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Investigating the Effects of Chronic Amphetamine Treatment on the Midbrain Superior Colliculus

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Investigating the effects of chronic amphetamine treatment on the midbrain superior colliculus

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A thesis submitted for degree of Doctorate of Philosophy in the Discipline of Neuroscience

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ABSTRACT

Heightened distractibility, the reduced ability to filter out irrelevant information, is a disruptive symptom found in numerous conditions and healthy ageing. It is most effectively treated with psychostimulant drugs, such as amphetamine, which are given chronically in attention deficit hyperactivity disorder (ADHD). There is converging evidence linking the superior colliculus (SC) to the regulation of distractibility, and acute amphetamine administration is known to suppress collicular responsiveness, however relatively little is known about its mechanism of action and long lasting effects when administered chronically. It is therefore the aim of this thesis to investigate the effects of chronic treatment with amphetamine on the SC, as a neural correlate of distractibility. To achieve this, adolescent Hooded Lister rats were treated orally with amphetamine (2, 5 or 10 mg/kg) or a control (vehicle or untreated) for one month. The effects of treatment were then explored by investigating: collicular-dependant and locomotor behaviour; visual responsiveness of the superficial SC; responses to an acute amphetamine challenge; and the morphology of the superficial SC.

At high doses, oral amphetamine treatment resulted in the development of locomotor tolerance, in contrast to previous research using i.p. administration which report sensitisation. Evidence of suppression of activity in the SC was identified from a reduced ability to perform collicular dependant behaviours, and from weaker light responsiveness measured from multi-unit activity in the superficial SC following treatment with high doses of amphetamine. Evidence was also found of a potential compensatory mechanism involving synaptophysin expression and enhanced peri-synaptic activity. This thesis also investigated the types of dendritic spines prevalent in the superficial SC for the first time, and found that these structures were unaffected by amphetamine treatment. These results indicate that the therapeutic effects of amphetamine may stem from suppression of collicular activity, and the SC is susceptible to amphetamine induced remodelling.

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LIST OF ABBREVIATIONS

ACC   Anterior Cingulate Cortex

ADHD   Attention Deficit Hyperactivity Disorder

Asf   Area sampling fraction

DA   Dopamine

DpG   Deep Grey

DpWh   Deep White

EEG   Electroencephalography

fMRI   Functional Magnetic Resonance Imaging

GH   Genetically Hypertensive

Hsf   Height sampling fraction

InG   Intermediate Grey

InWh   Intermediate White

i.p.   Intraperitoneal

i.v.   Intravenous

LFP   Local Field Potential

mcd   Milli-Candela

mEPSC   Miniature Excitatory Postsynaptic Currents

MRI   Magnetic Resonance Imaging

MUA   Multiunit Activity

NA   Noradrenaline

Op	Opticum
PB	Phosphate Buffer
PFC	Prefrontal Cortex
PPA	Parahippocampal Place Area
PSTH	Peristimulus Time Histogram
SEM	Standard Error Mean
SC	Superior Colliculus
SHR	Spontaneous Hypertensive Rat
SNr	Substantia Nigra Pars Reticulata
SNc	Substantia Nigra Pars Compacta
Ssf	Section sampling fraction
SUD	Substance Use Disorder
SuG	Superficial Grey
VTA	Ventral Tegmental Area
Zo	Zonal Layer
5-HT	Serotonin (5-Hydroxytryptamine)

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PUBLICATIONS

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CONFERENCE PRESENTATIONS

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CHAPTER 1. INTRODUCTION

1.1 Preface

The majority of people experience difficulty in maintaining their attention at some point, but for most people this is not a major problem. However, continual increased distractibility, that is a significantly reduced ability to filter out irrelevant information, is a disruptive symptom of a number of different mental health conditions, and also occurs during healthy ageing. Heightened distractibility can be treated with the administration of psychostimulant drugs. In cases where treatment is given these drugs are often prescribed for an extended period of time, however the mechanisms of action of the drugs are not yet fully understood, and the effects of chronic therapeutically relevant treatment is currently insufficiently researched. The aim of this thesis was to explore the effects of a chronic treatment paradigm which mimics the administration of psychostimulant drugs used to treat heightened distractibility in humans. The long-term effects of treatment on both structure and function was investigated in one region of the brain, superior colliculus (SC), which has been identified as both a neural correlate of distractibility and a potential therapeutic target in psychostimulant treatment.

1.2 Distractibility

1.2.1 INCREASED DISTRACTIBILITY

Increased, or heightened distractibility is the reduced ability to filter out irrelevant information in order to focus on relevant stimuli (Gaymard et al., 2003a). The level of distractibility a person experiences is highly variable both between different people and throughout an individual person's lifetime (Healey et al., 2008). For example, an increase in distractibility is associated with normal ageing (Gazzaley et al., 2005, Jonides et al., 2000). In this case, heightened distractibility is thought to be due to reduced ability to discriminate relevant from irrelevant information (Wascher et al., 2012). Evidence supporting this theory also comes from an functional magnetic resonance imaging (fMRI) study performed by Gazzaley et al. (2005) investigating top down suppression of activity, used to modulate attention. This study investigated modulation of activity within the parahippocampal place area (PPA) to explore the presence of age related changes to distractibility, due to inability to ignore stimuli irrelevant to the task (Healey et al., 2008). During each trial the participants were shown images from two categories: faces and landscape scenes. They were asked either to look at the images passively or remember one specified category while ignoring the other. When asked to ignore the images of scenes and remember the faces, activity within the PPA was suppressed in young adults, falling below the baseline level observed when the participants were asked to view the images passively, however, in older adults this suppression of PPA activity was not seen, which may signify the reduced ability of the older participants to filter out the irrelevant information as effectively (Gazzaley et al., 2005, Healey et al., 2008). It is also important to note that this effect is not exclusive to the PPA; this area was used to assess

differences in modulation between older and younger adults as the authors found it was the most robust marker of modulation of activity in young adults, in comparison to a face-selective region of interest also identified in the fusiform gyrus (Gazzaley et al., 2005) but the same mechanism could occur in different areas of the brain, depending on the stimulus presented.

As well as occurring with normal ageing, increased distractibility is also a commonly occurring symptom associated with a number of different conditions (Nunez Castellar et al., 2012, Facoetti and Molteni, 2001, Friedman-Hill et al., 2010, Lepistö et al., 2004) and damage to the brain (Chao and Knight, 1995). One condition heavily associated with heightened distractibility is attention deficit hyperactive disorder (ADHD) (Swanson et al., 1991, Overton, 2008, Brace et al., 2015a). Investigations into ADHD have been performed by a range of methods in order to precisely define the condition, and to identify the areas and networks within the brain affected by ADHD. Behavioural testing is one method commonly used in the investigation of increased distractibility associated with ADHD. These tests often involve the measurement of reaction times and errors made within a task in order to compare test groups to controls. The use of such studies has revealed potential implications in the top-down control of attentional networks. Friedman-Hill et al. (2010) performed a discrimination task with children with ADHD, healthy children, and healthy adults, in which the saliency of distractors surrounding the target was altered. It was found that children with ADHD performed as well as the control groups while performing the difficult discrimination task but performed significantly worse than the control groups during the easy discrimination tasks when the distractors were most salient. It was therefore hypothesised that one potential cause of attentional deficits in ADHD is due to a higher activation threshold for top-down processing, leading to a deficit

in distractor filtering during performance of a low-demand task (Friedman-Hill et al., 2010).

Neuroimaging studies investigating ADHD have provided evidence of dysfunction in the neural processing networks responsible for attention in children with ADHD (Fassbender et al., 2009, Konrad et al., 2006). This dysfunction may also relate to the heightened levels of distractibility that is experienced by individuals with ADHD. An fMRI study investigating affected networks within the brains of medication naive children with ADHD identified abnormalities in the networks controlling alerting, reorienting, and executive control of attention during an attentional network test task (Konrad et al., 2006). This study found that children with ADHD had significantly longer reaction times and made more mistakes during the task when compared to the healthy control group, and also each of the three attentional networks tested exhibited different areas of activation from the controls (Konrad et al., 2006). One example of differing activation patterns which could potentially underlie the increase in distractibility seen in ADHD was observed during alerting, where participants with ADHD had significantly reduced activation of the anterior cingulate cortex (ACC) in comparison to controls (Konrad et al., 2006), and there is evidence to suggest that the ACC is associated with focusing attention in the event of distracting stimuli (Weissman et al., 2004). It is, however, possible that some of this deviation in activation is due to attempted compensation during task completion since results from this study have shown that in some instances participants with ADHD have inverse activation networks to the control group. Although ACC activity was reduced in participants with ADHD, there was an increase observed in activity in the brainstem and locus coeruleus, whereas the control group experienced reduced activity in these areas during the task (Konrad et al., 2006). This evidence also supports the theory that there is

inhibition in top-down processing for people with ADHD (Friedman-Hill et al., 2010, Konrad et al., 2006).

Heightened visual distractibility is associated with dysfunction, or damage, in several different areas within the brain. Of these areas, the SC is frequently implicated in playing some role when an increase in distractibility is observed. This association has been linked to a reduction in the inhibition of visual saccades (Pierrot-Deseilligny et al., 1991) which are rapid eye movements that abruptly change the eye's fixation point from one part of the visual field to another (Purves et al., 2001, Wurtz and Optican, 1994). There is also evidence that the SC behaves abnormally in a spontaneous hypertensive rat (SHR) model of ADHD, in that SHRs lose the ability to modulate righting latency in a height dependent manner (Dommett and Rostron, 2011), an ability known to be reliant on a functioning superior colliculus (Dommett and Rostron, 2011, Pellis et al., 1989). Furthermore, there is evidence that a different animal model of ADHD, the genetically hypertensive (GH) rat, experiences sensory hyper-responsiveness in the superior colliculus (Clements et al., 2014).

Dyslexia is a condition which is often found to be comorbid with ADHD (Willcutt and Pennington, 2000, Overton, 2008) however it has also been associated with increased distractibility, irrespective of any comorbidity. This has been demonstrated in an investigation by Facoetti and Molteni (2001) into the reaction times and attention of participants diagnosed with dyslexia who had a specific reading difficulty but who did not have ADHD. It was found that where the control group had reaction times proportional to the difficulty of spotting the target appearance in the right visual field, the dyslexic group had no change in reaction time between targets appearing, regardless of relative

difficulty (Facoetti and Molteni, 2001). It was concluded that the results observed in the dyslexic group could be due to increased distractibility (Facoetti and Molteni, 2001). Increased distractibility has also been known to affect people with depression (Lepistö et al., 2004) and schizophrenia (Nunez Castellar et al., 2012), and has also been found in people who have suffered brain damage, such as in the case of stroke (Chao and Knight, 1995). Given the prevalence of heightened distractibility in numerous clinical groups and in healthy ageing it is important to better understand its neural basis.

1.2.2 NEURAL BASIS OF DISTRACTIBILITY

As indicated above, there are a number of areas within the brain linked to the regulation of healthy levels of distractibility but one area frequently noted is the SC. The SC is a highly conserved midbrain structure responsible for the organisation of eye movements in response to a novel or unexpected stimuli (Pierrot-Deseilligny et al., 1991, Overton, 2008, Krauzlis et al., 2013). The connection between the SC and altered distractibility can be observed from lesion studies in rats, where it was discovered that lesions to the SC resulted in a deficit in reflexive orienting towards novel stimuli (Goodale et al., 1978); yet the animals ability to perform visually guided running was unaffected by these collicular lesions. This finding led to the conclusion that, as a result of the SC function of controlling visual orientation towards a stimulus, it also has control over how focused or distractible an animal is (Goodale et al., 1978, Overton, 2008). This evidence is further supported by the observation of similar deficits in the orientation towards a stimuli found in hamsters (Mort et al., 1980), monkeys (Albano et al., 1982, Milner et al., 1978), and cats (Flandrin and Jeannerod, 1981) all following lesions to the SC. Furthermore ablation of the SC in

monkeys resulted in a decrease in distractibility during a fixation task (Wurtz and Albano, 1980).

In addition to the lesion studies described above, there is now increasing evidence that the SC is abnormal in ADHD, therefore further supporting the role of the SC as a neural correlate of distractibility. Evidence of this link is apparent from the observation that people with ADHD have been found to have difficulty inhibiting visual saccades (Klein et al., 2003, O'Driscoll et al., 2005) and shifts in covert attention (Swanson et al., 1991), which would be consistent with collicular dysfunction (Ignashchenkova et al., 2004, Katyal et al., 2010, Robinson and Bucci, 2014). Secondly, abnormal collicular behaviours and increased visual responsiveness within the SC have also been reported in rodent models of ADHD (Dommett and Rostron, 2011, Robinson and Bucci, 2014, Clements et al., 2014, Brace et al., 2015a).

Another area associated with the regulation of distractibility is the prefrontal cortex (PFC). Previous studies have found that there are alterations in the functional connections between the medial PFC and visual association cortex in individuals experiencing a natural increase in distractibility due to ageing, which resulted in a diminished ability to suppress irrelevant information in a visual task (Chadick et al., 2014). Further evidence to support the theory that the PFC is involved in mediating distractibility comes from investigations using tests of short term memory, which found young adults had greater activation of the rostral PFC, as well as the inferior parietal cortex, in comparison to the older adults, and again this reduction in activity in the PFC resulted in greater distractibility in the older participants (Campbell et al., 2012). Evidence that the PFC is involved in mediating levels of distractibility comes also from examinations using participants with focal lesions

located in the PFC. This investigation found that during an auditory task, participants with lesions in the PFC were significantly impaired in comparison to a control group following the introduction of distractors to the task, leading again to the conclusion that the PFC is essential for filtering irrelevant information (Chao and Knight, 1995)

Potential connections between areas thought to control distractibility become apparent when investigating distractibility associated with the SC in humans. There is evidence from human lesion studies to suggest that there is a pathway which runs between the SC and the PFC, and that this pathway is involved in attentional control (Gaymard et al., 2003b, Pierrot-Deseilligny et al., 1991). In one investigation it was found that small lesions to the parieto-collicular tract which interrupted the proposed pathway from SC to PFC resulted in a reduction in contralateral saccade accuracy towards an unpredictable target, but not to a predicted target (Gaymard et al., 2003b) so the interruption of the ascending pathway from the SC to the PFC resulted in the inhibition of reflexive shifts in visual attention. Further exploration into the connecting pathway between the SC and PFC in humans has been performed by observing the effects of lesions to the PFC on the generation of visual saccades. This study found that patients with lesions to the PFC made significantly more errors than control groups when performing an anti-saccade task (Pierrot-Deseilligny et al., 1991) which suggests the PFC has a role in the suppression of unwanted visual saccades (Pierrot-Deseilligny et al., 1991) and from these results it can be concluded that interruption of the pathway between the SC, the area responsible for generating visual saccades (Goldberg and Wurtz, 1972), and the PFC resulted in an increase in distractibility.

In addition to the PFC, the basal ganglia are another group of connected structures which have previously been associated with the inhibition of activity related to attentional control. Bari and Robbins (2013) have attempted to identify the regions of the brain responsible for inhibition of activity, to allow for better attentional control. A number of different areas were identified as a potential locus of inhibition within the brain, with the basal ganglia being commonly associated with inhibition of activity (Bari and Robbins, 2013). The basal ganglia is strongly linked to the SC (Redgrave et al., 2010), with ascending pathways from the SC able to provide important information relating to visual stimuli to the basal ganglia, so this pathway plays a significant role in the function of selection in the basal ganglia (Redgrave et al., 2010). There is further evidence that, in addition to the observed ascending projections from SC to basal ganglia, there are also returning neuronal projections from the basal ganglia to the SC (Hikosaka et al., 2000). It is theorised that this descending pathway has a role in the suppression of activity in the superior colliculus, in order to inhibit saccades and control the level of visual distractibility (Hikosaka et al., 2000).

The neural mechanisms involved in the expression of increased distractibility can be investigated with the use of animal models of diseases and conditions associated with an increase in distractibility. An example of this is the SHR, which is the most commonly used model of ADHD (Dommett and Rostron, 2011, Brace et al., 2015a). This model has been shown to exhibit inattention, hyperactivity and impulsivity, which are known to be the main symptoms of ADHD (Sagvolden and Johansen, 2011, Li et al., 2007). In addition to the SHR being a good behavioural model of ADHD, there is also evidence that the SHR has altered dopaminergic signalling in comparison to a control (Li et al., 2007, Linthorst et al., 1994) which is related to the model's eventual development of hypertension, but also

mimics similar dopaminergic signalling alterations found in humans with ADHD (Swanson et al., 2000).

The SHR model of ADHD has provided evidence of a link between the condition and dysfunction in the SC. As mentioned in section 1.2.1, the animals ability to height dependently alter air righting latency is lost (Dommett and Rostron, 2011) indicating dysfunction within the SC. The SHR has also demonstrated greater levels of distractibility in a visual orienting task and an increase in visual responsiveness in the SC (Brace et al., 2015a), indicating the SC is hyper-responsive to visual stimuli in the SHR. Areas connected to the SC which are similarly thought to be involved in controlling distractibility, such as the PFC, have also been associated with changes in the SHR. It has been found that there was significantly lower levels of specific dopamine (DA) receptor gene expression in the PFC of the SHR in comparison to the control strain (Li et al., 2007), and that $AMPA_R$-mediated synaptic transmission was reduced in pyramidal neurons in the PFC of the SHR (Cheng et al., 2017). Another animal model of ADHD also supports the link between dysfunction in the SC and increased distractibility. The response of the SC to a visual stimulus was greater in the New Zealand GH rat model of ADHD in comparison to a control strain (Clements et al., 2014), and furthermore acute administration of amphetamine, a drug used to treat increased distractibility in ADHD, was able to normalise this hyper-responsive activity in the GH rat (Clements et al., 2014).

