial interval between the acquisition and retention trials was necessarily $\leq$ 2 hours (Murai *et al.*, 2007), as mice could not discriminate the displaced object if the retention trial was substantially delayed. Nevertheless, most groups did not seem to acquire the task, as vehicle-treated groups were not able to recognize the displaced object. It has to be emphasized that the performance in the object location test could be influenced by gender and breeding method. Whereas outbred male and female CD-1 mice showed significant location memory performance (Murai *et al.*, 2007; Hikichi *et al.*, 2010), only inbred male C57BL/6J mice; the same strain used in these experiments, exhibited significant preference for the displaced object (Frick and Gresack, 2003). Nonetheless, male C57BL/6 mice acquired the novel object location task using different and complex protocols which involved more than 2 objects, 2 trials and simultaneously tested the novel object recognition and location memory (Frick and Gresack, 2003; Dere *et al.*, 2005). Interestingly, our results showed that the two sulpiride doses increased the total exploration times of running mice during the

acquisition trial compared to corresponding sedentary groups, and Sul50-treated exercising mice also exhibited an increase in the total exploration times during the acquisition trial, compared to vehicle-treated running mice. However, LIs of sulpiride-treated running groups tended to be lower than the vehicle-treated group and their respective sedentary groups, suggesting that the increase in the exploration times is not an indicator for the memory improvement. Overall, the current results suggest that C57BL/6J mice need more complex protocols to acquire the object location task, although it could be useful to show improvement, as some corticosterone self-administered groups demonstrated significant performance in this memory test, Chapter 2-Part 2.

GRs were found to be important for spatial learning in the Morris water maze (Oitzl *et al.*, 1997) and, therefore, this might explain why MIF attenuated the slight improvement of the alternation rate of mice, which were observed in other groups, compared to the pre-treatment alternation test. However, a 60 minute pre-testing microinjection of corticosterone or a GR agonist (RU 28362) into the prefrontal cortex impaired the rewarded delayed-alternation performance in rats (Barsegyan *et al.*, 2010). In contrast, a post-training systemic corticosterone injection enhanced the novel object location memory of rats in a 24-hour retention test (Roozendaal *et al.*, 2010), suggesting that the alternation behaviour and the location memory are influenced differently by glucocorticoids. Previous work showed that chronic exposure to high corticosterone levels either had no effects on spontaneous alternation (Luine *et al.*, 1993; Coburn-Litvak *et al.*, 2003), or impaired this natural behaviour (Bardgett *et al.*, 1994; Coburn-Litvak *et al.*, 2003), as a result of negative effects on the hippocampus (Sapolsky *et al.*, 1985; Pavlides *et al.*, 1993); which was also found to be important for spatial working memory (O'Keefe and Dostrovsky, 1971; Conrad *et al.*, 1996; Eichenbaum *et al.*, 1999; Broadbent *et al.*, 2004).

Although spatial memory has been considered as a hippocampus-dependent task, a previous study reported that rats with poor alternation performance showed no impairment of hippocampal LTP (Ueno *et al.*, 2002); which was found to be correlated with the alternation rate (Nakao *et al.*, 2001), indicating the involvement of other brain regions in this behaviour. For example, the medial prefrontal cortex was

reported to be involved in the delayed alternation task and working memory performance (Barsegyan *et al.*, 2010). Thus, our results suggest that although chronic corticosterone elevation induced by exercise was not accompanied by significant improvement of the spontaneous alternation performance, chronic GR blockade prevented the slight increase in the percentage of correct alternation compared to other groups.

In the novel object recognition test, mice in all groups spent a longer time exploring the novel object than the familiar one during the retention trial which was performed 4 hours after the acquisition trial. RIs of sedentary and running vehicle-treated groups were not significantly different, and RIs of the no-wheel group also did not differ from the vehicle-treated sedentary mice, suggesting that repeated 1-hour running sessions or just exploring the static wheel were not sufficient to induce significant recognition memory improvement compared to the sedentary group. Therefore, the inability of the exercise protocol used in this experiment to improve memory of running mice could be either due to insufficient exercise in the light phase or chronic excessive running as a result of limited access to wheel running, both conditions were unable to improve memory (Shaw *et al.*, 2003; Holmes *et al.*, 2004; Garcia-Capdevila *et al.*, 2009).

Previous work showed that access (12-24 hours) to running wheels even for few days improved spatial memory (Ebrahimi *et al.*, 2010) and enhanced hippocampal BDNF levels (Shaw *et al.*, 2003) in rats. Also, running in the dark phase even for short distances abolished memory deficits in rats (Shaw *et al.*, 2003). Moreover, C57BL/6J had to run at least 3 hours at night to demonstrate significant improvement of hippocampal neurogenesis compared to non-exercising mice (Holmes *et al.*, 2004), and it was reported that hippocampal neurogenesis improved spatial (Dupret *et al.*, 2007) and recognition memory (Lafenetre *et al.*, 2010). All these findings suggest that there is a threshold in the exercise level which must be reached to enhance memory performance compared to sedentary animals.

Using the same exercise schedule as in the present study but on treadmills enhanced hippocampal neurogenesis and this was accompanied by corticosterone-induced MR

downregulation and BDNF upregulation in the hippocampus (Chang *et al.*, 2008), and it was found that enhanced neurogenesis and hippocampal BDNF levels were associated with improved memory performance (Dupret *et al.*, 2007; Lafenetre *et al.*, 2010). In the passive avoidance task, mice exercising for 1 hour/day on treadmills outperformed continuously running mice in the wheels and this memory enhancement was independent of the running distances (Liu *et al.*, 2009), suggesting that different modes of exercise induced different effects on cognitive performance.