1.3 Superior colliculus

1.3.1 STRUCTURE

The SC is a laminated midbrain structure (Figure 1.1 (A)) comprised of 7 alternating cellular and fibrous layers (Sparks and Nelson, 1987, Kanaseki and Sprague, 1974) which are often functionally subdivided into the superficial (layers I-III) and deep (layers IV-VII) SC as seen in Figure 1.1 (B). The division between superficial and deep SC represents differences between each area in both afferent and efferent projections, neuronal morphology, and physiological properties of each area (Stein and Meredith, 1993, Casagrande et al., 1972).
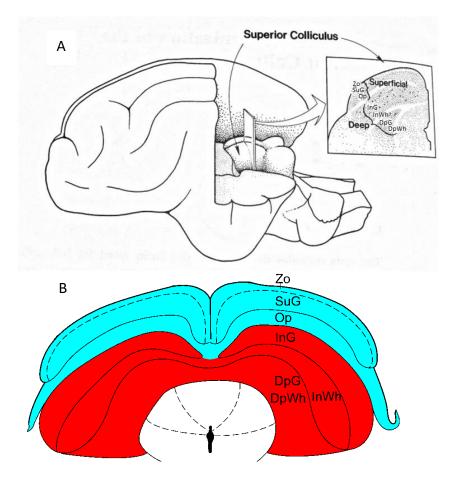
Figure 1.1 A diagram to show (A) the location of the superior colliculus in the human brain, adapted from Stein and Meredith (1993), and (B) structure of the superior colliculus in the rat brain at -6.3mm from bregma, with the superficial superior colliculus (comprised of the zonal layer (zo), superficial grey (SuG), and the optic nerve layer (Op)) coloured blue, and the deep SC (comprised of the intermediate grey (InG), intermediate white (InWh), deep grey (DpG), and deep white (DpWh)) coloured red. Adapted from Paxinos and Watson (1998).

The superficial layers of the SC are comprised of the zonal layer, superficial grey, and the optic nerve layer (opticum) (Wurtz and Albano, 1980); functionally these superficial layers are involved in visual processing and receive direct input from the retina (Casagrande et al., 1972, Overton, 2008). As well as directly from the retina, the superficial layer of the SC

receives afferent projections from the primary visual cortex (Collins et al., 2005) and the cortical eye fields (Bruce and Goldberg, 1985). As the superficial layers are primarily involved in visual processing, their efferent projections are not as extensive as those found in the deep layers which are involved more in multimodal and motor processing (Stein and Meredith, 1993, Grantyn and Grantyn, 1982). The ascending efferent pathways from the SC project to the thalamus and may transmit information on the functions of the deep layers of the SC (Stein and Meredith, 1993), however the more extensive descending pathways which project to the brainstem and spinal cord are involved in initiating behavioural motor responses for orientation and repositioning of the appropriate body part (e.g. eyes and head) (Hopkins and Niessen, 1976, Dean et al., 1989, Stein and Meredith, 1993). There are also extensive efferent projections from the superficial to the deep layers of the of the SC (Stein and Meredith, 1993).

Neuronal cells within the SC may have different functions. It has been found that some cells have in increased discharge rate immediately preceding a visual saccade, whereas other cells display increased firing during visual fixation (Wurtz and Optican, 1994). The increased firing rate has been shown not only to be a visual response, as increased fixation firing occurs regardless of whether there is a target for fixation present. It is hypothesised that the purpose of this increased firing rate in fixation related cells is to inhibit activity in the saccade related cells (Wurtz and Optican, 1994), which could be a cellular representation of a mechanism of attentional control. The saccade related cells can be further categorised into burst cells and build up cells (Wurtz and Optican, 1994, Munoz and Wurtz, 1995). Burst cells exhibit very little activity during fixation, but discharge a large amount of action potentials directly before the onset of a saccade, and these types of cells are found in the more superficial layers of the SC, however build up

cells display a more gradual increase in frequency of firing rate preceding saccade onset, and these cell types are associated more with deeper layers of the SC (Sparks, 1978, Wurtz and Optican, 1994). As well as differing neuronal cells, glial cells are also present in the SC and play an important role in the function of the superficial SC. Recent findings have indicated that in a rodent model of ADHD there was a reduced glia: neuron ratio (Brace et al., 2015a). This ratio has been shown to increase relative to neuronal size (Herculano-Houzel, 2014) therefore a lower ratio could indicate smaller neurons in this area.

1.3.2 FUNCTIONS

The superior colliculus is a midbrain structure which is highly conserved in vertebrates (Krauzlis et al., 2013). Although it is named the SC in mammals, it is also known as the optic tectum in non-mammalian vertebrates (Stein and Meredith, 1993, Krauzlis et al., 2013). The SC is centrally involved in detecting and responding to novel, unexpected and salient stimuli, involving the control of visual spatial attention and making saccadic eye movements (Goldberg and Wurtz, 1972, Krauzlis et al., 2013).

Saccadic eye movements can be either voluntary or reflexive and occur in order to assess a target stimulus using the fovea, which gives the greatest visual resolution (Purves et al., 2001, Shen and Paré, 2007). Saccades are therefore interspersed with periods of fixation and can be used to rapidly build up a topographic representation of the surrounding area. The SC is crucial for the generation of saccades (Wurtz and Albano, 1980). Saccadic eye movements triggered by the SC are caused when a stimulus activates a corresponding area in the retinotopic map of the superficial SC, and this activation is of a high enough

salience to trigger the motor representation of the map (Lee et al., 1988). Ablation of the SC in monkeys resulted in deficits in the generation of visually guided saccades. Specifically it reduced accuracy of fixation to peripheral targets, saccade frequency, and velocity of the saccade (Schiller et al., 1980).

The role of the SC in saccade generation means that it has it already has a large influence in controlling spatial attention and target selection. For this reason, the SC is also strongly implicated as having an important role in the regulation of distractibility. As previously discussed, a decrease in distractibility has been observed in primates following removal of the SC (Wurtz and Albano, 1980). Further evidence of the link between the SC and the regulation of distractibility comes from observations that damage to the SC can disrupt the orienting reflex of the rat towards a novel stimulus when it was presented outside the broad central area of the visual field (Goodale et al., 1978). Studies on lesions in humans have also found that damage to the SC has an effect on distractibility. A small lesion to the top of the brainstem, which interrupted a direct tract connecting the prefrontal cortex to the SC, resulted in an increase in distractibility while performing an anti-saccade task (Gaymard et al., 2003a). The connecting pathways between the SC and the PFC (Pierrot-Deseilligny et al., 1991), and also between the SC and basal ganglia (Redgrave et al., 2010) have also been implicated in the regulation of distractibility. From these findings there is clear evidence that the SC represents an essential part of the network controlling distractibility within the brain. Finally, as previously discussed in section 1.2.2, links can also be observed between the SC and the occurrence of heightened distractibility, with evidence of this apparent from behaviour and responsiveness of animal models of ADHD (Dommett and Rostron, 2011, Brace et al., 2015a, Clements et al., 2014) and from the observation that people with ADHD have a reduced ability to

suppress visual saccades (Klein et al., 2003, O'Driscoll et al., 2005). Dyslexia is another condition associated with poor saccadic control (Biscaldi et al., 1998) which has also been linked to heightened visual distractibility (Facoetti and Molteni, 2001).

It is also important to acknowledge that any role the SC in attentional control need not be limited to visual processing; this area is involved in multiple sensory systems, including auditory and somatosensory systems, as well as the visual system (Stein and Meredith, 1993). The layers of the SC each contain a topographic map of the sensory field, with the superficial layers containing a map of the visual field, and deeper layers are responsible for somatosensory and auditory sensory mapping (Wurtz and Albano, 1980, Lee et al., 1988, Overton, 2008).

1.4 Psychostimulants

1.4.1 EFFECTS OF PSYCHOSTIMULANTS ON DISTRACTIBILITY

Currently psychostimulant drugs, such as methylphenidate and amphetamine, are the most effective treatments to alleviate increased distractibility and both are used to treat ADHD (Himelstein et al., 2000, Overton, 2008, Teicher et al., 2000). More recently, non-stimulant drugs such as atomoxetine have also been found to be effective in ADHD and therefore may reduce increased distractibility (Accardo et al., 1999). For both psychostimulants and non-stimulants, the mechanisms of action within the brain are not fully understood. Similarly, the effects of long-term treatment are unclear. Behavioural sensitization has been observed following intermittent intraperitoneal (IP) injections of amphetamine (Robinson, 1984) which may be linked to an increase in drug self-administration (Lorrain et al., 2000, Chiodo et al., 2008), and so subsequently there have been concerns raised that treatment with psychostimulant drugs may increase drug abuse liability in adolescents (Marco et al., 2011). It may, therefore, be expected that treatment of ADHD using psychostimulants would result in increased risk of drug dependency or substance use disorders (SUD), however it has been observed that although adolescents with ADHD who have not received treatment were at increased risk of developing SUDs in comparison to a non-ADHD control group (Biederman et al., 1999), adolescents with ADHD who were treated with stimulants were actually at a significantly reduced risk of SUDs relative to their untreated counterparts (Biederman et al., 1999). This discrepancy may be due to differing routes of administration between the studies inducing sensitization and medical treatment of ADHD.

Although psychostimulant drugs, such as methylphenidate and amphetamine, are commonly prescribed to alleviate the increased distractibility experienced by people with ADHD, the effects of these drugs are also exploited for their cognitive enhancing effects by individuals not suffering from heightened distractibility (Ilieva et al., 2013, Smith and Farah, 2011). While some investigations have found that amphetamine treatment had no significant effect on measures of cognitive ability of healthy individuals (Ilieva et al., 2013, Lakhan and Kirchgessner, 2012), other studies have found that low to moderate acute doses of amphetamine decreased impulsive behaviours in healthy participants (De Wit et al., 2002).

Psychostimulants are also used recreationally at high doses. There is evidence that abuse of amphetamine resulted in an increase in impulsivity (Clark et al., 2006), which is contradictory to the reduction in impulsivity observed when a cognitive enhancing/therapeutic dose was used (De Wit et al., 2002). Chronic psychostimulant drug users were also found to have significant impairment in memory and executive function, which persisted following several years of abstinence (Ersche et al., 2006), indicating chronic use of high doses of psychostimulants caused long lasting changes within the brain. Behavioural responses to repeated psychostimulant administration are known to change over time; enhanced locomotor sensitization following administration of methylphenidate has been observed in rats (Tirelli et al., 2003, Andersen et al., 2002). Long lasting changes within the brain, and changes in behaviour following chronic treatment may signify underlying neuroadaptations in response to psychostimulant drug administration.

Further evidence that amphetamine treatment can reduce heightened distractibility is seen in an investigation into the links between foetal alcohol syndrome and ADHD in rats. This study found that exposing animals prenatally to ethanol resulted in super sensitivity of somato-dendritic dopaminergic receptors in the ventral tegmental area (VTA); however by chronically treating ethanol exposed animals with amphetamine, the super sensitive response found in untreated animals was counteracted (Shen et al., 1995).

As mentioned earlier, atomoxetine is a non-psychostimulant drug used to treat increased distractibility associated with ADHD (Turner et al., 2013, Accardo et al., 1999). At low doses atomoxetine inhibits the reuptake of noradrenaline (NA); it is believed that the increase in extracellular NA, particularly as it is localised to the PFC, provides one potential mechanism for the therapeutic actions of atomoxetine (Bymaster et al., 2002). At higher doses atomoxetine is also known to increase the extracellular levels of DA in the PFC, which could also be a contributing factor in the efficacy of the drug in the reduction of ADHD symptoms if a non-selective dose is used (Turner et al., 2013). When the effects of atomoxetine are compared to those of methylphenidate one identified advantage is that atomoxetine is unlikely to have the same abuse liability observed in psychostimulant drug studies (Bymaster et al., 2002, Heil et al., 2002), and there is evidence that atomoxetine may be effective in treating ADHD in methylphenidate non-responders (Newcorn et al., 2008, Bymaster et al., 2002) however overall response rates to psychostimulants are greater than the response rates to atomoxetine (Newcorn et al., 2008). For this reason, it can be concluded atomoxetine is less effective as a treatment for ADHD than psychostimulants, and therefore it is of greater priority to better understand the mechanisms used by psychostimulants in reducing distractibility, in order to ensure treatment is being utilised in the most effective manner.

1.4.2 MECHANISMS OF ACTION IN THE BRAIN

From research into the effects of psychostimulants it is known that they act on monoamine neurotransmitters (Sulzer and Rayport, 1990). The link between DA and amphetamine has been well established. For example, amphetamine treatment has been found to reduce spontaneous activity in dopaminergic neurons in the substantia nigra and VTA (Bunney and Aghajanian, 1973). Furthermore, as mentioned previously chronic amphetamine treatment counteracted the super sensitive response of somato-dendritic dopaminergic receptors in the VTA of animals prenatally exposed to ethanol (Shen et al., 1995).

Studies have also found that amphetamine inhibits the vesicular and synaptosomal accumulation of DA and NA (Easton et al., 2007) and the resulting increase of synaptic levels of these monoamines has been linked to the efficacy of amphetamine in the treatment of ADHD (Solanto, 1998, Easton et al., 2007). There is further evidence that amphetamine treatment also increases extracellular levels of serotonin (5-HT), particularly at high doses (Kuczenski and Segal, 1989). In addition to its effects on monoamines, amphetamine has also been found to decreases the number of transient voltage gated sodium channels in the PFC, resulting in an increase in the threshold for the generation of action potentials (Peterson et al., 2006). This effect may be one mechanism by which neuroadaptation to chronic amphetamine treatment occurs.

Although it has been established from previous research that psychostimulant drugs work by increasing both the function and the availability of DA and NA within the brain (Heal et al., 2009, Lakhan and Kirchgessner, 2012) and at high levels they can also act via 5-HT

mechanisms (Dommett et al., 2009). The target area of action within the brain is currently still under debate. A number of potential target areas for amphetamine have been identified; since amphetamine is known to affect dopaminergic signalling, the dopaminergic neurons of the midbrain have been proposed as one potential target site. Previous studies have found that i.p. injections of amphetamine reduced the sensitivity of dopaminergic neurons in the VTA to agonists, there was also a decrease in the area's auto-regulation ability, which was believed to be related to the mechanisms of behavioural sensitization (White and Wang, 1984). Administration of i.p. amphetamine was also found to increase the dendritic spine densities of medium spiny neurons located in the nucleus accumbens, an area of the brain which receives dopaminergic inputs from the VTA (Li et al., 2003). This increase in spine densities was localized to the distal dendrites which are known to be the site of convergence of dopaminergic and glutamatergic synapses in the striatum (Li et al., 2003).

It has also been hypothesised that the SC could be a therapeutic target for psychostimulants, since an observable dose dependent decrease in visual activity has been found within the superficial layers of the SC following acute amphetamine administration (Dommett et al., 2009, Gowan et al., 2008). In the SC there is limited dopaminergic input (Weller et al., 1987), however the SC receives more extensive NA and particularly 5-HT innervation (Weller et al., 1987, Parent et al., 1981), which seem to preferentially target the superficial visual layers (Parent et al., 1981, Gowan et al., 2008). Given that 5-HT is the main monoamine found in the SC, changes in visual activity found following amphetamine administration are likely mediated by 5-HT mechanisms (Gowan et al., 2008, Dommett et al., 2009).

In vitro administration of amphetamine was found to suppress weak activations within the SC, but retain strong activations, and so the signal to noise ratio in the SC was increased (Dommett et al., 2009). A higher signal to noise ratio would cause the target of a saccade to make a comparatively larger bid for a motor movement against an irrelevant stimuli, therefore this could be the mechanism utilised by psychostimulant drugs, in order to reduce distractibility (Dommett et al., 2009, Spencer et al., 2001, Gowan et al., 2008). It has also been found that acute amphetamine administration dose dependently decreases the visual responsiveness in the SC (Gowan et al., 2008). Acute amphetamine administration also reduced the emission of "high voltage waves" observed in the SC in both light and dark conditions (Chee, 1991).

Investigations into the effects of acute dosing with amphetamine on the superior colliculus found that rats with lesions to the SC exhibited consistently higher levels of activity in comparison to control groups. Conversely, levels of stereotypy (a behaviour commonly seen in animals treated with amphetamine) were significantly reduced at high doses of amphetamine when compared to the controls (Pope et al., 1980). This suggests that the SC is connected to the expression of behaviours associated with amphetamine treatment.

As mentioned earlier, the method by which psychostimulant drugs are administered has a great influence on the pharmacokinetics of the drugs in the body, as well as the pharmacodynamics. Clinical treatment of ADHD in humans is typically performed using orally administered therapeutic doses of psychostimulants (Kuczenski and Segal, 2005). Investigation into the effects of oral treatment with methylphenidate has found that when low therapeutic doses are delivered chronically (4 weeks) there was no difference

in locomotor activity, so no observable sensitization occurred (Kuczenski and Segal, 2002). It is also known that oral psychostimulant treatment results in a longer drug half-life, and lower peak drug concentrations than those observed following i.p.injections (Kuczenski and Segal, 2005). Exploration of peak activity and blood plasma levels following oral amphetamine administration in humans found that amphetamine treatment resulted in relatively uniform absorption, with doses of 0.5 mg/Kg resulting in peak levels slightly less than double the peak levels following a dose of 0.25 mg/Kg (Angrist et al., 1987). A study by Van Der Marel et al. (2014) also found that chronic (3 week) oral treatment with psychostimulant drugs had a small but significantly different effect on the structure of the adolescent rat brains in comparison to the adults.

The effects of continual amphetamine administration have also been investigated via the use of subcutaneous mini pumps. In this case it was found that animals experiencing continual low doses of amphetamine administration developed behavioural tolerance and not sensitization, but this effect was only seen during their least active (day/light) time period (Martin-Iverson and Iversen, 1989). Another study by Paterson et al. (2000) into the effects of continual amphetamine administration in rats found that administration of the drug resulted in decreased response latencies and lower reward thresholds, but drug withdrawal resulted in a subsequent increase in both reward thresholds and response latency. Furthermore, a second exposure to continual amphetamine administration resulted in greater lowering of the reward threshold and latency in comparison to the first administration. It was concluded that these results could represent sensitization to the amphetamine treatment, but tolerance to the effects of withdrawal (Paterson et al., 2000).

A number of investigations into the effects of treatment with amphetamine and methylphenidate have used i.p. injections as a method of drug delivery. When i.p. injections are used it is often found that behavioural sensitization can occur as a result of repeated treatment (Robinson and Kolb, 1999), this effect can persist even after the cessation of regular treatment patterns (Adriani et al., 2006), and behavioural sensitisation can also be observed in response to treatment with different stimulants, such as cocaine (Adriani et al., 2006, Marco et al., 2011). When treatment is delivered to adolescent rats, permanent changes to the structure and plasticity within the brain are also often observed (Robinson and Kolb, 1999, Adriani et al., 2006). These changes include persistent upregulation of specific transcripts causing altered expression of ionotropic and G-coupled receptors, resulting in an increase in extracellular DA (Adriani et al., 2006). Furthermore, long-lasting changes in synaptic connectivity within the nucleus accumbens and PFC have also been observed, including increases in dendritic spine density and the number of dendritic branches (Robinson and Kolb, 1999). However, these changes were found following repeated injections of psychostimulants, and it is not known whether these effects would also be found following oral treatment.

1.5 Research objectives

Despite the fact that psychostimulant drugs are often taken for an extended period of time, including in the treatment for ADHD, relatively little is known about their mechanism of action within the brain, and the potential long-term effects of treatment when taken chronically. Due to the links discovered previously between the SC and distractibility, and since psychostimulant drugs are known to be able to reduce the effects of distractibility, it is important to investigate whether these drugs have any effect on the SC when taken for an extended period of time, and what neuroadaptations if any result from prolonged exposure to the drug which may contribute to their therapeutic actions.

1.5.1 EXPERIMENTAL APPROACH

Since acute amphetamine administration has been shown to suppress activity in the superficial layers of the SC in both healthy animals (Dommett et al., 2009) and a rodent model of ADHD (Clements et al., 2014), it is anticipated that the SC, as a suspected neural correlate of distractibility, will be one target involved in the psychostimulant drugs' mechanism of action, and that chronic amphetamine treatment will cause alterations in structure and function of the superior colliculus. The impact of chronic oral amphetamine treatment on the SC will be examined using three levels of investigation:

1. SC-dependent behaviours

2. Electrophysiological measures of SC function

3. Measures of morphology within the SC

In addition, in order to ensure that SC dependent behaviours are not confounded by the psychomotor effects of amphetamine, the effects of chronic treatment with the drug on general activity levels will also be investigated.

1.5.2 RESEARCH AIMS

This thesis therefore aims to investigate:

- the effects of chronic amphetamine treatment on behaviour, specifically measures of collicular dependent behaviour;

- the effects of chronic amphetamine treatment on visual responsiveness within the superficial SC;

- the effects of chronic amphetamine treatment on the cellular and dendritic structure of the superficial visual layers of the SC;

- the effects of chronic amphetamine treatment on locomotor activity.

CHAPTER 2. EFFECTS OF AMPHETAMINE ON LOCOMOTOR ACTIVITY

2.1 Introduction

Repeated intermittent administration of psychostimulant drugs such as amphetamine to animals is known to generate sensitization, the enhancement of drug-induced locomotor activity (Robinson and Berridge, 1993). At low-to-moderate doses, acute injections of amphetamine result in an increase in locomotor activity. When administration is repeated intermittently, this effect sensitizes with each successive dose (Robinson, 1984). It is believed that this behavioural sensitization is caused by hypersensitivity within the dopaminergic systems (Robinson, 1984). The adaptation underlying the increase in psychomotor response observed is also thought to underlie the sensitization of the incentive motivational properties of the drug and this effect might be responsible for reinforcing certain aspects of drug addiction (Robinson and Berridge, 2001, Wise and Bozarth, 1987). Additionally, the treatment regimens known to cause locomotor sensitization are also associated with increased drug self-administration in animals (Chiodo et al., 2008, Lorrain et al., 2000). The mechanisms of action of these abuse level responses and behaviours in response to self-administration with amphetamine have been linked to dopaminergic neurons in the VTA (Deminiere et al., 1984, Koob and Bloom, 1988). Lesions to the dopaminergic projections in the forebrain resulted in a faster discrimination between an active and inactive lever used or self-administration of amphetamine (Deminiere et al., 1984). From this result it was determined that dysfunction in these dopaminergic projections in the brain could result in increased

vulnerability to the drugs (Deminiere et al., 1984) and sensitization to the reinforcing actions of amphetamine (Koob and Bloom, 1988).

Since these drugs are routinely prescribed to treat people with ADHD, concern has previously been raised that such long term treatment with psychostimulant drugs could increase potential drug abuse liability in children treated with these medications (Marco et al., 2011) especially when the treatment is prescribed to adolescents who may have enhanced vulnerability in comparison to adults, due to the ongoing maturation of the brain, which results in greater risk-taking behaviours in adolescents (Marco et al., 2011, Kelley et al., 2004). Contrary to this expectation, however, research actually indicates that, although un-medicated children with ADHD have a significantly increased risk for any substance use disorders compared to those without ADHD, those who are medicated with psychostimulants have a reduced risk in comparison to those who are un-medicated (Biederman et al., 1999).

This discrepancy in findings may be due to the route of drug administration used for people with ADHD. Investigations which utilise methods of continual drug delivery, such as mini-pumps, have found that treatment resulted in the development of tolerance to its effects (Chiodo et al., 2008, Chiodo and Roberts, 2009) which indicates reduced responsiveness to the drug in rodents. The method of drug delivery typically used for the treatment of ADHD is by oral administration of psychostimulants. This method of drug delivery is known to result in a slower initial rise in blood plasma levels of the drug and more sustained concentration when compared to injections (Kuczenski and Segal, 2005). Furthermore, the heightened drug concentrations appear to also continue for longer when oral drug treatment is used (Pashko and Vogel, 1980). For these reasons, it is

possible that the oral route of administration used in the treatment of ADHD results in a blood plasma profile more similar to methods of drug delivery known to result in drug tolerance as opposed to sensitization, which could explain the reduced risk of substance use disorders seen in those medicated with psychostimulants.

There has been no research conducted to date which aims to examine the impact of a therapeutically-relevant oral treatment regimen on locomotor activity in the rat. For this reason, and because locomotor sensitization could also become a confounding factor when investigating behavioural measures of distractibility, it is important to establish whether chronic oral administration of amphetamine results in sensitization or tolerance.

HYPOTHESIS

- It is hypothesized that chronic orally administered amphetamine treatment would result in tolerance to the locomotor effects of the drug.

2.2 Methods

2.2.1 ANIMALS

Male Hooded Lister rats (N=82), bred in-house as part of an on-going breeding colony, and aged six weeks (Mean ± standard error mean (SEM), 247g ± 2.75g) at the start of experiments were used. Animals were housed in groups of 2 – 3, with standard lab chow (RM3 diet, Special Diet Services, Witham, UK) and water available ad libitum within the home cages. Cages were kept in scantainers held at a temperature of 21-23 °C, and humidity of approximately 50 %. The holding room was on a 12 hr reverse dark-light cycle with lights turning on at 8 pm. All procedures were approved by an ethical committee, and were carried out in accordance with the Animal (Scientific Procedures) Act (1986). A timeline of chronic amphetamine treatment and all subsequent investigations into its effects on the SC can be seen in Figure 2.1.
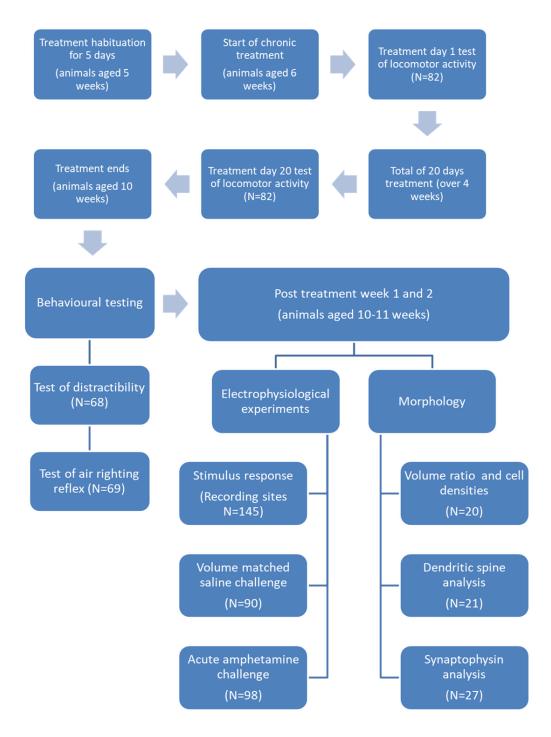
Figure 2.1 A timeline and overview of chronic treatment and all experimental procedures performed.

2.2.2 CHRONIC DRUG TREATMENT

Drugs were administered orally rather than by injection to more closely reflect how these drugs are taken by humans (Kuczenski and Segal, 2002). For the oral treatment drugs were mixed with apple juice (Just Juice, DME, Middlesex, UK) and fed to animals using a pipette to allow precise volumes to be administered (Wheeler et al., 2007). As well as allowing precise administration in the microlitre range, this method has few health risks compared to oral gavage which can result in damage to the oesophagus, or accidental drug delivery to lungs (Wolfensohn and Lloyd, 2003). Prior to chronic treatment animals were habituated to oral administration using 200 µl of apple juice for 5 days. Drugs were then administered every day for 4 weeks (excluding weekends) for a total of 20 days (Kuczenski and Segal, 2002).

Amphetamine (Sigma Aldrich, Gillingham, UK) was dissolved in distilled water, at a stock concentration 10 times greater than the actual dose and stored at -20 °C. Immediately prior to use the required amount of stock solution was thawed and diluted 1-in-10 with 100 % apple juice on the day of use. Three doses of amphetamine were used: 10 mg/kg, 5 mg/kg, and 2 mg/Kg. These doses were selected in order to ensure clinical relevance. Clinically used doses of amphetamine range from 5 to 60 mg (Greenhill et al., 2001, Wilens et al., 2004); these doses are thought to result in blood plasma concentrations between 120 and 140 ng/ml in people receiving treatment for ADHD (McGough et al., 2003, Ricaurte et al., 2005). When administered orally to rats, a dose 0.067 mg/Kg gives a peak plasma concentration of 4 ng/ml (Pashko and Vogel, 1980) therefore if linear scaling of plasma concentrations is assumed, a dose of 2 mg/Kg would amount to a blood plasma level of approximately 120 ng/ml. It was on this basis that our lower dose was chosen. The two higher doses were then selected to allow comparison with other existing

literature. Although this approach makes assumptions about linear scaling, it is generally accepted that the use of blood plasma levels is preferable to extrapolation on a milligram per kilogram basis from clinical doses when translating from humans to laboratory animals (Kuczenski and Segal, 2005). The vehicle solution consisted of the same volume of distilled water, also previously frozen and diluted 1-in-10 in apple juice. The drug treatment was performed blind, with randomly assigned letters representing each group. A fifth untreated group was also used as an additional control to ensure that the administration procedure itself did not have any effect, as have been found previously, albeit for injections (Berridge et al., 2006).

2.2.3 BEHAVIOURAL TESTING

The animals (untreated N = 16; vehicle N = 16; 2 mg/Kg N = 16; 5 mg/Kg N = 18; 10 mg/Kg N = 16) were kept on a reverse dark-light cycle, so all behavioural habituation and testing took place in the dark phase, and during the middle of the day (between 10am and 5pm). Performing testing at this time ensured the animals were in their most active period of the day.

Recording of locomotor activity was performed using automated Activity Monitoring Chambers (Med-Associates, Sandown Scientific, Hampton, Middlesex, UK). These consisted of a clear chamber surrounded by two levels of infrared transmitters and receivers, which recorded breaks of the infrared beams in order to measure the animal's horizontal and vertical activity as shown in Figure 2.2.

Figure 2.2 Activity monitoring chambers used to record locomotor activity.

Activity chambers were cleaned after each test, before a new animal entered the chamber, in order to remove all olfactory cues. The animals were habituated to the chambers for 15 minutes on two consecutive days immediately prior to the first locomotor activity test to ensure familiarity with the chambers. Locomotor activity was then recorded for one hour on the first and the final day of chronic treatment beginning 30 minutes after administration. This time period was chosen to ensure that the peak psychostimulant activity, which occurs approximately 1 hour post drug administration, was recorded (Kuczenski and Segal, 2002, Martínez-Clemente et al., 2013, Sakai et al., 1983).

2.2.4 DATA ANALYSIS

All data were stored as 5 minute epochs and the categories of activity, and therefore, dependent variables, recorded for offline analysis were: the number of jumps, horizontal distance travelled, horizontal activity, stereotypic activity and vertical activity. The horizontal activity was measured by the number of x and y-axis infrared beams broken as the animal moved about the chamber. Furthermore the animals movements were classified as horizontal activity (as opposed to stereotypic activity) once the animal had moved outside of a predetermined box of four horizontal beams. Similarly vertical activity was measured whenever there were beam breaks of both the z-axis, which are the higher level beams seen in Figure 2.2, and either the x or y-axis. Jumps differ from vertical activity as they are measured whenever there were no x or y-axis beams broken. One limitation of these measurements however, is that a jump may be recorded in instances of wall rearing where the horizontal beams pass between the animals' legs without being broken, and so there may be instances of correlation between the number of jumps and vertical activity recorded for this reason. Both variables have been analysed separately to provide a comprehensive overview of their locomotor activity, since there is no way of further differentiating jump behaviour offline. Finally, instances of stereotypic behaviour were defined and counted as the number of beam breaks within a 4 beam by 4 beam box. This encompasses behaviours such as stereotyped head waving, sniffing, licking, and gnawing which all occur within a discrete area (Pope et al., 1980). However, it should be acknowledged that these measurements may also include some non-stereotyped grooming behaviours, and exclude any stereotypic behaviour exhibited over a wider area (Inglis et al., 1994). In order to rule out that latter, the map of each animals' movement around the chamber, which is created automatically was examined for a subset of

animals. A sample of 25 maps (N=5 for each dose group; 2, 5 and 10 mg/Kg and each control group; treated and untreated) were accessed and visually examined for instances of repetitive behaviour over a wider region. No such instances were identified . Each of these measures were checked for normality using the Kolmogorov-Smirnov test and measures of skewness and kurtosis, and were deemed suitable for use with parametric tests. Following normality testing each measure of activity was analysed in two ways. Firstly, the Day 1 data was analysed in order to see what effect acute amphetamine had on behaviour. For this analysis a repeated measures ANOVA was run for the Day 1 data for each of the different chronic treatment groups with TIME as the within subjects factor to investigate the time course of drug effects on activity. For all repeated measures ANOVAs, if the assumption of sphericity was violated, the Greenhouse-Geisser results were reported (Greenhouse and Geisser, 1959). In addition, the total activity for each parameter was calculated by summing data across the entire 60 minute period for each experimental subject. This was then used to conduct a One-Way ANOVA to compare each activity parameter between the different treatment groups.

Secondly, the Day 20 data was analysed, and compared to the data obtained on Day 1 in order to investigate the effect of chronic amphetamine treatment. This was analysed in three ways for each of five parameters measuring activity. Initially, for each drug dose, a repeated measures ANOVA was run, with TIME as the within subjects factor, to investigate the time course of drug effects on activity. Next, the total activity for each parameter was calculated by summing data across the entire 60 minute period for each experimental subject. This was then used to conduct a One-Way ANOVA to compare each activity parameter between the different chronic treatment groups. Finally, in order to investigate the presence of behavioural sensitization or tolerance, each drug condition

was analysed using a Two-Way repeated measures ANOVA to investigate whether the responses over the 60 minute period differed between Day 1 and Day 20.

2.3 Results

2.3.1 ACUTE EFFECTS OF AMPHETAMINE TREATMENT

As mentioned in the methods, data from the first day of treatment was analysed in order to determine the effects that acute amphetamine treatment had on locomotor activity, with the view to checking for sensitization after chronic treatment. After normality was confirmed and prior to the main analyses outlined above, a T-test was run to compare the untreated and treated (0 mg/Kg) control groups, the results of which can be seen in Table 2.1. As there are significant differences between the untreated and treated controls, both control groups will be used in the main analysis.

	Untreated (N=16)	Treated control (N=16)	T-test
Jumps	14.86 ± 1.14	18.38 ± 1.92	$t_{(24.41)} = -1.58$, $p = 0.127$
Distance travelled	446.33 ± 36.86	658.48 ± 51.55	$t_{(27.16)} = -3.35$, $p = 0.002$
Horizontal activity	235.30 ± 21.80	255.05 ± 23.91	$t_{(29.75)} = -0.61$, $p = 0.546$
Stereotypic activity	798.43 ± 61.60	608.73 ± 46.88	$t_{(28.01)} = 2.45$, $p = 0.021$
Vertical activity	16.24 ± 1.20	36.21 ± 5.00	$t_{(16.73)} = -3.89$, $p = 0.001$

Table 2.1 For both control groups the mean ± SEM is shown for each locomotor measure, and results of independent samples T-test reveal significant differences for three of the five measures.

The results of the main analysis are displayed in Figure 2.3. These graphs reveal a general pattern which distinguishes both control groups and the 2 mg/Kg group, which all decreased over the hour the animals were being monitored for, presumably as the animals habituated to their novel environment, from the other two groups. The 5 and 10

mg/Kg groups, however, showed less decline in activity, with high activity levels continuing throughout the monitoring period.
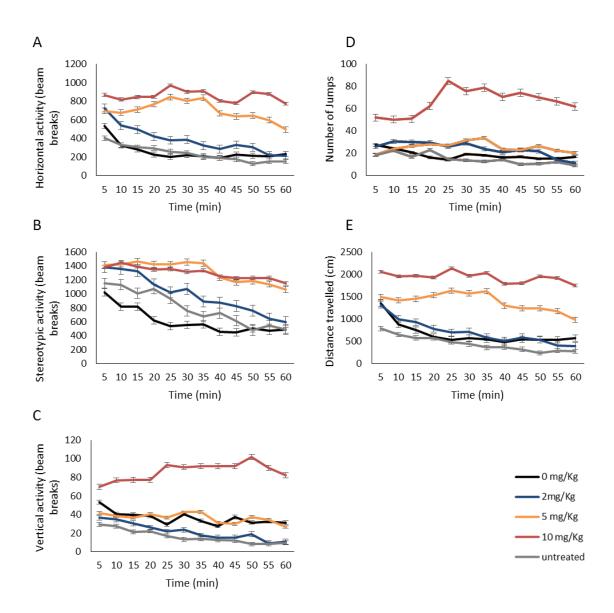
Figure 2.3. The effects of acute amphetamine treatment over one hour of activity monitoring, measuring A) horizontal activity, B) stereotypic activity, C) vertical activity, D) jumps and E) distance travelled. All data is shown as mean ± SEM.

A repeated measures ANOVA with TIME as the within measures factor and DOSE as the between measures factor for Day 1 revealed significant main effects of TIME for all measures (horizontal activity: $F_{(4.595, 353.794)} = 12.381$, $p < 0.001$, $\eta^2 = 0.14$, Power =

1.00 ; vertical activity: F (4.161, 320.413) = 3.309, p = 0.010, η^2 = 0.04, Power = 0.85 ;

stereotypic activity: F (5.421, 417.390) = 50.591, p < 0.001, η^2 = 0.40, Power = 1.00 ;

number of jumps: F (4.414, 339.876) = 2.977, p = 0.016, η^2 = 0.04, Power = 0.82 F (4.896,

377.017) = 21.243, p < 0.001, η^2 = 0.22, Power = 1.00). For horizontal activity within-

subjects difference contrasts revealed there were significant decreases between all

consecutive time points except between 20 and 35 minutes when the main effect of time

is considered across doses. For vertical activity within-subjects difference contrasts

revealed there were significant decreases in activity between 35 and 40 minutes, then

again from 50 to 55 minutes and 55 to 60 minutes. Further investigation into stereotypic

activity revealed there were significant decreases in activity between each consecutive

time point except from the 5 to 10 minute epoch. For the number of jumps, within-

subjects difference contrasts found a significant difference in activity only from 50 to 55

minutes, and 55 to 60 minutes when the effect of time was considered across all

treatment groups. Finally, for the distance travelled, within-subjects difference contrasts

found a significant decrease in activity between every consecutive time point.