As with the running performance and corticosterone levels, there were also no differences in recognition and location memory performance between the non-injected and vehicle-treated exercising mice, suggesting that the stressful effects of injections on the cognitive performance were also minimal.

Our results also showed that recognition memory impairment which was observed in MIF-treated sedentary mice was ameliorated in MIF-treated running mice compared to corresponding vehicle-treated groups, although there were no significant differences in RIs of the two MIF-treated groups. In agreement with this, unlimited wheel running for 13 days rescued short-term recognition memory deficits, and enhanced neurogenesis and BDNF levels in the mouse hippocampus (Lafenetre *et al.*, 2010). Also, it was reported that metyrapone or adrenalectomy attenuated exercise-induced enhancements of spatial memory in rats, suggesting a significant role of corticosterone in the cognitive effects of wheel running (Hajisoltani *et al.*, 2011). Previous research showed that GRs were implicated in recognition memory performance (Mesripour *et al.*, 2008; Roozendaal *et al.*, 2010). In addition, the activation of membrane-bound GRs by post-training injection of corticosterone-bovine serum albumin complex into the insular cortex was found to enhance the consolidation of recognition memory via the activation of the cAMP/protein kinase A pathway (Roozendaal *et al.*, 2010). However, MIF reversed corticosterone-dependent recognition memory deficits in morphine-withdrawn mice (Mesripour *et al.*, 2008). Also, MIF abolished memory deficits in diabetic mice (Revsin *et al.*, 2009). It was found that corticosterone levels improved memory performance in an inverted U-shaped manner (Diamond *et al.*, 1992; Akirav *et al.*, 2004; Okuda *et al.*, 2004), suggesting that memory performance is dependent on the degree of GR activation.

Also, it was reported that MRs were downregulated in the hippocampus of mice chronically exercising in the wheel (Droste *et al.*, 2003) and on treadmills (Chang *et al.*, 2008). Additionally, previous work reporting that exercise-induced memory improvement *via* corticosterone secretion (Hajisoltani *et al.*, 2011) suggested that GRs; which are activated by high corticosterone levels (Reul and de Kloet, 1985), at least partially mediate exercise effects on cognition. However, both MRs and GRs were found to be important for memory performance (Oitzl and de Kloet, 1992; Brinks *et al.*, 2007). MIF is a competitive GR antagonist (Cadepond *et al.*, 1997), and, thus, running-induced corticosterone elevation probably attenuated memory deficits induced by the chronic GR blocking effects through displacing MIF from GR binding sites. In support of this, dexamethasone, a GR agonist, blocked MIF-induced elevation of basal ACTH and cortisol levels in humans (Raux-Demay *et al.*, 1990). Conversely, MIF blocked dexamethasone-induced cortisol suppression (Bertagna *et al.*, 1984). This suggests that MIF-induced effects can be reversed with exogenous glucocorticoid administration or with exercise-evoked corticosterone increase as in the present experiment.

As with the alternation test, Sul25 and Sul50 produced opposing effects on the recognition memory performance in sedentary and running mice. Whereas recognition memory performance was attenuated in Sul50-treated sedentary mice compared to vehicle-treated sedentary mice, Sul25 impaired recognition memory of running mice compared to vehicle-treated exercising mice and Sul25-treated sedentary mice. As mentioned earlier, the two sulpiride doses imposed opposite effects on the alternation performance. Thus, improvement of working memory was accompanied by impairment of recognition memory and *vice versa*. In two recent studies, activation of the cAMP-dependent protein kinase A pathway resulted in impairment of the delayed alternation performance (Barsegyan *et al.*, 2010) and enhancements of recognition memory performance (Roozendaal *et al.*, 2010)**.** Also, it was found that a D1 agonist (SKF 81297), injected 10 minutes before a 4 hour retention test, improved object recognition and location memory *via* enhancement of the levels of phosphorylated cAMP response element binding protein (pCREB); a downstream mediator of D1/cAMP/protein kinase A, in the prefrontal cortex of rats (Hotte *et al.*, 2005; Hotte *et al.*, 2006). Given that chronic sulpiride treatment probably induced D2

receptor upregulation and, subsequently, reduced D1 receptor activation, this hypothesis may explain why chronic Sul25 treatment improved the alternation performance and impaired recognition memory of running mice.

In the novel object recognition test, there was a significant increase in the total exploration times of Sul25 and Sul50 treated running mice compared to vehicle-treated running group during the acquisition trial. Also, Sul50 increased the total exploration times of mice during the retention trial, compared to vehicle-treated running mice. The effect of sulpiride on exploration times was clearly controlled by exercise since sulpiride-treated sedentary groups did not show the same motivation to explore objects. On the other hand, vehicle-treated sedentary mice spent a longer time exploring objects during the two trials than mice in the no-wheel group, but neither group showed any differences in their RIs. All in all, the differences in the length of exploration times appear not to be important for mice's performance in the novel object recognition memory test. On the other hand, Sul25-treated exercising mice moved shorter distances than vehicle-treated exercising mice during the habituation session. This suggests that the reduced locomotor activity probably interfered with the effective exploration of the objects and underlay the memory impairment detected in the Sul25-treated exercising mice.