There were also significant main effects of DOSE for each measure of activity (horizontal

activity: F (4, 77) = 13.936, p < 0.001, η^2 = 0.42, Power = 1.00; vertical activity: F (4, 77) =

16.590, p < 0.001, η^2 = 0.34, Power = 1.00; stereotypic activity: F (4, 77) = 8.253, p <

0.001, η^2 = 0.46, Power = 1.00; number of jumps: F (4, 77) = 8.253, p < 0.001, η^2 = 0.30,

Power = 1.00; horizontal distance travelled: DOSE F (4, 77) = 26.222, p < 0.001, η^2 = 0.58,

Power = 1.00).

Post hoc Tukey testing (Table 2.2) revealed that the two higher dose groups consistently

displayed significantly higher levels of activity in comparison to the 2 mg/Kg group and

both control groups. The 2 mg/Kg group also displayed significantly higher levels of stereotypic behaviour (Figure 2.3(B)) in comparison to the 0 mg/Kg group (p = 0.008) but not the untreated control (p = 0.417).

		5 mg/Kg	10 mg/Kg
Horizontal activity	untreated	$p < 0.001$	$p < 0.001$
	0 mg/Kg	$p < 0.001$	$p < 0.001$
	2 mg/Kg	$p = 0.027$	$p < 0.001$
Vertical activity	untreated	$p = 0.470$	$p < 0.001$
	0 mg/Kg	$p = 1.000$	$p = 0.001$
	2 mg/Kg	$p = 0.741$	$p < 0.001$
Stereotypic activity	untreated	$p < 0.001$	$p < 0.001$
	0 mg/Kg	$p < 0.001$	$p < 0.001$
	2 mg/Kg	$p = 0.023$	$p = 0.040$
Distance Travelled	untreated	$p < 0.001$	$p < 0.001$
	0 mg/Kg	$p < 0.001$	$p < 0.001$
	2 mg/Kg	$p = 0.001$	$p < 0.001$
Jumps	untreated	$p = 0.833$	$p < 0.001$
	0 mg/Kg	$p = 0.957$	$p < 0.001$
	2 mg/Kg	$p = 1.000$	$p = 0.001$

Table 2.2 Results of post hoc Tukey HSD testing between the high dose groups, and the controls and low dose group on the repeated measures ANOVA analysis performed on data collected from activity monitoring following an acute (Day 1) treatment with amphetamine (or control).

In addition to the main effects, significant interaction effects between TIME and DOSE were found for each measure (horizontal activity: $F_{(18.379, 2.740)} = 2.842$, $p < 0.001$, $\eta^2 = 0.13$, Power = 1.00; vertical activity: $F_{(16.645, 320.413)} = 2.342$, $p = 0.002$, $\eta^2 = 0.11$, Power = 0.99; stereotypic activity: $F_{(21.683, 417.39)} = 3.503$, $p < 0.001$, $\eta^2 = 0.15$, Power = 1.00; number of jumps: $F_{(17.656, 339.876)} = 2.842$, $p < 0.001$, $\eta^2 = 0.13$, Power = 1.00; horizontal distance travelled: $F_{(19.585, 377.017)} = 2.714$, $p < 0.001$, $\eta^2 = 0.12$, Power = 1.00).

For horizontal activity restricted ANOVAs were run between each possible combination of group pairings (the results of restricted ANOVAs are reported in full in Appendix A), and found significant interaction effects in each combination except between the 0 mg/Kg and 10 mg/Kg groups, untreated and 2 mg/Kg groups, and the 5 mg/Kg and 10 mg/Kg groups, which may be due to small overlaps between the different chronically treated groups over time, as seen in Figure 2.3 (A). For vertical activity restricted ANOVAs found significant interaction effects when investigating the untreated and 5 mg/Kg groups, untreated and 10 mg/Kg groups, 2 mg/Kg and 5 mg/Kg groups, and also the 2 mg/Kg and 10 mg/Kg groups. These interactions may be due to the steady decrease in activity over time observed in the 2 mg/Kg and untreated groups, in comparison to the generally constant level of activity observed in the 5 mg/Kg and 10 mg/Kg groups (Figure 2.3 (C)). It can also be observed from the data that vertical activity (Figure 2.3 (C)) and jumps (Figure 2.3 (D)) have very similar patterns of activity across groups, with the comparatively high levels of activity in the 10 mg/Kg groups particularly noticeable in both. As mentioned in chapter 2.2.4 these similarities may be due in part to some wall righting being attributed to jumps as opposed to vertical activity when the infrared beam travels unbroken between the animals' legs. For stereotypic activity restricted ANOVAs found significant interaction effects in each combination except between the 2 mg/Kg and untreated groups, and the 5 mg/Kg and 10 mg/Kg groups, from Figure 2.3 (B) it can be seen that the activity levels of the 2 mg/Kg and 5 mg/Kg groups run in parallel to the untreated and 10 mg/Kg groups respectively, which may explain the lack of interaction for these specific comparisons. For the number of jumps, there were significant interaction effects found using restricted ANOVAs in each combination except between the untreated groups and either the 0 mg/Kg group or the 2 mg/Kg, and also no interaction found between the 5

mg/Kg group and either the 2 mg/Kg group or the 10 mg/Kg group. For the distance travelled, significant interaction effects were found when investigating the 0 mg/Kg and untreated groups, 0 mg/Kg and 5 mg/Kg groups, 0 mg/Kg and 10 mg/Kg groups, 2 mg/Kg and 5 mg/Kg groups, and also the 5 mg/Kg and untreated groups. The interaction effects associated with the 0 mg/Kg group may be due to the comparatively steeper decline in activity of this group over the initial 15 minutes as seen in Figure 2.3 (E).

In order to compare the level of activity between dose groups across the whole period a One Way ANOVA was run on the data averaged over the total 60 min, and in line with the effects seen above, there were significant differences between the five chronic treatment groups for each of the five recorded variables (horizontal activity: $F_{(4, 77)}$ = 13.936, $p < 0.001$, η^2 = 0.42, Power = 1.00; vertical activity: $F_{(4, 77)}$ = 9.816, $p < 0.001$, η^2 = 0.34, Power = 1.00; stereotypic activity: $F_{(4, 77)}$ = 16.590, $p < 0.001$, η^2 = 0.46, Power = 1.00; jumps: $F_{(4, 77)}$ = 8.253, $p < 0.001$, η^2 = 0.30, Power = 1.00; distance travelled: $F_{(4, 77)}$ = 26.222, $p < 0.001$, η^2 = 0.58, Power = 1.00). Tukey HSD post hoc testing revealed a large number of differences between groups as seen in Figure 2.4.
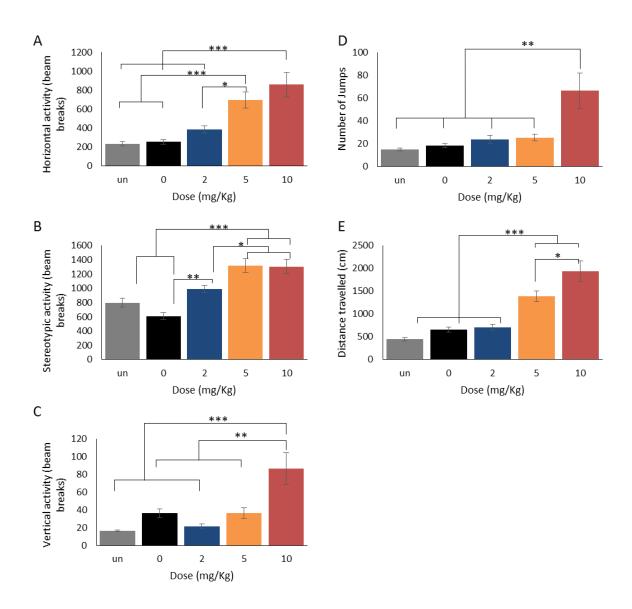
Figure 2.4. The effect of acute amphetamine treatment on the amount of A) horizontal activity, B) stereotypic activity, C) vertical activity, D) jumps and E) distance travelled per 5 minute bin (averaged over 60 minutes), for each pre-treated group on the first day of treatment (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). All data is shown as mean ± SEM. (un = untreated).

2.3.2 CHRONIC EFFECTS OF AMPHETAMINE TREATMENT

Following the chronic treatment period the above analysis was repeated using data collected on the final day of drug treatment in order to investigate the effects of chronic amphetamine treatment on the rats' level of activity. Using the Kolmogorov-Smirnov test the data was determined to be normally distributed, and again a T-test between the two control groups revealed significant differences, therefore the main analysis was performed using both control groups, the results on the T-test can be seen in Table 2.3.

	Untreated (N=16)	Treated control (N=16)	T-test
Jumps	15.26 ± 1.92	17.45 ± 3.05	t (25.30) = -0.61, p = 0.548
Distance travelled	48.68 ± 2.73	175.54 ± 19.40	t (21.251) = -3.94, p = 0.001
Horizontal activity	48.15 ± 1.83	128.50 ± 17.67	t (22.09) = -2.29, p = 0.032
Stereotypic activity	446.33 ± 36.86	658.48 ± 51.55	t (22.874) = -0.84, p = 0.412
Vertical activity	410.23 ± 34.97	735.63 ± 74.85	t (20.685) = -2.77, p = 0.012

Table 2.3 The mean ± standard error mean, and results of independent samples T-test for both control groups using data from the final day of pre-treatment.

The effect of time in each parameter is illustrated in Figure 2.5. Levels of activity are initially high and decrease over time as the animal habituated to its environment, as was the case for the control groups and the 2 mg/Kg group following the initial acute dose of amphetamine (Figure 2.3). Following chronic treatment, however, the activity levels of both the 5 mg/Kg and 10 mg/Kg groups also decrease overtime, whereas they had previously stayed consistently higher following the acute dose.
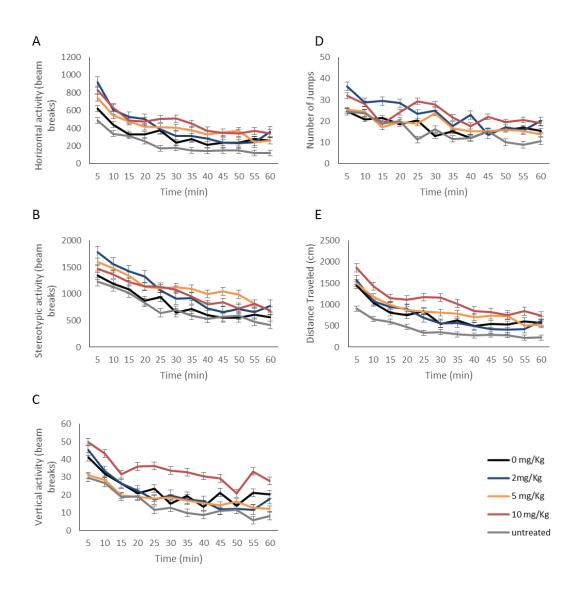

Figure 2.5. The effects of chronic amphetamine treatment over one hour of activity monitoring, measuring A) horizontal activity, B) stereotypic activity, C) vertical activity, D) jumps and E) distance travelled. All data is shown as mean ± SEM.

Once again a repeated measures ANOVA was run with TIME as the within measures factor and DOSE as the between measures factor. This analysis revealed significant main effects of TIME for each measure (horizontal activity: $F_{(6.352, 489.110)} = 78.145$, $p < 0.001$, $\eta^2 = 0.50$, Power = 1.00; vertical activity: $F_{(7.806, 601.099)} = 49.174$, $p < 0.001$, $\eta^2 = 0.39$, Power = 1.00; stereotypic activity: $F_{(6.917, 532.631)} = 107.895$, $p < 0.001$, $\eta^2 = 0.58$, Power = 1.00; Jumps: $F_{(8.434, 649.431)} = 12.891$, $p < 0.001$, $\eta^2 = 0.14$, Power = 1.00; horizontal distance travelled: $F_{(6.997, 538.744)} = 98.814$, $p < 0.001$, $\eta^2 = 0.56$, Power = 1.00). Within-subjects difference contrasts revealed there were significant decreases in activity between all consecutive time points for horizontal activity, vertical activity, stereotypic activity, and distance travelled. For the number of jumps, within-subjects difference contrasts found a significant difference in activity over all time points except from 5 to 10 minutes, 15 to 20 minutes, and 25 to 30 minutes.

Significant main effects of DOSE were found for all measures except for the number of jumps (horizontal activity: $F_{(4, 77)} = 6.968$, $p < 0.001$, $\eta^2 = 0.27$, Power = 0.99; vertical activity: $F_{(4, 77)} = 6.866$, $p < 0.001$, $\eta^2 = 0.26$, Power = 0.99; stereotypic activity: $F_{(4, 77)} = 7.129$, $p < 0.001$, $\eta^2 = 0.27$, Power = 0.99; Jumps: $F_{(4, 77)} = 2.120$, $p = 0.086$, $\eta^2 = 0.10$, Power = 0.60; horizontal distance travelled: $F_{(4, 77)} = 11.934$, $p < 0.001$, $\eta^2 = 0.38$, Power = 1.00). The significant results of post hoc Tukey testing is shown in Table 2.4. Significant differences, when present, were found particularly between the control groups and amphetamine treated groups, and the 10 mg/Kg group had significantly higher levels of vertical activity in comparison to all other chronic treatment groups (Figure 2.5 (C)).

	Horizontal		Vertical	Stereotypic		Distance	
	untreated	0 mg/Kg	10 mg/Kg	untreated	0 mg/Kg	untreated	10 mg/Kg
untreated		n.s.	$p < 0.001$		n.s.		$p < 0.001$
0 mg/Kg	n.s.		$p = 0.036$	n.s.		$p = 0.013$	$p = 0.008$
2 mg/Kg	$p = 0.005$	n.s.	$p = 0.014$	$p = 0.006$	n.s.	$p = 0.018$	$p = 0.005$
5 mg/Kg	$p = 0.003$	n.s.	$p = 0.001$	$p < 0.001$	$p = 0.006$	$p < 0.001$	n.s.
10 mg/Kg	$p < 0.001$	$p = 0.045$		$p = 0.015$	n.s.	$p < 0.001$	

Table 2.4. All significant results found using post hoc Tukey HSD testing on the repeated measures ANOVA analysis performed on data collected from activity monitoring following chronic (Day 20) treatment with amphetamine (or control). (n.s. = non-significant)

Finally, there were also significant interaction effects between TIME and DOSE for each measure other than the number of jumps (horizontal activity: $F_{(25.408, 489.110)} = 2.163$, $p = 0.001$, $\eta^2 = 0.10$, Power = 1.00; vertical activity: $F_{(31.226, 601.099)} = 1.844$, $p = 0.004$, $\eta^2 = 0.09$, Power = 1.00; stereotypic activity: $F_{(27.699, 532.631)} = 2.252$, $p < 0.001$, $\eta^2 = 0.11$, Power = 1.00; Jumps: $F_{(33.731, 649.431)}$, $p = 0.197$, $\eta^2 = 0.06$, Power = 0.96; horizontal distance travelled: $F_{(27.987, 538.744)} = 1.988$, $p = 0.002$, $\eta^2 = 0.09$, Power = 1.00). For horizontal activity, restricted ANOVAs found significant interaction effects for each combination involving the 2 mg/Kg group. As seen in Figure 2.5 (A) this group has a steeper initial decline in activity, which may explain the interaction effects found. For vertical activity there were significant interaction effects for the 0 mg/Kg group when in combination with the untreated, 2 mg/Kg and 5 mg/Kg groups, there was also a significant interaction effect observed for the combination of 2 mg/Kg and 5 mg/Kg groups. For stereotypic activity significant interaction effects were found for the 2 mg/Kg group when paired with the untreated, 5 mg/Kg, and 10 mg/Kg groups. In Figure 2.5 (B) it appears that the activity of the 2 mg/Kg group continues to decrease steeply after 25 minutes, whereas the activity levels of the untreated, 5 mg/Kg and 10 mg/Kg groups seem to plateau at this point, which may be the reason for the interaction effects observed.

There was also a significant interaction effect observed for the combination of 0 mg/Kg and 5 mg/Kg groups. Finally for the distance travelled, significant interaction effects were found when investigating the 0 mg/Kg and untreated groups, 2 mg/Kg and untreated groups, 10 mg/Kg and untreated groups, and the 2 mg/Kg and 5 mg/Kg groups. The distance travelled for the untreated group has a more gradual decrease over time in comparison to the 0 mg/Kg, 2 mg/Kg, and 10 mg/Kg groups, as seen in Figure 2.5 (E). No restricted ANOVAs were performed for the number of jumps, because there was no interaction effect found between DOSE and TIME.