It is interesting that sulpiride-treated sedentary and running groups demonstrated opposing performance in memory tests. Changes in exploration times and motor activity probably underscored the interference with acquisition of memory tasks. For example, sedentary Sul25-treated mice did not show alterations in the exploration times or motor activity compared to vehicle-treated sedentary mice, indicating low motor activity of Sul25-treated running mice interfered with memory performance. On the other hand, running ameliorated recognition memory impairment induced by Sul50, but Sul50-treated running or sedentary groups showed no changes in the motor activity during the habituation session. As mentioned earlier, it was found that chronic wheel running for 6 weeks induced D2 receptor downregulation in the nucleus accumbens core of rats (Greenwood *et al.*, 2011), and, therefore, chronic sulpiride treatment triggering D2 receptor upregulation led to insufficient activation of D1 receptors *via* reduced dopamine release, Fig 4.9. Altogether, results suggest that

exercise regulates dopamine neurotransmission, and sulpiride modifies exercise-induced cognitive effects, Fig 4.9.

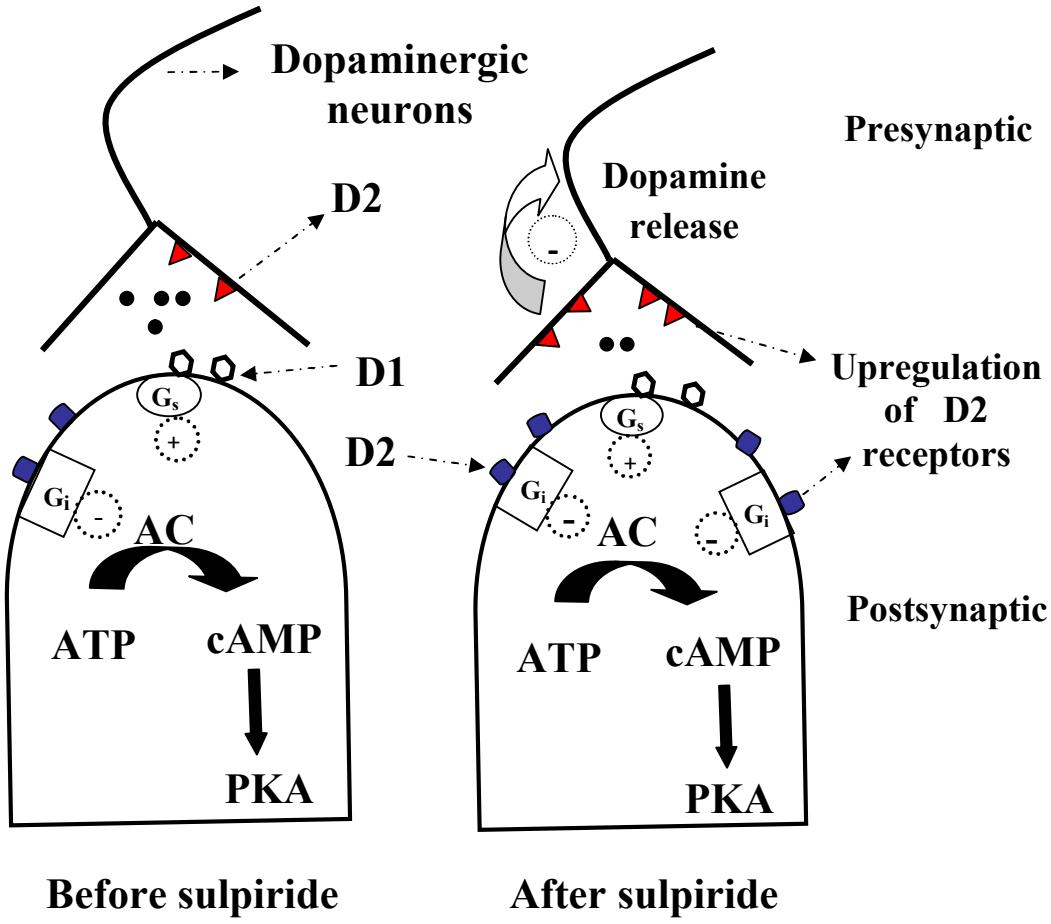

Figure 4.9 Running enhanced chronic sulpiride induced D2 receptor upregulation. Whereas Sul25 seems to have enhanced presynaptic D2 receptor expression, Sul50 is suggested to have promoted D2 receptor upregulation at pre-synaptic and postsynaptic sites. The increase in presynaptic D2 receptors reduced dopamine release and activation of D1 receptors with a subsequent decrease in locomotor activity and recognition memory performance, and improved alternation performance. In contrast, these effects were attenuated by upregulation of postsynaptic D2 receptors in Sul50-treated mice. AC = adenylyl cyclase, G proteins: stimulatory Gs and inhibitory Gi, PKA = protein kinase A.

As stated earlier, chronic sulpiride treatment was associated with D2 receptor upregulation (Stefanini *et al.*, 1991; Motohashi *et al.*, 1992; Parsons *et al.*, 1993; Hurley *et al.*, 1996), suggesting that D2 receptor-mediated effects were enhanced while the memory tests were undertaken. This conclusion is supported by the finding that sulpiride-treated groups and particularly Sul25-treated mice travelled short distances during the habituation session and/or the memory tests, indicating a decrease in dopaminergic neurotransmission and enhanced sensitivity of D2 autoreceptors which negatively regulate dopamine release (Jaworski *et al.*, 2001; Hahn *et al.*, 2006; Marsden, 2006). Subsequently, Sul25 impaired recognition memory but improved the alternation performance of running mice. Although levels of D2 receptors were not estimated, the reduced activity of sulpiride-treated groups, in particular Sul25-treated exercising mice during the habituation session suggests that D2 receptors had been upregulated during the memory tests which were performed within 3 to 5 days after the treatment ended.

Dopamine has been suggested to influence learning and memory especially when a motivated behaviour is involved (Shohamy and Adcock, 2010), since dopaminergic neuronal terminals and receptors are abundant in the striatum as well as in the hippocampus and frontal cortex (Shohamy and Adcock, 2010). Also, it was found that the blockade of D1 or D2 receptors did not impair recognition memory, but activation of D1 receptors enhanced recognition memory performance (de Lima *et al.*, 2011). For example, a post-training intraperitoneal injection of SKF38393, a D1 receptor agonist, enhanced consolidation of recognition memory of rats (de Lima *et al.*, 2011). In contrast, post-training injection of quinpirole, a D2 receptor agonist, or pre-training injection of raclopride, a D2 receptor antagonist, did not influence recognition memory (de Lima *et al.*, 2011). Nevertheless, it has been thought that blocking D2 receptors can be translated into more activation of D1 receptors, since the two receptors have opposite effects on adenylyl cyclase (Marsden, 2006). As stated earlier, the activation of D1 receptors had an inverted U-shaped relationship with memory performance (Goldman-Rakic *et al.*, 2000; Williams and Castner, 2006), and therefore, moderate activation of D1 receptors is required for optimum memory performance.

4.6 Conclusion

Results of the present experiment demonstrated that GR activation was not required for mice to sustain high running performance, but long-term intermittent exercise-induced corticosterone elevation provided protection from MIF-induced recognition memory deficits. More work is needed to investigate the involvement of corticosterone in the rewarding effects of voluntary wheel running in a standard paradigm such as the conditioned place preference, and to test whether GR blockade could attenuate the acquisition of wheel running behaviour. In agreement with the previous work, our findings indicated that D2 receptors were involved in the running performance of mice and cognitive effects of voluntary wheel running, suggesting that the activation of the reward pathway by wheel running could influence cognition.

Chapter 5

General Discussion

5.1 Summary of findings

The main objectives of the work described in this thesis were to investigate the potential rewarding properties of corticosterone in male C57BL/6J mice and how their cognitive performance could be influenced by the hedonic effects of the stress hormone. To address this hypothesis, two models were employed in separate experiments: 1- An oral self-administration model (the two-bottle free choice test) to establish the rewarding properties of the stress hormone, 2- Voluntary wheel running, a rewarding stimulus which enhances corticosterone secretion and, thus, represents an appropriate model to study the link between its rewarding and cognitive effects.

To the best of our knowledge, this is the first work carried out to study the rewarding effects of corticosterone in mice. Results demonstrated that mice acquired and reinstated oral corticosterone self-administration at high doses indicating its rewarding effects. In particular, the marked preference for the corticosterone solution was observed when the unflavoured corticosterone solution was offered *versus* the IMP solution as a result of IMP's initial aversive taste which accelerated and

facilitated corticosterone self-administration. Results also suggest that corticosterone self-administration protected mice to some extent from memory impairment induced by a surprisingly low concentration (1%) of ethanol and also enhanced object location memory. Moreover, levels of BDNF and its downstream target pERK2 in the hippocampus and frontal cortex were significantly altered in some or all corticosterone-treated groups compared to control groups, suggesting that they may underlie the rewarding and cognitive effects of corticosterone. Furthermore, corticosterone self-administration was accompanied by changes in dopamine turnover in the striatum, which suggests a role of dopamine in the corticosterone-seeking behaviour. These findings led us to the idea of using a stressful natural reward, such as voluntary wheel running which promotes corticosterone secretion and modulates cognitive performance, in order to examine the influences of corticosterone-related reward on memory.

Whereas the exercise protocol used in the present studies increased corticosterone levels after single or repeated sessions, it was unable to enhance memory performance compared to non-exercising mice. Acute inhibition of exercise-induced corticosterone elevation decreased running distances, but chronic GR blockade had no effect. Nonetheless, running prevented MIF-induced recognition memory deficits. Finally, results showed that D2 receptors were involved in the running performance of mice and the effects of exercise on memory, suggesting that cognitive effects of voluntary wheel running could be controlled by its rewarding properties.

5.2 Is corticosterone rewarding?

The current results demonstrate that inbred male C57BL/6J mice are prone to self-administer corticosterone, indicating its rewarding properties. Also, corticosterone-withdrawn mice were motivated to drink primarily from the corticosterone tube, suggesting that it has addictive potential. In conjunction with these findings, others reported that corticosterone was self-administered by male Sprague-Dawley rats in the intravenous operant-conditioning and two-bottle free choice tasks (Deroche *et al.*, 1993; Piazza *et al.*, 1993), and they relapsed to self-administer corticosterone in the two-bottle choice test (Deroche *et al.*, 1993). However, the same rat strain did not exhibit CPP to corticosterone (Dietz *et al.*, 2007). This could be dose or task related,

as corticosterone was only self-administered at high doses (Piazza *et al.*, 1993), and, thus, corticosterone administered at low doses did not induce conditioned place preference because GRs, which have been found to mediate corticosterone-facilitating effects on drug-seeking behaviour (Fiancette *et al.*, 2010), are activated mainly during stress or circadian corticosterone peaks (Reul and de Kloet, 1985). Also, some abused drugs such as pentobarbital and phencyclidine were self-administered, but they did not induce CPP in rats (Collins *et al.*, 1984; Marquis *et al.*, 1989; Acquas *et al.*, 1990; Lew and Parker, 1998), indicating that operant self-administration procedures and CPP probably test different aspects of drug-seeking behaviour.