A One-Way ANOVA was also run on the data averaged over the full 60 minutes in order to further investigate differences between treatment groups. As was shown in the above analysis, there were significant differences between doses in each recorded parameter (horizontal activity: $F_{(4, 77)} = 6.968$, $p < 0.001$, $\eta^2 = 0.27$, Power = 0.99; vertical activity: $F_{(4, 77)} = 6.866$, $p < 0.001$, $\eta^2 = 0.26$, Power = 0.99; stereotypic activity: $F_{(4, 77)} = 7.129$, $p < 0.001$, $\eta^2 = 0.27$, Power = 0.99; distance travelled: $F_{(4, 77)} = 11.934$, $p < 0.001$, $\eta^2 = 0.38$, Power = 1.00) except for jumps ($F_{(4, 77)} = 2.120$, $p = 0.086$, $\eta^2 = 0.10$, Power = 0.60). To identify which dose groups differed significantly post hoc Tukey HSD tests were conducted and the results are displayed in Figure 2.6 below. Although there are still significant differences in most activity levels between the different treatment groups, seen particularly between the high dose group and controls, the actual levels of activity are far more similar following chronic treatment when compared to the acute data (Figure 2.4) where there was a very clear dose dependent increase in activity as dose strength increased.
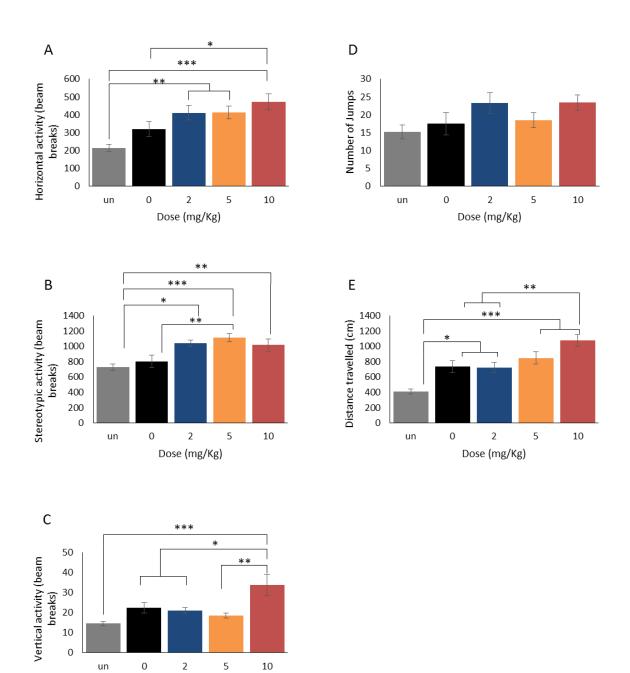
Figure 2.6. The effect of chronic amphetamine treatment on the amount of A) horizontal activity, B) stereotypic activity, C) vertical activity, D) jumps and E) distance travelled per 5 minute bin (averaged over 60 minutes), for each pre-treated group on the first day of treatment (* p< 0.05, ** p< 0.01, *** p<0.001). All data is shown as mean ± SEM. (un = untreated)

2.3.3 BEHAVIOURAL SENSITIZATION OR TOLERANCE

To determine whether any changes in responsiveness had taken place, such as the development of either behavioural sensitization or tolerance following the chronic amphetamine treatment, a two-way repeated measures ANOVA was run for each of the five parameters, using DAY and TIME as within subjects measures, and DOSE as a between subjects measure. As the significant main effects of TIME and DOSE and the interaction between them has been reported above for both the first and final days of treatment, just the main effects of DAY and any interactions from the two way ANOVA are reported here.

For each measure of activity except stereotypic activity there was a significant main effect of DAY, and there were also significant interaction effects for every measure (horizontal activity: DAY $F_{(1, 77)} = 13.070$, $p = 0.001$, $\eta^2 = 0.15$, Power = 0.95, interaction effects between DAY and DOSE $F_{(4, 77)} = 7.355$, $p < 0.001$, $\eta^2 = 0.28$, Power = 1.00, TIME and DAY $F_{(5.896, 453.976)} = 9.750$, $p < 0.001$, $\eta^2 = 0.11$, Power = 1.00, and TIME, DAY, and DOSE $F_{(23.583, 453.976)} = 1.882$, $p = 0.008$, $\eta^2 = 0.09$, Power = 0.99; Vertical activity: DAY $F_{(1, 77)} = 27.739$, $p < 0.001$, $\eta^2 = 0.27$, Power = 1.00, interaction effects between DAY and DOSE $F_{(4, 77)} = 8.115$, $p < 0.001$, $\eta^2 = 0.30$, Power = 1.00, TIME and DAY $F_{(5.120, 394.254)} = 4.442$, $p = 0.001$, $\eta^2 = 0.06$, Power = 0.97, and TIME, DAY, and DOSE $F_{(20.481, 394.254)} = 1.943$, $p = 0.009$, $\eta^2 = 0.09$, Power = 0.99; Stereotypic activity: DAY $F_{(1, 77)} = 2.476$ $p = 0.120$, $\eta^2 = 0.03$, Power = 0.34 interaction effects between DAY and DOSE $F_{(4, 77)} = 4.894$, $p = 0.001$, $\eta^2 = 0.20$, Power = 0.95, TIME and DAY $F_{(7.184, 553.130)} = 11.301$, $p < 0.001$, $\eta^2 = 0.13$, Power = 1.00, and TIME, DAY, and DOSE $F_{(28.734, 553.130)} = 2.151$, $p = 0.001$, $\eta^2 = 0.10$, Power = 1.00; Jumps: DAY $F_{(1, 77)} = 11.789$, $p = 0.001$, $\eta^2 = 0.13$, Power = 0.92, interaction effects between DAY and DOSE $F_{(4, 77)} = 7.654$, $p < 0.001$, $\eta^2 =$

0.29, Power = 1.00, TIME and DAY F (6.683, 514.609) = 4.647, p < 0.001, η^2 = 0.06, Power

= 0.99, and TIME, DAY, and DOSE F (26.733, 514.609) = 1.587, p = 0.032, η^2 = 0.08, Power

= 0.98; distance travelled; DAY F (1, 77) = 23.989, p<0.001, η^2 = 0.24, Power = 1.00,

interactions between DAY and DOSE F (4, 77) = 11.240, p < 0.001, η^2 = 0.37, Power = 1.00,

TIME and DAY F (6.358, 489.592) = 8.757, p < 0.001, η^2 = 0.10, Power = 1.00, and TIME,

DAY, and DOSE F (25.433, 489.592) = 1.943, p = 0.001, η^2 = 0.10, Power = 1.00). In each

case where there was a significant main effect of day, the activity levels on the final day

were found to be lower than the activity levels observed on the first day.

Restricted repeated measures ANOVAs were performed for each combination of pairs for

chronic treatment. For horizontal activity interactions between DAY and DOSE were

found for each combination except for 0 mg/Kg and 2 mg/Kg, 0 mg/Kg and untreated, 2

mg/Kg and untreated, and 5 mg/Kg and 10 mg/Kg. Interaction effects between TIME, DAY

and DOSE were found for the combination of 0 mg/Kg and 5 mg/Kg, and 2 mg/Kg and 5

mg/Kg. For vertical activity interactions between DAY and DOSE were found for every

combination except for 0 mg/Kg and 5 mg/Kg, and 2 mg/Kg and untreated. Interaction

effects between TIME, DAY and DOSE were only found for the restricted ANOVA

performed with the untreated and 10 mg/Kg groups. For stereotypic activity there were

interaction effects found between DAY and DOSE when investigating 0 mg/Kg and

untreated, 0 mg/Kg and 5 mg/Kg, 0 mg/Kg and 10 mg/Kg, and 2 mg/Kg and 10 mg/Kg.

Interactions between TIME, DAY and DOSE were found when investigating the

combination of untreated and 0 mg/Kg, untreated and 2 mg/Kg, untreated and 10 mg/Kg,

0 mg/Kg and 5 mg/Kg, 2 mg/Kg and 5 mg/Kg, and 5 mg/Kg and 10 mg/Kg. For the number

of jumps, the significant interaction effect between DAY and DOSE was retained for the

combinations 10 mg/Kg with every other group, and also for untreated and 5 mg/Kg.

Significant interactions between TIME, DAY and DOSE were found in restricted ANOVAs using 0 mg/Kg and 5 mg/Kg, 0 mg/Kg and 10 mg/Kg, and 2 mg/Kg and 5 mg/Kg. Finally for horizontal distance travelled significant interaction effects between DAY and DOSE were found for each combination except untreated and 0 mg/Kg, untreated and 2 mg/Kg, 0 mg/Kg and 2 mg/Kg, and 5 mg/Kg and 10 mg/Kg. There were also significant interactions between TIME, DAY and DOSE for the combinations of 0 mg/Kg and 5 mg/Kg, 0 mg/Kg and 10 mg/Kg, 2 mg/Kg and 5 mg/Kg, and for the 5 mg/Kg and untreated groups. Many of these interactions effects can be explained by the acquired reduction in activity levels over time exhibited by the 5 mg/Kg and 10 mg/Kg groups which was present on the final day, but absent on the first day. The differences in the level of activity between the first and final day can be seen in Figure 2.7. In this figure a positive value represents an increase in activity levels on the final day in comparison to the first, thus signifying locomotor sensitization had occurred, whilst a negative value indicates a decrease in activity on the final day indicating the presence of tolerance. As can be seen in the figure, for the higher dose groups there is a decrease in activity levels following repeated chronic treatment, signifying the presence of locomotor tolerance.
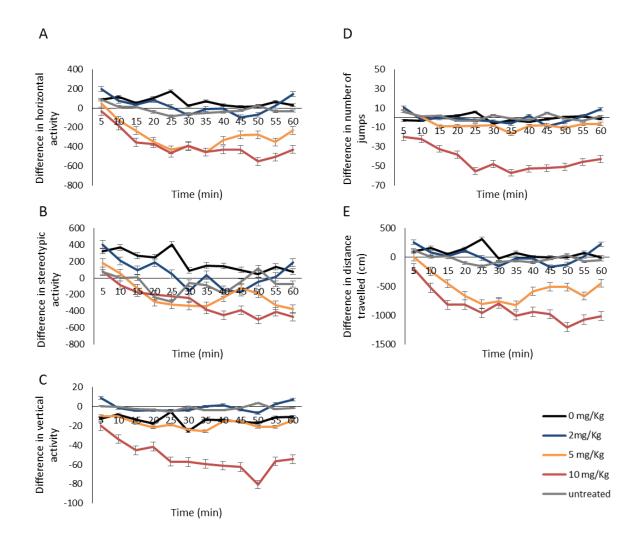
Figure 2.7. The difference in amount of A) horizontal activity, B) stereotypic activity, C) vertical activity, D) jumps and E) distance travelled on the final day in comparison to the first day of chronic treatment, for each pre-treated and untreated. All data is shown as mean ± SEM.

2.4 Discussion

While in the activity monitoring chambers for the 60 minute recording period it is assumed that a normally behaving animal would have initially high levels of activity as they have been placed in a novel environment, however the level of activity is expected to decrease over time as the animal habituates to its surroundings. This pattern was observed on the first day of treatment in both control groups and in the 2 mg/Kg group for each measured parameter. The higher dose groups (5 mg/Kg and 10 mg/Kg) showed a different behaviour pattern on the first day of treatment, with generally continual high levels of activity throughout the full 60 minutes. This high level of activity was also apparent when investigating the levels of activity averaged over the entire monitoring period, where large significant differences were observed mainly between the high dose groups (5 mg/Kg and 10 mg/Kg) and the low dose (2 mg/Kg) and control groups. These differences are likely due to the fact that amphetamine is a psychomotor stimulant, therefore causing increased levels of activity when large doses were administered (Casanova et al., 2013, Morales-Mulia et al., 2007).

By the final day of drug treatment, the pattern of initially high activity which decreases as the animal habituates could be observed in all treatment or control groups. The level of activity for the 10 mg/Kg and 5 mg/Kg dose groups appeared to become more similar to the control groups, with no or fewer significant differences between the low and high dose groups in comparison to the first day when levels of activity were averaged over the entire 60 minute period. The reduction in the level of activity on the final day of treatment could also be seen clearly in all parameters when comparing data obtained on the first and final day for both the 5 and 10 mg/Kg groups, whereas the activity levels of

the low dose and control groups have remained mostly similar to the levels of activity observed on the first day. If behavioural sensitization had occurred, a significant increase in activity would be expected following chronic drug treatment (Casanova et al., 2013, Morales-Mulia et al., 2007, Jaber et al., 1995), however from the results reported above the opposite has been observed, with significant decreases in the activity of the groups treated with higher doses of amphetamine. This result indicates that chronic treatment with amphetamine using the paradigm described in the methods resulted in the development of a drug tolerance in line with the hypothesis. Tolerance to amphetamine and other psychostimulants such as methylphenidate have been similarly observed in studies using chronic implantation or a constant method of drug delivery (Davidson et al., 2005, Zimmer et al., 2014). It is possible that the tolerance developed following chronic oral treatment due to the long half-life of psychostimulant drugs when ingested in this manner (Kuczenski and Segal, 2002, Davidson et al., 2005).

Significant differences were also found between the untreated and treated control groups on both the first and final day of treatment during preliminary testing, however these differences did not appear during post hoc testing in the full analysis. The initial differences observed are unlikely to be due to the sugar in the apple juice producing increased levels of activity in the treated control, since drinking sucrose has not been found to have a significant effect on locomotor activity (Serafine et al., 2015). Therefore the differences found between the controls may have been caused by differing stress levels as a result of the apple juice administration in the treated group.

There is also evidence that there may be a link between the superior colliculus and expression of stereotypic behaviour, with previous research demonstrating reduced

amphetamine-induced stereotypy in SC lesioned animals (Pope et al., 1980). Stereotypic behaviour can be broadly characterised as repetitive, purposeless movements within a discrete area, which can interrupt normal, goal-directed behaviours (Wolgin, 2012), but may also include sniffing and rearing behaviour performed over a wide area, or pacing (Inglis et al., 1994). It is particularly expressed in the head, snout, and orofacial area following amphetamine administration (Kelley, 1998). This typically results in increases in sniffing and head weaving following the administration intraperitoneal injection of amphetamine albeit only at higher doses (>8.0 mg/kg) (Pope et al, 1980). If the SC is lesioned a reduction in all measures of stereotypy has been found (Pope et al, 1980). Given the possible relationship between stereotypic behaviours and collicular functioning it was important to measure stereotypic behaviours to ensure they were not confounding variables in our collicular-dependent measures of interest.

From the above results, stereotypic activity did not appear to be greatly increased in response to chronic amphetamine treatment compared to controls. This finding is in line with other previous investigations which found long term amphetamine treatment may reduce some stereotypic behaviours even when injected at moderate doses (≤5.5 mg/kg) (Rebec and Segal, 1980), and so stereotypic activity should not be a confounding factor when examining collicular dependent behaviours. As interest in stereotypic behaviour in the rats was initially just in relation to whether the behaviour may have an effect on subsequent behavioural testing performed, stereotypic behaviours were not further categorised during the recording of locomotor activity beyond the movements within a discrete area as described in Section 2.2.4. While these gross measurements of stereotypic behaviour would encompass a large number of head movements associated with amphetamine induced stereotypy (Pope et al., 1980), any stereotypic activity

performed over a larger area (Inglis et al., 1994) could not be classified. We examined the overall maps of activity to check for any clear patterns of stereotypy over wider areas but to be certain of this video recording of animal behaviour may be considered in the future to accurately determine and categorise stereotypic behaviour following chronic oral amphetamine treatment.

There was also concern that the locomotor tolerance exhibited by the amphetamine treated groups could be a confounding factor in later behavioural testing, however since levels of activity in the amphetamine treated groups was reduced to a level similar to the control groups, and did not fall below control groups, it was determined that the tolerance observed would not affect the results of further behavioural testing.

CHAPTER 3. THE EFFECTS OF CHRONIC AMPHETAMINE TREATMENT ON BEHAVIOUR

3.1 Introduction

As discussed in Chapter 1, heightened distractibility refers to a reduced ability to discriminate relevant from irrelevant information. Increased distractibility often occurs naturally as a result of normal aging (Wascher et al., 2012), but is also a common symptom of a number of different medical conditions, including ADHD (Friedman-Hill et al., 2010, Konrad et al., 2006), schizophrenia (Castellar et al., 2012) and depression (Lepistö et al., 2004).

Increased distractibility normally goes untreated when it occurs during aging or as a symptom of many disorders, however since increased distractibility is a core symptom of ADHD, treatments for this condition often focus on reducing distractible behaviours (Himelstein et al., 2000). In these cases the most common treatment for increased distractibility is psychostimulant drugs such as amphetamine or methylphenidate (Himelstein et al., 2000, Teicher et al., 2000).

There is evidence that distractibility is mediated by a large network of areas within the brain (Fassbender et al., 2009, Campbell et al., 2012). One area of the brain that has been repeatedly linked to distractibility is the superior colliculus (SC). An important function of the SC is to detect and respond to novel stimuli, particularly visual stimuli as the superficial layers of the SC receive direct input from the retina, allowing it to drive orienting behaviour towards novel stimuli in the visual field (Sprague et al., 1973, Bruce

and Goldberg, 1985). Deeper layers of the SC are also able to respond to auditory or somatosensory input and stimuli (Wise and Irvine, 1983). Previous studies have found that lesioning the SC in several different species, including rats, resulted in a decrease of distractibility (Goodale et al., 1978, Milner et al., 1978, Flandrin and Jeannerod, 1981). Furthermore, previous work has investigated the air righting reflex in rats, which is known to be reliant on a functioning SC (Pellis et al., 1991). One study found that the air righting reflex was abnormal in an ADHD model rat, with these animals unable to modulate their air righting in a height dependent manner, indicating abnormalities in the SC in this strain (Dommett and Rostron, 2011). This same strain show heightened responsiveness to repeated visual stimuli indicative of increased distractibility and increased activity in the SC (Brace et al., 2015a). Together, these results suggest that an undamaged, normally functioning colliculus is critical for maintaining healthy levels of distractibility.

Despite being a relatively commonly occurring phenomenon, the neurobiological causes of increased distractibility are still not fully understood, nor are the mechanisms of action of the psychostimulant drugs that reduce levels of distractibility. If distractibility is reduced with suppression of collicular activity, it is possible that amphetamine acts to bring about this suppression, as hypothesized by Overton (2008).

In the present study, we investigated the effect of chronic treatment with the psychostimulant amphetamine on two distinct behaviours i) responsiveness to repeated visual stimuli where heightened distractibility would result in responding to more presentations of the stimulus (Brace et al., 2015a, Clements et al., 2014), and ii) air righting reflexes as a measure of collicular integrity (Brace et al., 2015a, Clements et al.,

2014). For the latter, we also measured static righting reflexes to be sure any results were not confounded by gross motor deficits (Dommett and Rostron, 2011).

HYPOTHESES

It is hypothesized that:

- There would be a significant reduction in distractibility following chronic amphetamine treatment, demonstrated by reduced number of stimuli responded to and/or a reduced response duration to individual stimuli.

- There would be a significant reduction in the ability of rats to air right in a height dependent fashion following chronic amphetamine treatment.

3.2 Methods

As stated in chapter 2, all behavioural habituation and testing took place in the dark phase, and during the middle of the day (between 10am and 5pm). Because the animals were kept on a reverse dark-light cycle, performing testing at this time ensured the animals were in their most active period of the day. All tests of collicular dependant behaviour were completed within one week following the end of chronic treatment.