An important point is whether rewarding effects of corticosterone are strain-dependent. A considerable number of studies have shown that C57BL/6J mice have a tendency to self-administer abused drugs (Elmer *et al.*, 2010; Fiancette *et al.*, 2010; Gieryk *et al.*, 2010). On the other hand, DBA/2J mice were found to be less vulnerable to abused drugs-related reward (van der Veen *et al.*, 2007; Glatt *et al.*, 2009; Elmer *et al.*, 2010). Being inbred strains may explain the differences in the propensity for drug craving. For example, it was found that differences in genetic expression of prodynorphin or proenkephalin in the nucleus accumbens regulated morphine-induced CPP in C57BL/6J and DBA/2J mice (Gieryk *et al.*, 2010). Interestingly, Sprague-Dawley rats which are outbred self-administered corticosterone (Deroche *et al.*, 1993), suggesting that rewarding effects of corticosterone are not restricted to inbred strains with homogeneous genetic background.

Similarly, it has been reported that inbred C57BL/6J mice are motivated to run spontaneously in the wheel, although other inbred strains, such as CBA and C3H, displayed better running performance (Brene *et al.*, 2007). In addition, our results demonstrated a wide variability in the running performance between individual mice of the same strain. This variability was previously observed in C57BL/6 mice (Klaus *et al.*, 2009). In addition, the running performance of male Long-Evans (an outbred strain) rats was considerably different (Tarr *et al.*, 2004), suggesting that the variability in motivation to run is not species- or strain-dependent. Also, corticosterone levels correlated with running distances, indicating that exercise-evoked corticosterone elevation depends on running performance, or increased corticosterone levels motivate mice to run. In line with this, corticosterone levels were

found to function as a determining factor for vulnerability to amphetamine self-administration in rats (Piazza *et al.*, 1991). Likewise, corticosterone levels could be a determining factor for the voluntary running performance in mice.

There is a possibility that the rewarding effects of corticosterone or voluntary wheel running are independent, and this may explain the lack of significant chronic effects of GR blockade on the running performance of mice. First, our results suggest that D2 receptors are implicated in the running performance of mice. In accordance with this, chronic continuous wheel running lowered D2 receptor mRNA expression in the nucleus accumbens core of rats (Greenwood *et al.*, 2011). Second, it has been reported that the endogenous opioidergic system is modulated by chronic wheel running. Mice over-expressing Δ FosB in the striatal dynorphin neurons ran more than the control running group (Werme *et al.*, 2002b), but the running activity was decreased in mice which extensively expressed Δ FosB in the striatal enkephalin neurons (Werme *et al.*, 2002b). Also, Δ FosB levels increased in the striatum (Werme *et al.*, 2002b) and nucleus accumbens (Greenwood *et al.*, 2011) of running rats. Moreover, naloxone (an opioid receptor antagonist) blocked CPP induced by wheel running, suggesting the involvement of the opioidergic system (Vargas-Perez *et al.*, 2008). In addition, naloxone also attenuated the acquisition of the wheel running behaviour (Vargas-Perez *et al.*, 2008). Third, a recent study showed that exercising men had high plasma levels of endocannabinoids and β-endorphins (Heyman *et al.*, 2011), suggesting other mechanisms of exercise-induced reward. Fourth, Sprague-Dawley rats running continuously for 5 weeks exhibited initial corticosterone elevation during the first week, but its levels declined until running was not accompanied by an increase in corticosterone levels (Fediuc *et al.*, 2006). This suggests that the same rat strain which self-administered corticosterone (Piazza *et al.*, 1993) retained high running performance without an increase in corticosterone levels (Fediuc *et al.*, 2006; Sasse *et al.*, 2008), indicating rewarding effects of wheel running are GRs-independent. The lack of association between running activity and the increase in corticosterone levels in Sprague-Dawley rats (Fediuc *et al.*, 2006; Sasse *et al.*, 2008) can be explained as rats kept running at high rates for corticosterone-independent rewarding effects of wheel running.

Several studies have indicated that wheel running modifies rewarding effects of abused drugs. Cocaine-induced reinstatement in rats was attenuated by wheel running (Zlebnik *et al.*, 2010). Additionally, wheel running enhanced ethanol preference in rats (Werme *et al.*, 2002a). On the other hand, C57BL/6J mice which had drunk 10% ethanol increased their running performance in a wheel when no alcohol was available, but mice ran less when ethanol access was regained, and this occurred after mice exhibited stable intermittent running activity with continuous ethanol access, suggesting that rewarding effects of wheel running substitutes for ethanol-related reward (Ozburn *et al.*, 2008). Given that rewarding effects of wheel running are probably mediated *via* multiple mechanisms, it is likely that MIF-treated mice ran at high rates; after the first week's mild decrease in their running activity compared to the training level, in order to substitute for the reduced corticosterone-related reward induced *via* ongoing GR blockade. In contrast to these assumptions, adrenalectomized and metyrapone-treated Lewis rats ran slightly less than control exercising rats, when they had only 10 days of unlimited access to wheel running (Hajisoltani *et al.*, 2011), and, therefore, it is not conclusive as HPA axis adaptation might have been induced by long-term wheel running.

As discussed earlier, D2 receptor blockade attenuated the running performance of mice, and corticosterone self-administration enhanced dopamine turnover. Thus, it is inferred that alterations of dopamine neurotransmission influenced rewarding effects of the wheel running and the stress hormone. Taken together, although corticosterone elevation induced by wheel running was not required for male C57BL/6J mice to sustain high running performance, this does not preclude the possibility that corticosterone is rewarding, since dopamine and other mechanisms are suggested to mediate rewarding effects of wheel running.

5.3 Implications of rewarding effects of corticosterone in psychiatric disorders.

Elevated glucocorticoid levels have been found to accompany certain mood disorders such as major depression (Souetre *et al.*, 1988) and psychoses (Collip *et al.*, 2011). Indeed, the persistent increase in endogenous secretion or chronic administration of glucocorticoids leads to the development of Cushing's syndrome; a hormone disorder characterized by hypercortisolemia which is either ACTH-dependent or -independent

(Fink, 2010), which may precipitate depressive symptoms (Kelly *et al.*, 1980) and psychotic episodes (Wada *et al.*, 2001; Myhill *et al.*, 2008). Interestingly, a case study of a patient who had a history of Cushing's syndrome presented with various psychotic symptoms including euphoria, but these complications were improved after reducing salivary and urinary cortisol levels using metyrapone and ketoconazole (Myhill *et al.*, 2008), indicating rewarding effects of cortisol.

Reduced glucocorticoid-induced negative feedback on HPA axis activity has been found to underscore dysregulation of HPA axis, as high cortisol levels in depressed patients were either unaffected (Rush *et al.*, 1996) or increased (Heuser *et al.*, 1994) in response to the dexamethasone (a GR agonist) suppression test; in which an oral dose of dexamethasone decreases the secretion of ACTH and cortisol *via* the activation of GRs which mediate negative feedback of HPA axis activity (Carroll *et al.*, 1981). This suggests that dysfunction of GRs underlies hyperactivity of the HPA axis in mood disorders (Abraham *et al.*, 2003; Thomson and Craighead, 2008). Conversely, it was reported that depressed patients treated acutely with mifepristone exhibited rapid symptomatic improvement (Belanoff *et al.*, 2001a). This could be related to the ability of mifepristone to enhance GR expression, resulting in enhancement of negative feedback inhibition on HPA axis activity and reduction of elevated glucocorticoids (Thomson and Craighead, 2008).

Several studies have addressed the involvement of stress and glucocorticoids in the development of depression (Johnson *et al.*, 2006; Detanico *et al.*, 2009). It is important to distinguish between the influences of acute or chronic exposure to stress or glucocorticoid treatment. Whereas acute footshock had antidepressant effects, chronic stress exposure induced depression-like behaviour in mice in the forced swim test (Swiergiel *et al.*, 2008), suggesting that short-term elevation of glucocorticoids improves mood status. On the other hand, repeated corticosterone injections were reported to induce a depression-like state in mice (Zhao *et al.*, 2008) and rats (Kalynchuk *et al.*, 2004; Johnson *et al.*, 2006). In contrast, acute corticosterone treatment did not increase rats' immobility in the forced swim test (Johnson *et al.*, 2006). Nevertheless, there have been a number of conflicting studies which indicate that glucocorticoids have mood-enhancing effects. For instance, acute corticosterone intake in the drinking water significantly enhanced mice's performance in the forced

swim test compared to the vehicle and desmethylimipramine-treated groups (Stone and Lin, 2008). Similarly, young male C57BL/6 mice ingesting corticosterone chronically at low doses in drinking water outperformed the control group in the tail suspension and forced swim tests, and this was accompanied by reduced MR expression in the hippocampus (Xu *et al.*, 2011). This suggests that corticosterone in drinking water even at low doses is able to improve depression-like behaviour. Furthermore, rats treated with a low dexamethasone dose for 1 week exhibited attenuated sepsis-induced anhedonia (Cassol-Jr *et al.*, 2010), indicating a role of GRs in decreasing depression-like behaviour. Previous work has provided some evidence that exercise improves the mood of depressed patients (Schuch *et al.*, 2011; Tordeurs *et al.*, 2011). Also, running has been reported to attenuate depression-like behaviour in experimental animals (Brene *et al.*, 2007; Marais *et al.*, 2009). Interestingly, long-term wheel running protected against stress-induced depression-like behaviour *via* the increase in BDNF levels, but corticosterone levels were also elevated (Adlard and Cotman, 2004; Zheng *et al.*, 2006), suggesting that corticosterone probably contributes to rewarding effects of exercise or, at least, did not aggravate depression-like behaviour. In fact, chronic running was reported to induce adaptation status in HPA axis in rodents (Droste *et al.*, 2003; Fediuc *et al.*, 2006), and, therefore, regular exercise probably improves depressive symptoms *via* regulation of HPA axis activity.

It is widely accepted that glucocorticoid secretion increases in response to stress to inhibit further activation of the HPA axis and to maintain homeostasis. Thus, terms such as allostasis and allostatic overload have been introduced to describe how glucocorticoids mediate the remission of stress-evoked HPA axis hyperactivity. Homeostasis can be defined as a self-regulating process which is required to maintain the internal environment of an individual close to a set point *via* limited operations important for survival (Fink, 2010). On the other hand, allostasis reflects the alteration in the levels of mediators such as glucocorticoids required to generate energy and modulate behaviour to cope with changes in the external environment (Fink, 2010). Consequently, an inability to maintain homeostasis may lead to allostatic overload which comprises overexposure to stress hormones (Fink, 2010), with subsequent harmful effects. Altogether, the elevations of glucocorticoids which accompany certain psychiatric disorders are probably vital to restore homeostasis or to compensate anhedonia *via* their rewarding effects.

In brief, elevation of corticosterone levels accompanies both aversive and rewarding stressors, and habituation of the HPA axis is associated with motivated behaviours, suggesting that the initial increase in glucocorticoid levels in mood disorders is likely to counteract the associated anhedonia (allostasis), but chronic exposure to underlying causes (allostatic overload) probably stimulates HPA axis to induce further adverse effects on the mood status.