3.2.1 DISTRACTIBILITY

Visual distractibility was assessed by placing the animals (Untreated N=16; Vehicle N=14; 2 mg/Kg N=13; 5 mg/kg N=12; 10 mg/kg N=13) into a large circular arena with a centrally placed light stimulus inside a Perspex podium. A white noise machine was also placed centrally to mask sounds occurring outside the room and avoid bias in the rat's movements. Each animal was habituated to the arena for 15 minutes for two consecutive days immediately prior to testing. The experimental set-up is shown in Figure 3.1.
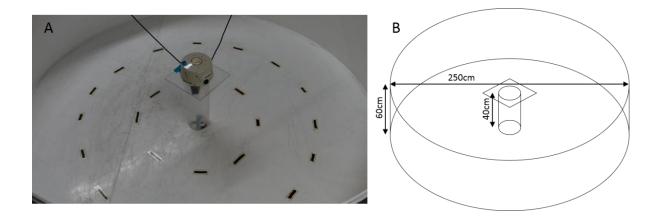
Figure 3.1 A: A photograph and B: a diagram of the arena in which distractibility testing took place. The light stimulus and white noise machine were placed centrally, positioned on a Perspex podium.

For the test of distractibility the light stimulus was operated remotely, and the rat was exposed to 10 light stimulations, lasting 5 seconds. The stimulations occurred at 5 minute intervals, randomised to jitter around the 5 minutes by ± 1 minute in order to prevent the animal from anticipating stimulus onset. The animals' behaviour was recorded throughout using a Samsung VP-HMX20C camcorder and the 5 seconds prior, during, and following the light stimulus were analysed (a total of 15 seconds analysed per stimulus). Analysis of behaviour resulted in three dependent variables for each stimulus presentation: i) whether an animal responded to each stimulus (yes/no), ii) the type of response (freezing/orienting towards the light/interaction with the podium) and iii) the response duration.

For each of the time periods (before, during and after the stimulus) the same analysis was conducted to ensure any response was due to the light flash and not the presence of the stimulus object. The first two dependent variables provided categorical data. For the first dependent variable i.e. whether a response was made, data for the first stimulus was analysed using Chi-Square test of independence to determine whether the animals' response to a novel stimulus was affected by prior chronic amphetamine treatment. The second dependent variable, that is the type of response made, was also analysed in this manner by calculating the number of occurrences of each type of response summed across all stimulus presentations for each trial. Using the first dependent variable it was possible to derive the number of responses made before habituating and this, along with the duration of responses were confirmed as normally distributed using the Kolmogorov-Smirnov test prior to statistical analysis. For the number of stimuli responded to a One-Way ANOVA was performed to ascertain if there was a significant difference in the number stimuli responded to before habituation between the different chronic

amphetamine treatment groups. Finally, the response duration was examined was analysed using a repeated measures ANOVA with STIMULUS NUMBER as the within measures factor and CHRONIC TREATMENT as the between measures factor. Where Mauchly's tests of sphericity was significant, results are reported for the Greenhouse Geisser correction (Greenhouse and Geisser, 1959).

Note that prior to these analyses, the treated and untreated control groups were compared directly using Chi-Square tests and independent samples t-tests where appropriate. In all cases there were no significant differences between the treated and untreated control groups, and so only the treated control group was used in the main analysis.

3.2.2 AIR RIGHTING REFLEX

While falling, a rat is able to modulate the onset air righting depending on the height of the drop (Pellis et al., 1991). This height dependency is visually modulated and dependent on a functional superior colliculus (Dommett and Rostron, 2011, Yan et al., 2010, Pellis et al., 1991). The rats' air righting reflex was tested by holding the animals (Untreated N=18; Vehicle N= 14; 2 mg/Kg N= 14; 5 mg/Kg N=11; 10 mg/Kg N=12) in a supine position and dropping them from heights of 50 cm and 10 cm onto a cushion. Drops were repeated 4 times at each height and heights were alternated to prevent the rats from using tactile landing cues to judge the appropriate righting speed, thus ensuring only visual cues are used for modulating righting speed (Pellis et al., 1989).

All trials were recorded using a Samsung VP-HMX20C camcorder at a frame rate of 50 fps. The footage was then analysed frame by frame to assess the number of animals able to successfully right at each height, that is reach a point where all four paws faced towards

the floor before landing, the latency from the animals release to successful air righting (measured in ms to the nearest 20ms frame), and the plane in which the rotation took place (whether the animals turned longitudinally or laterally). The latency and rotation measurements are illustrated in Figure 3.2.
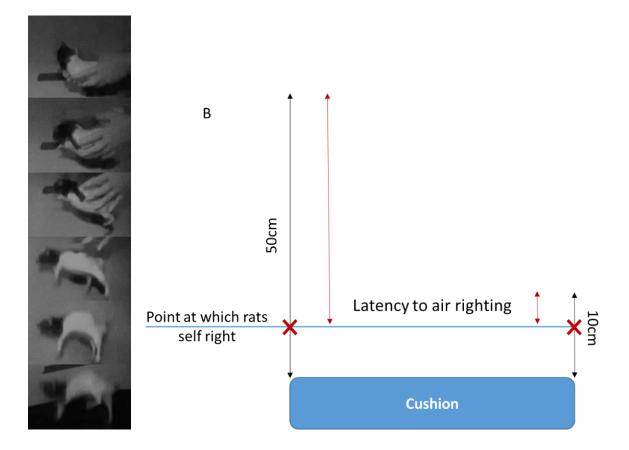
Figure 3.2 A: Illustration of typical longitudinal righting movements. B: Diagram to illustrate measurement of righting latency.

This task, therefore, produced three dependent variables: i) the proportion of trials with successful righting, ii) the dominant righting style (longitudinal, lateral, or no preference) and iii) the average righting latency at each height. The two categorical variables (i and ii) were analysed using a Chi-Square test of independent to establish whether chronic amphetamine treatment had impacted on the ability to right or righting style. The latency

data was tested for normality using a Kolmogorov-Smirnov test and, once confirmed as having a normal distribution, a repeated measures ANOVA was performed with HEIGHT as the within subjects factor and CHRONIC TREATMENT as the between subjects factor.

In order to ensure any group differences in air-righting reflexes were specific to air righting and not due to gross motor deficits, a test of the rats' static righting ability was also performed, in which the animal was held supine against a flat surface and released. This test was repeated 4 times for each animal and the same dependant variables were measured for static righting as were used in the test of the rats air righting ability. These were: the proportion of animal able to right, the dominant plane of rotation used by each animal across the four trials, and the average righting latency across the four trials. The categorical data was analysed as described above. The latency data was tested for normality as above and a One-Way ANOVA was performed to check for differences between chronic amphetamine treatment groups. For both types of righting, as with the test of visual distractibility, initial analyses compared the untreated and treated control group and revealed no significant differences between them and as such only the treated control group is considered in the main analysis.

3.3 Results

3.3.1 DISTRACTIBILITY

There were no responses to the stimulus object in the 5 seconds prior to or immediately after the light stimulus. This indicates that the responses made during the light flash are a response to the stimulus rather than the object. Given no responses were made, no statistical analysis is reported. For the period when the light was on, there were no significant differences in the number of animals responding to the initial light flash between the chronic treatment groups (χ^2 (3) = 1.46; p=0.691; V = 0.168, observed power = 0.82), which suggests visual responsiveness was not affected by chronic amphetamine treatment. It was determined that for the initial stimulus 50% of the 0 mg/Kg group, 54% of the 2 mg/Kg group, 67% of the 5 mg/Kg group, and 69% of the 10 mg/Kg group responded. This initially low proportion responding is potentially due to the fact that non-responding animals were oriented in the opposite direction to the podium for the duration of light flash, or were otherwise stationary at the onset of the stimulus, and so it was not possible to conclude whether a freezing response had occurred. The average number of responses before habituation were calculated and a dose dependent decrease in the number of stimuli responded can be observed in Figure 3.3, however the One-Way ANOVA revealed no significant differences in stimulations taken to habituate between the treatment groups (F(3, 48)=1.44, p=0.244; η^2 = 0.082, Observed power = 0.36).
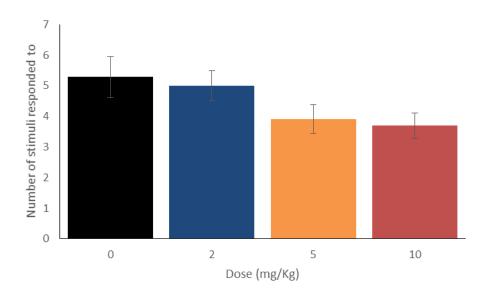
Figure 3.3 The number of stimuli (mean ± SEM) animals responded to before ceasing to respond for each pre-treatment group.

A Chi-Square test performed on the response type data found no significant differences between the chronically treated groups (χ^2 (9) = 7.48; p = 0.486; V = 0.075, observed power = 0.0.708), as shown in Figure 3.4.
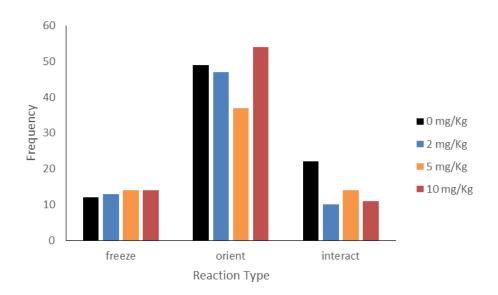
Figure 3.4 The frequency of reaction types for each chronic treatment group.

Data was also collected for the duration of response to the light flash, and it was anticipated for normally behaving animals that the length of response would initially be longer, but the duration would decrease over time as the animal habituated to the stimuli. A repeated measures ANOVA showed that there was a significant main effect of STIMULUS PRESENTATION ($F_{(9, 369)} = 2.40$, $p = 0.012$, $\eta^2 = 0.06$, Observed power = 0.92), verifying that the length of response did decrease over time, as expected. Difference contrasts showed that these decreases occurred between stimulus 4 and 5 ($p = 0.044$) and between stimulus 9 and 10 ($p = 0.001$). This effect can be seen in Figure 3.5. However, the repeated measures ANOVA also indicated that there were no significant main effects of CHRONIC DOSE on the length of response ($F_{(3, 41)} = 0.57$, $p = 0.637$, $\eta^2 = 0.04$, Observed power = 0.12), nor any significant interaction effects between CHRONIC DOSE and STIMULUS PRESENTATION ($F_{(27, 369)} = 0.90$, $p = 0.608$, $\eta^2 = 0.06$, Observed power = 0.79).
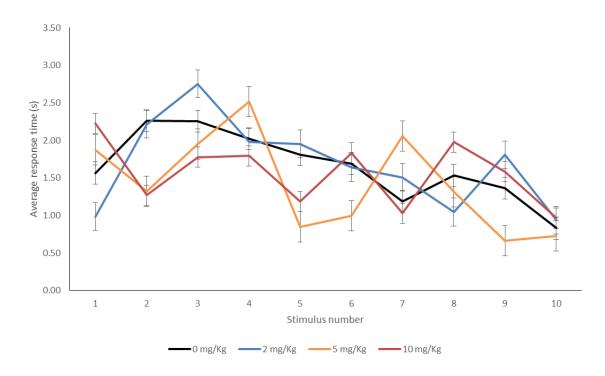
Figure 3.5 The average length of response (mean ± SEM) in seconds to each light flash stimulus per chronic treatment group.

3.3.2 AIR RIGHTING BEHAVIOUR

A test of the animals' height dependant air righting reflex was performed to further investigate collicular dependant behaviour, because although air righting is not directly related to distractibility, it is reliant on a functional, healthy superior colliculus (Dommett and Rostron, 2011, Pellis et al., 1991). As previously described the rats were dropped from heights of 50 cm and 10 cm. The ability of the rat to self-right before landing was then assessed, and in successful cases the time taken for the animal to right with all four paws pointing down was calculated. The orientation in which the rat righted was also recorded, either longitudinally or laterally, however all rats from each group consistently rotated longitudinally, therefore no statistical analysis of this data was performed.

All animals were consistently able to right when dropped from 50 cm, so no statistical analysis was performed on this data. The rats' ability to right at the 10 cm height was investigated using Chi-Square analysis. This showed that there was no significant relationship between the chronic treatment doses received and whether they were able to right from a 10cm drop height (χ^2 (12) = 10.33; p=0.587; ϕ = 0.262, observed power = 0.83). This data can be seen in Figure 3.6.
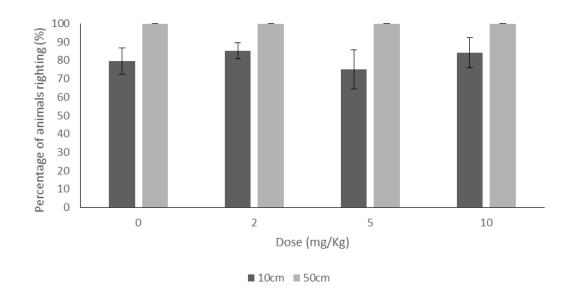
Figure 3.6 The percentage of animals (mean ± SEM) which were able to self-right in each dose group when dropped from either 10 cm or 50 cm.

The animals' righting latencies were analysed with a repeated measures ANOVA with HEIGHT as the within-subjects variable and DOSE as the between-subjects variable. The analyses revealed a significant main effect of HEIGHT ($F_{(1, 44)} = 127.69$; $p < 0.001$; $\eta^2 = 0.744$, Power = 1.00) with the animals' righting latency at the longest on average when dropped from the greatest height. There was also a significant main effect of DOSE ($F_{(3, 44)} = 4.03$; $p = 0.013$; $\eta^2 = 0.215$, Observed power = 0.81). Post hoc Tukey tests revealed there was a significant difference between the 2 mg/Kg and 10 mg/Kg doses ($p = 0.01$), with the latter having a shorter latency to right. Finally, there was a significant interaction effect between HEIGHT and DOSE ($F_{(3, 44)} = 5.09$; $p = 0.004$; $\eta^2 = 0.258$, Observed power = 0.90). To determine the cause of this interaction a series of restricted repeated measures ANOVAs were conducted between just the vehicle group and individual amphetamine dose groups. A significant interaction was found for the vehicle and 10 mg/Kg comparison ($F_{(1, 23)} = 9.39$; $p = 0.005$; $\eta^2 = 0.29$, Observed power = 0.84).

Although there appears to be a similar interaction between the vehicle and 5 mg/Kg from observation of the data in Figure 3.7, no significant interaction effect was found ($F_{(1, 21)}$ = 3.02; p = 0.097; η^2 = 0.13, Observed power = 0.38). This comparison, however, had lower power, which could explain why significance was not reached in this case. Finally there was no significant interaction found between the vehicle and 2 mg/Kg groups ($F_{(1, 25)}$ = 0.06; p = 0.812; η^2 = 0.002, Observed power = 0.06). Figure 3.7 shows that the animals treated with 10 mg/Kg amphetamine have a reduced ability to modulate the latency of their righting by height compared with the vehicle group, which supports the hypothesis made in section 3.1. From Figure 3.7 it can also be seen that the 5 mg/Kg group appeared to have an intermediate ability to modulate their latency, falling between the abilities of the vehicle and 10 mg/Kg group.
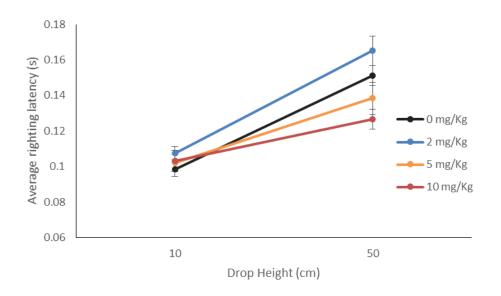
Figure 3.7 The average righting latency (mean ± SEM) at a 10cm and 50cm drop for each chronic treatment group.

To ensure the differences observed in the air righting reflex were due to difference in the superior colliculus as opposed to gross motor deficits, a test of the rats' static righting

reflex was performed. All animals successfully righted in this paradigm so no statistical analysis of righting ability was performed. A One-Way ANOVA found no significant differences in the latency of the static righting reflex between the dose groups ($F_{(3, 49)}$ = 0.796, p = 0.503; η^2 = 0.049, Observed power = 0.70) as shown in Figure 3.8. This indicates that the significant differences observed in the air righting reflex are due to collicular differences in the rats' treated with the highest (10 mg/Kg) concentration chronic treatment group.
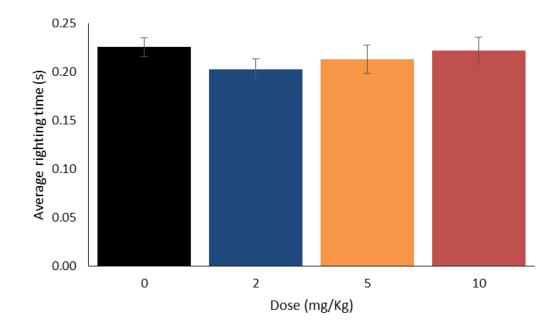
Figure 3.8 The average time (mean ± SEM) taken for to self-right during a static righting test for each pre-treatment group.

3.4 Discussion

3.4.1 DISTRACTIBILITY

The test of visual distractibility revealed no significant differences between different dose groups for either the response to the light flash stimulus, nor the duration of the response. A dose-dependent decrease in the number of stimuli responded to following the initial response was observed, and this overall trend is in line with studies showing a decrease in collicular activity in the presence of amphetamine (Dommett et al., 2009, Gowan et al., 2008, Clements et al., 2014). However, this effect did not reach statistical significance. The analyses indicated a medium-to-large effect size and reduced power, which may suggest potential differences between the groups were present, but that the power of the analysis prevented this reaching significance. Furthermore, the average number of stimulations before the animals had habituated revealed that habituation occurred quite late in most groups, after around 9 stimulations, making it difficult to differentiate between groups, as habituation appeared to occur over the final two stimulations for all groups. Further stimulus presentations may have facilitated in differentiating responses and response durations between groups. Another possible explanation for the lack of effect of amphetamine on the number of orienting responses is that when administered chronically, cortical effects may mask the effects found within the colliculus itself. Previous research has shown that acute systemic amphetamine, whilst depressing visual responses in the colliculus (Gowan et al., 2008, Clements et al., 2014) also causes cortical desynchronization (Contreras et al., 2013) which would have a facilitatory effect on the colliculus. This facilitation could then potentially counteract the expected depressive effects. However, so far this research has only been conducted in

response to acute amphetamine administration, and it is not known whether chronic amphetamine administration causes the occurrence of the same cortical effects, and this matter may warrant further investigation. Another possibility is that the SC may have become desensitized to the depressive effects of amphetamine when exposed to repeated administration. This explanation would seem unlikely since sensitization is known to occur in response to repeated administration of amphetamine in other paradigms (Robinson, 1984) but it cannot be ruled out without further investigation.