5.4 Do rewarding effects of corticosterone have positive influences on memory?

It has been believed that glucocorticoids are implicated in memory processing. Whereas MRs are abundant in the hippocampus, GRs are widely expressed in the brain (Reul and de Kloet, 1985), suggesting that corticosterone could influence different forms of memory such as recognition, spatial and fear memory (Roozendaal *et al.*, 2008; Barsegyan *et al.*, 2010; Roozendaal *et al.*, 2010). However, effects of corticosterone on memory depend on their levels (Diamond *et al.*, 1992; Akirav *et al.*, 2004; Okuda *et al.*, 2004) and duration of exposure (Coburn-Litvak *et al.*, 2003). Also, corticosterone has a differential impact on different phases of memory processing. Acute corticosterone elevation was reported to enhance consolidation of spatial and recognition memory (Sandi *et al.*, 1997; Roozendaal *et al.*, 2010), but it impaired the delayed alternation performance (Barsegyan *et al.*, 2010) and retrieval of memory (Roozendaal *et al.*, 2001; Roozendaal *et al.*, 2008).

It is important to distinguish between influences of stress and glucocorticoids on cognition. For instance, spatial memory of rats was impaired by restraint stress for 21 days (Luine *et al.*, 1994), but unaffected by exogenous corticosterone treatment for the same period (Coburn-Litvak *et al.*, 2003) in male Sprague-Dawley rats which self-administered the stress hormone (Piazza *et al.*, 1993). Whereas exposure to corticosterone for 3 weeks induced reversible hippocampal morphological changes such as atrophy of apical dendritic branches in the CA3 region of the rat hippocampus (Woolley *et al.*, 1990; Watanabe *et al.*, 1992), hippocampal neuronal loss was observed after a 3-month treatment (Sapolsky *et al.*, 1985) in male Fischer rats (an inbred strain), suggesting that long-term exposure to corticosterone could impair hippocampus-dependent memory. In agreement with this, the current results suggest that short-term exposure to significant corticosterone self-administration did not

impair spontaneous alternation or recognition memory, but it improved the object location memory of mice and protected from ethanol-induced memory impairment. Furthermore, restraint stress for 1 month was reported to trigger loss of hippocampal neurons in 9-month-old castrated Wistar rats (Mizoguchi *et al.*, 1992). In contrast, none of these effects were detected in the hippocampus of aged macaques monkeys treated for 12 months with high oral doses of cortisol (Leverenz *et al.*, 1999). Moreover, chronic moderate elevation of corticosterone levels for 3 months enhanced spatial memory of mountain chickadees (Pravosudov, 2003). This suggests that although the duration and intensity of corticosterone exposure have distinct effects on cognition, other factors including species, strain and stress should be considered.

It has been reported that HPA axis dysregulation underlies cognitive disorders such as Alzheimer's disease (Swanwick *et al.*, 1998). For example, dexamethasone treatment for 1 week increased amyloid β-peptide levels in the whole brains of male 3X-TG-AD mice (Green *et al.*, 2006). Also, dexamethasone accelerated accumulation of tau-laden neurofibrillary tangles in the hippocampus and amygdala of mice (Green *et al.*, 2006). In addition, immobilization stress accelerated the onset and progress of memory impairment of a transgenic mouse model of Alzheimer's disease (Jeong *et al.*, 2006). Overall, this suggests that disturbances of HPA axis activity are implicated in Alzheimer's-related pathological features and cognitive decline. However, all of these studies used transgenic mice which already have or are prone to develop cognitive impairment. In effect, Alzheimer's mouse models demonstrated HPA axis hyperactivity (Green *et al.*, 2006), which could be induced by disease-related destruction of hippocampal neurons (Ball *et al.*, 1985; Braak *et al.*, 1999), thereby contributing to negative feedback regulation of HPA axis activity (Sapolsky *et al.*, 1984). Interestingly, the exercise protocol involving 1-hour wheel running (5 days/week) for 16 weeks improved memory performance in a transgenic mouse model of Alzheimer's disease (Yuede *et al.*, 2009), and this effect could be due to habituation of the HPA axis. As discussed earlier, corticosterone was reported to mediate cognition-enhancing effects of voluntary wheel running (Hajisoltani *et al.*, 2011), and this could be *via* GRs as shown in the present work. In contrast, unlimited access to running wheels for 8 weeks did not enhance memory performance of mice with Alzheimer's-like deficits (Richter *et al.*, 2008), suggesting that intermittent

exercise, as in the former study (Yuede *et al.*, 2009), could be better than continuous exercise. Overall, this suggests that elevation of glucocorticoids which accompanies Alzheimer's disease is a consequence rather than an underlying cause, but this does not rule out the possibility that chronic glucocorticoid elevations worsen the pathology and memory deterioration in Alzheimer's disease.

The use of imaging studies has indicated that drug-seeking behaviour does not only influence the reward system, but also other brain regions including memory circuits are involved (Volkow *et al.*, 2011; Volkow *et al.*, 2012), suggesting that the effects of corticosterone on the reward pathway may modify its effects on cognition. Abused drugs have been shown to induce modifications of learning and memory. For example, it has been reported that the majority of addictive drugs induce CPP (Tzschentke, 1998), which is a learning paradigm to associate the rewarding effects of a drug with the drug-paired environment, suggesting that motivated behaviour facilitates learning and enhances adaptive memory *via* enhancements of dopamine neurotransmission (Shohamy and Adcock, 2010). It has been found that nicotine improves memory and, thus, nicotine patches may have a therapeutic use in retarding cognitive deterioration in Alzheimer's disease and schizophrenia (Levin and Rezvani, 2000). In contrast, repeated exposure to methamphetamine impaired recognition memory in mice (Mizoguchi and Yamada, 2011). Also, other psychostimulants including cocaine could modulate learning and memory (Harvey, 1987). Addiction is accepted as the negative side of reward, suggesting that rewards without significant dependency probably have cognition-enhancing effects. For example, spatial memory of mice was improved by post-training self-administration of sugar which is a natural reinforcer (Dalm *et al.*, 2009). In agreement with this, our results showed that significant corticosterone self-administration for a limited time enhanced spatial memory and did not impair recognition memory.

In summary, moderate exposure to rewarding effects of corticosterone could improve memory performance and mediate cognitive effects of exercise which has recently been demonstrated as a novel strategy to treat cognitive dementia.

5.5 Conclusion

The present thesis demonstrates that corticosterone has rewarding effects in male C57BL/6J mice. Whereas acute inhibition of exercise-induced corticosterone elevation attenuated the mice's motivation to run, chronic blockade of GRs did not influence running activity of mice. Moderate continuous corticosterone self-administration or long-term intermittent voluntary wheel running did not impair memory, but corticosterone self-administered groups outperformed the control groups in the object location memory and were protected from ethanol-induced memory impairment. In addition, exercise-evoked corticosterone rise rescued recognition memory deficits induced by MIF in sedentary mice. All in all, the data from this thesis suggest that rewarding effects of corticosterone improve cognition. Results also showed that corticosterone-withdrawn mice exhibited alterations in synaptic plasticity involving BDNF/pERK2 pathway in the hippocampus and frontal cortex, indicating long-lasting neural adaptation induced by the stress hormone. Also, dopamine turnover in the striatum was enhanced by corticosterone self-administration, suggesting that dopamine is involved in the rewarding effects of corticosterone.

On the other hand, results indicate that voluntary wheel running regulates dopamine neurotransmission, suggesting that dopamine is involved in the rewarding and cognitive effects of wheel running. Moreover, D2 receptor blockade suggests that cognitive effects of voluntary wheel running are controlled by the running performance of mice. Taken together, this work provided insights into the role of reward and motivated behaviour in the enhancement of cognitive performance.

5.6 Future work.

The present work provides more evidence that corticosterone is rewarding in mice. However, there is a need for more experiments to unravel the mystery beyond some findings.

First of all, it would be interesting to investigate the immediate effects of acute and chronic corticosterone self-administration on dopamine levels in the nucleus accumbens by means of microdialysis. Conversely, corticosterone-seeking behaviour could be challenged by different dopamine receptor antagonists.

Although previous work and the current results suggest that GRs have an indispensable role in the rewarding effects of corticosterone, the use of pharmacological interventions including GR and MR antagonists will enhance our knowledge regarding the role of each receptor in the rewarding effects of corticosterone.

In addition, studies involving the use of protein synthesis inhibitors such as anisomysin, or corticosterone-bovine serum albumin complex; which acts only at membrane-bound GRs and MRs, can be undertaken to investigate whether rewarding effects of corticosterone are transcription-independent, since flavour-guided mice drank almost uniquely from the corticosterone tube, which suggests that rewarding effects of corticosterone are rapid.

Memory tests used in the thesis have provided, to some extent, insights into the effects of corticosterone self-administration on memory performance of mice, but other tasks including contextual fear conditioning or inhibitory avoidance can alternatively be used, since corticosterone has been found to be important for consolidation of fear memory (Barsegyan *et al.*, 2010). Another good approach would be to test memory performance of mice at the time of corticosterone self-administration or wheel running, as this will eventually provide the opportunity to study the simultaneous relationship between reward experience and memory performance.

It would also be instructive to investigate whether the rewarding effects of corticosterone are accompanied by antidepressant-like effects in standard tests.

Research can be done to examine if transgenic mouse models of cognitive disorders have tendency to self-administer corticosterone. Then, effects on memory performance can be studied after blocking *de novo* corticosterone synthesis.

Finally, results of this thesis have expanded our knowledge concerning the euphoric effects of glucocorticoids, and the proposal that these rewarding effects could improve memory encourages the development of new therapeutic strategies for certain disorders, which involve dysregulation of HPA axis activity. For instance, rewarding

stressors, such as exercise and rewarded competitions or challenges, can be examined for their possible role in the prognosis and treatment of mood disorders and cognitive dysfunction. Although addiction to exercise is reported in certain individuals as a serious complication, controlled exercise could have a therapeutic potential for substance dependence, in particular during the drug-withdrawal period, to attenuate the risk of relapse. For example, effects of exercise or other forms of rewarding stressors can be added to the treatment protocol of drug addicts under supervision, especially when subjects are recovering, to study whether rewarding effects of stress could safeguard against drug reinstatement. In addition, retrospective or meta-analysis studies could be conducted to figure out the relationships between the risk of developing mood disorders or substance abuse and the lack of exercise, or if these health problems started after sufferers gave up exercising. Moreover, investigation could be extended to find out whether subjects who were simultaneously prescribed glucocorticoid therapy for any other diseases, such as bronchial asthma and arthritis, had their mood status or substance dependency altered.

Taken together, these findings have provided evidence about the involvement of glucocorticoids in the development of drug or behavioural addiction, suggesting that the use and development of drugs which could modulate mineralocorticoid or glucocorticoid receptor activity represent a promising therapeutic tool for substance or behavioural dependence.

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