The duration of the response to the stimulus did decrease following repeated stimulus presentations, as was predicted, with results showing a significant difference over time and a medium effect size. No significant differences were observed between the different dose groups, however this analysis had a small effect size and also a low power. There was also no significant interaction found between stimulus presentation and dose, despite moderate observed power. These findings imply that the duration of the response is unaffected by chronic amphetamine administration using the current paradigm, however the duration measures may also be influenced by cortical activation or desensitization as described above.

3.4.1 AIR RIGHTING BEHAVIOUR

The rats' ability to air right and modulate righting latency depending on height is reliant on a normally functioning superior colliculus (Pellis et al., 1991, Dommett and Rostron, 2011). It was found that chronic amphetamine treatment did not significantly affect the rats' ability to air right at either a 10 cm or 50 cm drop, but since the rat's actual ability to right is not dependent on the SC, only the ability to modulate righting by height, this result is not surprising. From previous research it is known that the vestibular system is

responsible for overall righting (Pellis et al., 1989) and there is also evidence that amphetamine does not impact on this system (Vanspauwen et al., 2011). There were also no changes to the plane in which righting occurred. Previous investigation has found that rotational plane changes do occur with SC-lesioned animals (Yan et al., 2010). However the lack of change found in the present investigation may be because the colliculus was not removed, but activity levels were likely just suppressed by amphetamine (Chee, 1991, Clements et al., 2014, Gowan et al., 2008).

There was, however, a significant difference found between dose groups when investigating the difference in righting latency between the 10 cm and 50 cm drops i.e. the height dependency of this righting. The control group showed a larger difference in righting latency, with rats falling from 10 cm righting faster than when they fell from 50 cm, as would be expected in a healthy rat with a normal SC. The rats treated with 2 mg/Kg amphetamine also showed similar results and did not differ significantly from the control group, however the 10 mg/Kg group had a significantly reduced ability to modulate righting latency according to height righting latency, with animals righting at a similar speed when dropped from either 10 cm or 50 cm. This reduction in ability to modulate righting latency according to height could indicate reduced functionality in the SC following chronic amphetamine treatment at high doses (Pellis et al., 1989), indicating potential suppression of activity in the SC (Clements et al., 2014, Gowan et al., 2008). The 5 mg/Kg group also showed a slight reduction in ability to height dependently control righting latency, however this reduction was not significantly different to the control group. This may be explained by the reduction in observed power of the analysis for the 5 mg/Kg group in comparison to the analysis for the 10 mg/Kg group, although cortical activation countering collicular effects, as described previously, cannot be ruled out. The

fact that no significant differences were found when performing a test of the animals' static righting reflex further supports the hypothesis that the significant differences observed above are due to amphetamine suppressing activity in the SC (Pellis et al., 1989, Pellis et al., 1991), as opposed to causing gross motor deficits.

The presence of effects of the drug on the ability to modulate righting by height have been observed suggests that at least the higher doses of amphetamine are having an impact on the colliculus, which would indicate that desensitization is unlikely to explain the lack of effects on orienting. There were no changes in righting latency observed for the 2 mg/Kg dose when compared to the control. A dose of 2 mg/Kg is the most closely associated with typical therapeutic doses of amphetamine, and so the therapeutic effects of amphetamine may have different underlying effects on the SC. However, to be sure of this further research using blood plasma levels for additional dose validation would be necessary.

The reasons underlying how height-dependent modulation could be impacted by suppression of activity in the colliculus following amphetamine are unclear, but one possible explanation could be that amphetamine is affecting the time-to-impact calculation, believed to be computed in the colliculus (Nakagawa and Hongjian, 2010, Pellis et al., 1996). The exact mechanisms of these calculations are not fully known but it is suggested that it involves binocular collicular cells in other species (Pellis et al., 1996) and these cells are also known to exist in rats (Diao et al., 1983, Diao et al., 1984, Kondo et al., 1993, Van Camp et al., 2006). Further to this, acute injections of amphetamine have previously been shown to change receptive field size in the visually-responsive layers of the SC (Grasse et al., 1993). These alterations diminished after eight hours when

amphetamine was administered acutely (Grasse et al., 1993) however it is possible that effects on the receptive fields would be longer lasting following a chronic treatment regime. Consequently, it is possible that amphetamine induced increases in receptive field size may result in a reduced ability to calculate time-to-right and so reduce the animals' ability to right in a height-dependent manner. Longer lasting effects of amphetamine, as reasoned here, would be in line with research investigating the impact of comparable amphetamine treatment on brain structures other than the SC, in which changes were found to persist for 3.5 months after cessation of treatment (Kolb et al., 2003, Li et al., 2003). Furthermore, this research suggests that chronic amphetamine treatment may also increase dendritic spines and branching, and this has been previously reported to be positively correlated with receptive field size in the colliculus (Mooney et al., 1993). Further investigation into how long amphetamine induced changes last following cessation of chronic treatment may also be needed since clinical literature is currently unclear about the occurrence or timings at which symptoms return if amphetamine treatment in ADHD is stopped (Johnston et al., 1988).

In summary, the data reported show partial support of our hypotheses. Although no significant reduction in distractibility was observed following chronic amphetamine treatment, it was found that chronic treatment significantly altered collicular-dependent modulation of air-righting. This effect is consistent with findings showing that acute amphetamine administration had the ability to suppress activity in the visually-responsive superficial layers of the colliculus. It is, however, important to also acknowledge the limitations of this investigation. Although effort was made to ensure doses were of therapeutic relevance and were administered using a method designed to emulate treatment of humans, the animals blood plasma levels were not measured. This should be

considered in future research to ensure accurately that a therapeutic dose was received. Furthermore, for some of the analyses reported, the observed power was under the recommended value of 0.8. Low power was observed specifically for the number of stimuli oriented to in the analysis of the orienting task (Observed power = 0.36), and for the main effect of dose (Observed power = 0.132) and interaction effect in the response duration (Observed power = 0.779), although the latter was only just short of the recommended power. In the analyses of the air-righting task, the only key comparison which failed to reach the recommended observed power was the restricted ANOVA comparing the control and 5 mg/Kg in terms of their ability to modulate their righting by height. Lower observed power indicates that there is an increased risk of Type II errors, and so an increased possibility that the null hypothesis was not rejected despite being false. Therefore, lack of power does not invalidate the findings of this investigation but may suggest an underestimation of the impact of amphetamine on the collicular dependent behaviours. Finally, it has been proposed that the observed effect of amphetamine on air-righting may be underpinned by changes in the receptive field size. This explanation is based on current literature and so future research may consider directly measuring the size of receptive fields in animals chronically treated with amphetamine.

CHAPTER 4. ELECTROPHYSIOLOGICAL RESPONSE TO CHRONIC AMPHETAMINE ADMINISTRATION

4.1 Introduction

As discussed in the introduction (chapter 1) psychostimulant drugs such as amphetamine are commonly used in order to reduce symptoms of ADHD, including reducing the increased distractibility and to improve sustained attention (Gowan et al., 2008, Sostek et al., 1980). This reduction in distractibility following treatment with psychostimulants occurs not only in cases where there are increased levels of distractibility, such as for people with ADHD, but also in people with normal baseline levels of distractibility (Overton, 2008, Sostek et al., 1980).

As a psychostimulant, amphetamine is known act on monoamine neurotransmitters, and when administered acutely, it has been found that amphetamine treatment has a number of different effects on the functions of the brain. Links have been found between amphetamine administration and alterations in the activity of dopaminergic neurons in the midbrain; for example previous research has found that administration of amphetamine reduces spontaneous activity in dopaminergic neurons in the substantia nigra pars compacta (SNc) and VTA (Bunney and Aghajanian, 1973). Furthermore infusion of amphetamine into the substantia nigra pars reticulata (SNr) rapidly reduced multiunit activity recorded within the SNr (Timmerman and Abercrombie, 1996). Additional effects of acute amphetamine administration on the midbrain function can be seen in the SC. Previous studied have found that acute amphetamine administration resulted in dose dependent depression in visual activity within the SC (Gowan et al., 2008) and there is

also evidence that the effects of amphetamine on the SC are mediated by 5-HT (Dommett et al., 2009).

As amphetamine is often administered over long periods of time in order to treat the symptom of increased distractibility, it is important to also understand the effects of chronic treatment as well as acute dosing. An investigation into foetal alcohol syndrome and its links to ADHD in rats found that exposing animals prenatally to ethanol resulted in super sensitivity of somato-dendritic dopaminergic receptors in the VTA; however chronically treating ethanol exposed animals with amphetamine negated this super sensitive response (Shen et al., 1995). Chronic amphetamine treatment via i.p. injections has also been found to result in a reduction in the ability of acute amphetamine administration to supress firing in dopaminergic neurons of the VTA (White and Wang, 1984) an effect that could be indicative of behavioural sensitisation. This also suggests that chronic amphetamine exposure may impact on later acute responsiveness to amphetamine.

As mentioned above, the SC is one area of the brain known to be affected by acute amphetamine treatment. There is also direct evidence of a link between the SC and distractibility in humans; a case study found that a lesion to the brainstem, interrupting a direct tract connecting the prefrontal cortex to the superior colliculus, resulted in the patient experiencing increased distractibility (Gaymard et al., 2003b). Additionally, the SC is known to be dysfunctional in the SHR, the best-validated rodent model of ADHD, with visual layers of the SC exhibiting hyper-responsiveness in both multiunit and local field potential recordings (Brace et al., 2015a). Acute treatment with amphetamine has also been found decrease responsiveness to a visual stimuli in the superficial visual layers of

the superior colliculus in another ADHD model (genetically hypertensive) rats (Clements et al., 2014).

So it is known that the function of the SC is affected by acute doses of amphetamine, and there are also links between the SC and distractibility. There is also evidence that the superior colliculus may be dysfunctional in models of ADHD, a condition commonly treated chronically with psychostimulant drugs such as amphetamine. For these reasons it is important to investigate what effects chronic amphetamine treatment has on the function of the SC. This was explored by observing the collicular response to a light flash stimulus, and also by investigating whether chronic treatment influenced the subsequent response to acute administration of amphetamine.

HYPOTHESES

It was hypothesised that:

- chronic treatment with amphetamine would suppress visual responses with the superior colliculus;
- prior chronic treatment with amphetamine would alter the effect of an acute amphetamine challenge on visual activity in the superior colliculus.

4.2 Methods

Within 2 weeks of the final administration of chronic treatment, a proportion of the animals underwent acute terminal electrophysiology (Untreated N=16; Vehicle N=34; 2 mg/Kg N=32; 5 mg/kg N=37; 10 mg/kg N=26). All remaining animals were used for anatomical measures, described in Chapter 5.

4.2.1 SURGICAL PREPARATION

Animals were initially anaesthetised with 4 % isoflurane (Abbott Laboratories, Maidenhead, UK) followed by an intraperitoneal (i.p.) injection of 30 % Urethane (5ml/kg, Sigma Aldrich, Gillingham, UK). Anaesthetic depth was determined using the pedal and eye blink reflexes. Supplementary i.p. injections of urethane were administered where needed until loss of reflexes demonstrated surgical anaesthesia. Once a suitable anaesthetic depth was reached the animal's eyelids were sutured open (Clinisut, Advena Ltd., Warwick, UK) and eye gel (Viscotears ®, Novartis Pharmaceuticals Ltd., Surrey, UK) was applied to prevent desiccation. In order to administer saline and amphetamine intravenously during electrophysiology animals were cannulated in one of two ways based on veterinarian guidance. Initially animals underwent cannulation of their left femoral vein (N=24). The cannula consisted of a 200 mm length of sterile tubing (Smith's Medical International Ltd, Ashford, UK), which was cut so one end was bevelled for easier insertion into the vein. The opposite end of the cannula had a 25 gauge needle inserted into the lumen, so that a syringe could be attached to administer the appropriate solution. Cannulation was performed by first shaving the surgery site, on the animal's lower abdomen and inner hind leg, and applying a local anaesthetic (Ethyl Chloride BP,

Cryogesic, Acorus Therapeutics Ltd., Chester, UK). An incision was then made just above the groin, and blunt dissection of the adductor muscles was used to locate the femoral vein and to separate it from the femoral artery and sciatic nerve. Once a sufficient length of vein was separated, 3 cotton sutures (Clinisut, Advena Ltd., Warwick, UK) were passed underneath the vein and the most distal suture from the heart was tied tightly to occlude blood flow from the hind leg. A vein clamp (Fine Scientific Instruments, Heidelberg, Germany) was then positioned on the vein in the location most proximal to the heart. Following the application of the vein clamp, a small incision was made in the vein using a 20 gauge needle, close to the tied off suture. The tip of the catheter could then be inserted into the incision and threaded along inside the vein, up to the location of the vein clamp. At this point the vein clamp was removed and it could be determined if the cannula was viable by drawing back on the syringe attached, to ensure blood was drawn back into the tubing. Following this the two remaining sutures were tied off to secure the cannula in the vein, and 0.1 ml heparinised saline (Tocris Biosciences, Bristol, UK) was administered through the cannula to prevent the formation of blood clots. The incision was then sutured shut and the cannula was secured to the rat's hind leg, outside of the incision area, by the application of Vetbond tissue adhesive (3M, Berkshire, UK). Following new guidance for our Named Veterinary Surgeon on a more robust technique for tail vein cannulations, the remaining animals underwent this method of cannulation instead which is a more refined technique (N= 122). This involved cleaning the tail with hibiscrub (BCM Ltd, Nottingham, UK) the lateral tail vein was then located by eye and a cannula (24G SurFlash, Terumo) inserted approximately two thirds along the length of the tail from the base. The metal needle portion was then removed and the cannula was deemed to be viable if blood flowed back into the plastic tip. Following this the cannula

was secured with fabric strapping tape (Tesco, Welwyn Garden City, UK) and 0.1 ml heparinised saline (Tocris Biosciences, Bristol, UK) was administered through the cannula to prevent the formation of blood clots.

The head was then shaved and the animal was positioned in a stereotaxic frame (Kopf Instruments, Tujunga, USA); the head was secured using ear bars and placed in the skull flat position with the incisor bar 3.3 mm below the interaural line. Local anaesthetic (Ethyl Chloride BP, Cryogesic, Acorus Therapeutics Ltd., Chester, UK) was applied and an incision made along the length of the head. Burr holes with a diameter of 3 mm were then drilled 6.3 mm posterior to bregma, and 2-3 mm from the midline on both sides of the skull to expose the cortex above the SC. An additional burr hole, 1 mm in diameter, was drilled 1 mm anterior to bregma and 1 mm from the midline for an electroencephalography (EEG) electrode (loop-tipped silver wire, 0.2 mm Ø; Intracel). These can be seen in Figure 4.1.
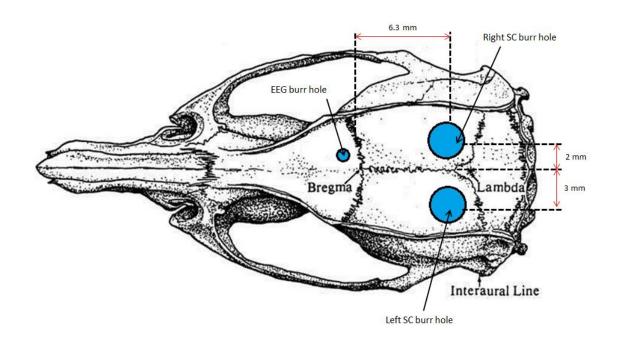
Figure 4.1. A diagram to show the position of burr holes and EEG electrode hole. Adapted from Paxinos and Watson (1998).

Respiration rate was recorded throughout as an aid to monitoring anaesthetic depth through comparison of respiration rates to relative stages of anaesthesia (Guedel, 1920, Friedberg et al., 1999) using a three-axis accelerometer IC (ADXL330KCPZ, Analog Devices, Norwood, MA, USA, device (Oxford University, UK)), attached to the animal's lateral abdomen (Devonshire et al., 2009). Body temperature was also monitored throughout via a rectal thermometer attached to a thermostatically-controlled heating blanket (Harvard Apparatus Ltd, Cambridge, UK), to maintain a temperature of 37°C.

4.2.2 ELECTRODE PLACEMENT

Tungsten electrodes (Parylene-C-insulated; 2 MΩ, A-M Systems Inc., Carlsborg, WA, USA) were positioned on each side for simultaneous recordings from both left and right SC. These electrodes were positioned -6.3 mm AP (Anterior Posterior) from bregma. The right electrode was vertical and positioned 2 mm right of the midline, directly above the location of the SC (Paxinos & Watson, 1998). To allow for simultaneous recording, the left electrode was angled at 25 °, and positioned 2.5 mm left of the midline. An EEG electrode was also positioned above the burr hole +1 mm AP from bregma. This electrode was used to aid the monitoring of anaesthetic depth which was assessed offline via comparison of EEG bands to relative stages of anaesthesia (Friedberg et al., 1999).

To locate the SC a light flash (green LED flashing at 0.5 Hz, 10 ms duration, 20 mcd positioned 5 mm anterior to the contralateral eye) stimulus was applied whilst the electrode was gradually lowered into the SC until a strong light response was detected in both the audio feed from the recording (NL120, The Neurolog System, Digitimer, Welwyn Garden City, UK) and visual feed via Spike2 (CED, Cambridge, UK). Once both the electrodes were positioned in the superficial layers, the animal was left in the dark for a

further 25 minutes to adapt to the darkness before actual recordings began with a stimulus response curve.

4.2.3 STIMULUS-RESPONSE CURVE DATA COLLECTION

Visual responses to 150 stimulations were then recorded at five different stimulus intensities (from minimum to maximum light: 4, 8, 12, 16 and 20 mcd) and the intensity for which a mid-range response was produced was used for the remainder of the experiment. Extracellular data incorporating low frequency (local field potential; LFP) and high-frequency (multi-unit activity; MUA) data was amplified (gain 1000), digitized at 11 kHz and recorded to PC using a 1401+ data acquisition system (Cambridge Electronic Design Systems, Cambridge, UK), running Cambridge Electronic Design Systems data capture software (Spike 2) and saved for offline analysis.

4.2.4 ACUTE DRUG CHALLENGE

Once the appropriate (mid-range) stimulus intensity had been determined, solutions could be delivered intravenously to the animal via the cannula. First a baseline of 300 stimulations was recorded, then 5 doses of either amphetamine (see Table 4.1) or volume matched 0.9 % saline were administered, with each subsequent dose being double the volume of the previous dose, thus the doubling the cumulative dose concentration. An additional 0.06 ml was added to the first dose to allow for solution remaining in the cannula. Doses were delivered every 12 minutes, with a 2 minute adaptation period immediately following each drug delivery. Responses to 300 stimulations were then recorded for each dose.

Cumulative Dose	Amphetamine (mg/Kg)
1st	0.5
2nd	1
3rd	2
4th	4
5th	8

Table 4.1: Cumulative doses of amphetamine administered as an acute drug challenge. Comparable volumes of saline were used as a vehicle condition.

Upon completion of the electrophysiology recordings a direct current of 10 µA was passed through the each of the electrodes for 5 seconds (Constant Voltage Isolated Stimulator DS2A MK2, Digitimer, Welwyn Garden City, UK) in order to mark the site of recording for later histological reconstruction of the site. The animal was then removed from the stereotaxic frame and sacrificed with 1 ml pentobarbatone (Animalcare, York, UK) and perfused with 0.9 % saline and fixed with 4 % paraformaldehyde in 0.1 M phosphate buffer (PB), the brain was subsequently removed and stored overnight in 4 % paraformaldehyde fixative for at least 24 hours before transfer into 20 % sucrose in 0.1 M PB solution for at least 36 hours.

4.2.5 RECONSTRUCTION OF RECORDING SITES

Brains were frozen to -20 °C in isopentane (VWR International, Lutterworth, UK) and coronal sections were sliced using a cryostat (CM1900, Leica, Milton Keynes, UK) at a thickness of 50 µm through the SC. Slices were mounted on slides before being dehydrated in alcohol, Nissl stained using 0.5 % cresyl violet (Sigma Aldrich, Gillingham, UK), and cover slipped with DPX (VWR International, Lutterworth, UK). The recording site was then verified using an Olympus microscope (Olympus BX61 SOP 57) photographed, as seen in Figure 4.2, and plotted (corelDRAW X6, Corel corporation Ottawa, Canada) onto reconstructed sections from Paxinos and Watson (1998).
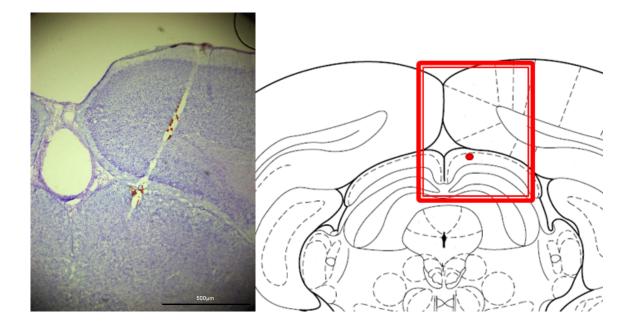
Figure 4.2. A: An image of a 50 μm thick Nissl stained coronal section through the superior colliculus. Anatomical structures and measurements were used to identify recording site. B: Location of recording site as indicated by the red marker. Adapted from Paxinos and Watson, (1998).

4.2.6 DATA ANALYSIS

Analysis of all electrophysiological responses to the light flash was performed using MatLab (Mathworks, Massachusetts, United States). Data was filtered using a low pass to give local field potentials and high pass filter to give multi-unit activity. A peristimulus time histogram (PSTH) was produced for the multiunit activity for each stimulus intensity of the stimulus response curve, and for each dose of the acute amphetamine trial and volume matched saline. Onset latency, peak latency, maximum amplitude (normalised against the baseline firing rate), area under the curve, and the baseline firing rate for each PSTH was then extracted. Waveform averages were plotted for the local field potential data, and used to calculate onset latency, peak latency, peak to peak amplitude, area

under the curve for each stimulus intensity of the stimulus response curve, and for each dose of the acute amphetamine trial and volume matched saline. The measured parameters for both PSTHs and waveform averages can be seen in Figure 4.3. All data was then exported to SPSS for statistical analysis.
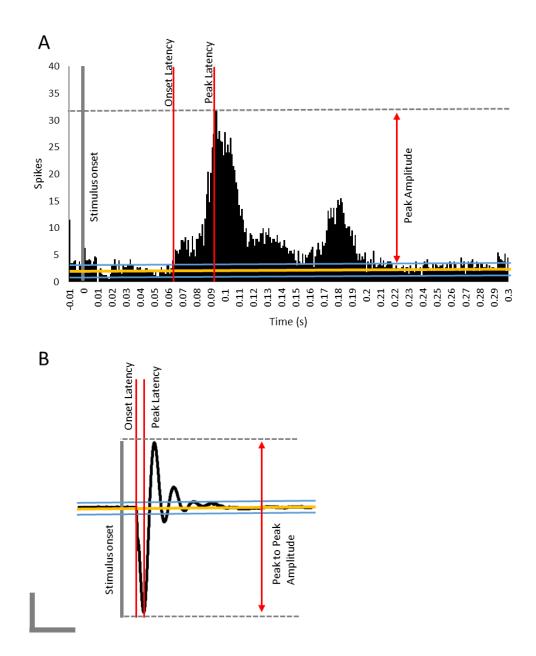
Figure 4.3 An example of the onset latency, peak latency and peak amplitude for A) a peristimulus time histogram and B) a waveform average extracted from light response data from the visual layers of the SC (scale bars, x: 50 ms, y: 400 μV) Yellow line represents baseline firing rate, blue lines represent 1.96 standard deviations from baseline.

This exported data was tested for normality using a Kolmogorov-Smirnov test and measures of skewness and kurtosis as appropriate. Once data was shown to be normally distributed comparisons between the treated (0 mg/Kg) and untreated control groups were performed. In each case there were no significant differences identified between the groups, and therefore only the treated control groups was reported in the main analysis. The results of statistical testing between the control groups can be found in Appendix B. Repeated measures ANOVAs were conducted for each parameter, with STIMULUS INTENSITY as the within measures factor, and CHRONIC TREATMENT as the between measures factor for analysis of the stimulus response curves. For analysis of the effects of the acute drug challenges ACUTE DOSE was used as the within measures factor, and CHRONIC TREATMENT as the between measures factor when repeated measures ANOVAs were run. If the assumption of sphericity was violated, the Greenhouse-Geisser results were reported. Saline data was analyses separately in the same manner.

It has also been observed that collicular responses to a visual stimulus are biphasic. This is due to the fact the SC receives direct retinal input, allowing a rapid initial response to a visual stimuli, followed by slower generalised cortical response input in response to the stimuli (McPeek and Keller, 2002, Lim and Ho, 1997). As it has not been previously investigated, it would also be interesting to investigate if either chronic or acute amphetamine treatment has differing effects on the two phases of visual responses in the SC.

Further analysis performed using Matlab to investigate potential multiphasic responses in the multiunit activity separated the data into two phases. The first phase ran for the first 200 ms following the light flash stimulus. This period was decided based on observations

of separate phases within the data collected. The initial phase isolated the collicular response to retinal input and allowed the peak to return baseline before the beginning of the second cortical input phase, which ran from 210 ms following the light flash stimulus to the termination of the response. PSTHs were produced for each phase and the area under the curve and maximum amplitude were recorded and exported to SPSS for statistical analysis. For this a Two-Way repeated measures ANOVA was used, with PHASE and either STIMULUS INTENSITY or ACUTE DOSE as the within measures factor, and CHRONIC TREATMENT as the between measures factor. Any significant interaction effects were explored using a series of simple effects tests.

In order to determine the animals' anaesthetic depth a power spectrum analysis was performed to calculate the dominant EEG frequency over the first 300 seconds of each recording, these frequencies were then categorised into Guedel's (1920) stages of anaesthesia (Friedberg et al., 1999) (See Table 2.2). Respiration rate was calculated by counting breaths taken in the first 30s of each recording and the final 30s of the last recording, with one breath characterised by the respiration rate rising above a predetermined level. This data was used to calculate an average respiration rate (breaths/min) and was categorised into Guedel's stages of anaesthesia (Table 2.2).

Guedel Stages of Anaesthesia	Kubicki's EEG bands	Friedbergs's Respiration rats (breaths/min)
III-1	10-13 Hz	NA
III-2	5-7 Hz	96-120
III-3	3-4 Hz	88-104
III-4	1-2 Hz	48-68
IV	Suppression	24-38

Table 4.2: Dominant EEG and respiration rates correlated to Guedel stages of anaesthesia in a rat. Adapted from Friedberg et al. (1999).

Following categorisation of both the dominant EEG frequency and rate of respiration, One-Way ANOVAs were performed to check for significant differences between chronic treatment groups, and a chi-squared analysis was performed to ensure there was no difference in anaesthetic depth.

4.3 Results

4.3.1 ANALYSIS OF ANAESTHETIC DEPTH

The depth of anaesthesia was also monitored during electrophysiological recordings and analysed offline to ensure consistency between the chronically treated groups. Analysis of the dominant EEG frequency found that the animals were categorised as being within stage III-3 or III-4 of Guedel's stages of anaesthesia (Table 4.2) (Friedberg et al., 1999). There were no significant differences in the dominant EEG frequency between the chronic treatment groups for the stimulus response curve ($F_{(4, 66)} = 0.24$, $p = 0.912$, $\eta^2 = 0.02$, observed power = 0.10), the acute amphetamine trial ($F_{(4, 61)} = 1.83$, $p = 0.136$, $\eta^2 = 0.12$, observed power = 0.52) and the saline trial ($F_{(4, 63)} = 0.88$, $p = 0.480$, $\eta^2 = 0.57$, observed power = 0.26). These results can be observed in Figure 4.4.
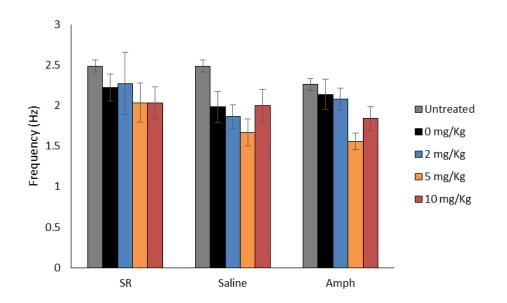
Figure 4.4 Average (mean ± SEM) dominant EEG frequency for each electrophysiology experiment (SR = stimulus response).

In addition to the analysis of EEG dominant frequencies, respiration rate was also calculated in order to confirm the correct anaesthetic depth was achieved. This analysis confirmed that the animals were in Guedel's anaesthesia stage III-3. There were also no significant differences in respiration rate between the chronic treatment groups during the stimulus response curve ($F_{(4, 49)} = 2.29$, $p = 0.074$, $\eta^2 = 0.17$, observed power = 0.62), the acute amphetamine challenge ($F_{(4, 44)} = 2.30$, $p = 0.075$, $\eta^2 = 0.19$, observed power = 0.61) and the saline challenge ($F_{(4, 43)} = 2.18$, $p = 0.089$, $\eta^2 = 0.19$, observed power = 0.59). The comparison of the respiration rate can be seen in Figure 4.5.
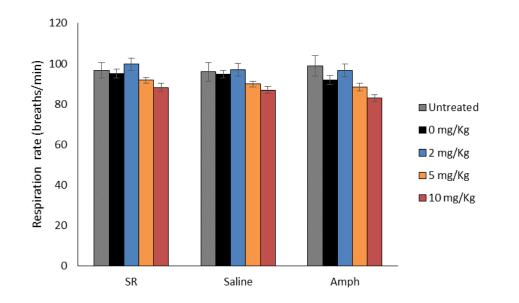
Figure 4.5 Average (mean ± SEM) respiration rate in breaths per minute per electrophysiology experiment (SR = stimulus response).

4.3.2 STIMULUS RESPONSE CURVES

Multiunit activity

Once the collicular responses to a light flash stimulus were recorded for the stimulus response curve, the data collected was high pass filtered in order to analyse the multiunit activity. As mentioned previously there were no significant differences between the treated and untreated control groups (Appendix B), and so only the treated control group was used for the main analysis. Visual responses were found in multiunit activity to at least one stimulus intensity in 94.5% of recordings (N = 145). There were no significant associations between chronic dose and whether a visual response was recorded at any stimulus intensity (4 mcd: χ^2 (4) = 3.67, p = 0.453, V = 0.159; 8 mcd: χ^2 (4) = 5.29, p = 0.259, V = 0.191; 12 mcd: χ^2 (4) = 7.736, p = 0.102, V = 0.231; 16 mcd: χ^2 (4) = 6.14, p = 0.189, V = 0.206; 20 mcd: χ^2 (4) = 4.99, p = 0.288, V = 0.185) which indicates that amphetamine does not impact on the occurrences of multiunit activity visual responses. PSTHs of the filtered data were plotted in order to measure the average onset latency, peak latency, maximum amplitude, and the area under the curve of the response to the light flash. Following tests for normality, repeated measures ANOVAs were then used to investigate what effect a light flash stimulus of increasing intensity had on collicular responses following chronic treatment. STIMULUS INTENSITY was used as the within measures factor, and CHRONIC TREATMENT was used as the between measures factor. The results of this testing revealed that there was a significant main effect of STIMULUS INTENSITY on onset latency (F (2.82, 214.40) = 445.33, p < 0.001, η^2 = 0.85, observed power = 1.00). Contrasts revealed the onset latency for each successive light intensity differed significantly to the previous stimulus (p < 0.001), and from Figure 4.6(A) it is evident that the onset latency decreases for all chronic treatment groups as the light

intensity increases. There was also a significant main effect of CHRONIC TREATMENT ($F_{(3, 76)}$ = 2.97, p = 0.037, η^2 = 0.11, observed power = 0.68), however post hoc Tukey testing did not reveal any significant differences between chronic treatment groups. From Figure 4.6(A) it can be seen that the largest difference between chronic treatment groups is between the 5 mg/Kg dose group and both the control group (p = 0.061), and 2 mg/Kg dose group (p = 0.081), with the onset latency being consistently shorter in the control and low dose groups. There was no significant interaction effect between the STIMULUS INTENSITY and CHRONIC TREATMENT ($F_{(8.46, 214.40)}$ = 1.78, p = 0.079, η^2 = 0.07, observed power = 0.77).

A significant main effect of STIMULUS INTENSITY was found for the peak latency data ($F_{(2.92, 245.42)}$ = 2.76, p = 0.044, η^2 = 0.03, observed power = 0.65). From within subjects difference contrasts the only significant difference was found between the third and fourth stimulus intensities, but from fig 4.3(B) it can be seen that there is a general decrease in the peak latency across the chronic treatment groups. There was no significant main effect of CHRONIC TREATMENT ($F_{(3, 84)}$ = 0.882, p = 0.454, η^2 = 0.03, observed power = 0.24), however there was a significant interaction effect between CHRONIC TREATMENT and STIMULUS INTENSITY ($F_{(8.77, 245.42)}$ = 2.36, p = 0.015, η^2 = 0.07, observed power = 0.91). Simple effects tests were used to assess the basis for the interaction effect; significant differences were found between 2 mg/kg and 10 mg/Kg dose groups at the second stimulus intensity (p = 0.016), further differences were found during the fifth stimulus intensity between the 5 mg/Kg group and all other dose groups (0 mg/Kg, p = 0.007; 2 mg/Kg, p = 0.006; 10 mg/Kg, p = 0.046).

Analysis of maximum amplitude revealed that there was a significant main effect of STIMULUS INTENSITY ($F_{(2.49, 201.77)} = 69.44$, $p < 0.001$, $\eta^2 = 0.46$, observed power = 1.00), but no significant main effect of CHRONIC TREATMENT ($F_{(3, 81)} = 0.74$, $p = 0.531$, $\eta^2 = 0.03$, observed power = 0.20) and no interaction effect ($F_{(7.47, 201.77)} = 1.56$, $p = 0.145$, $\eta^2 = 0.06$, observed power = 0.66). Within subjects contrasts revealed significant differences between each pair of consecutive stimulus intensities ($p < 0.001$). Figure 4.6(C) shows that the peak amplitude increased across all treatment groups up to the penultimate light intensity of 16 mcd, and then decreased for the final brightest light intensity (20 mcd).

Finally, there was also a significant main effect of STIMULUS INTENSITY on the area under the curve ($F_{(1.74, 146.12)} = 216.07$, $p < 0.001$, $\eta^2 = 0.72$, observed power = 1.00), and again within subjects contrasts found significant differences between each pair of consecutive stimulus intensities ($p < 0.001$), but there were no significant main effect of CHRONIC TREATMENT ($F_{(3, 84)} = 0.99$, $p = 0.402$, $\eta^2 = 0.03$, observed power = 0.26), and no interaction effect ($F_{(5.22, 146.12)} = 2.19$, $p = 0.055$, $\eta^2 = 0.07$, observed power = 0.72). Since the area under the curve of the PSTH incorporates both the duration and amplitude of a response, it can be used as an analogue for the strength of the response; it can therefore be seen from Figure 4.3(D) that as the light intensity increased, the response strength also increased across all treatment groups.
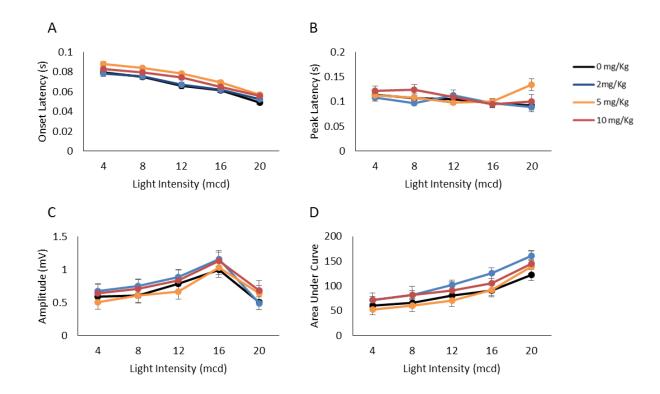
Figure 4.6 The A) onset latency, B) peak latency, C) maximum amplitude (normalised) and D) area under the curve (mean ± SEM) of PSTHs calculated from multiunit activity in response to increasing light intensity of a flashing stimulus, following chronic amphetamine treatment. Key shows the chronic amphetamine dose groups. Only recordings with responses to all stimuli were included in this analysis (0 mg/Kg, N = 24; 2 mg/Kg, N = 20; 5 mg/Kg, N = 26; 10 mg/Kg, N = 16).

Local field potentials

The data recorded from the superior colliculus for the stimulus response curve was also low pass filtered, visual responses were found in local field potentials to at least one stimulus intensity in 99.3% of recordings (N = 143). There were no significant associations between chronic dose and whether a visual response was recorded at any stimulus intensity (4 mcd: χ^2 (4) = 2.81, p = 0.590, V = 0.140; 8 mcd: χ^2 (4) = 2.63, p = 0.622, V = 0.136; 12 mcd: χ^2 (4) = 1.60, p = 0.809, V = 0.106; 16 mcd: χ^2 (4) = 4.65, p = 0.325, V =

0.180; 20 mcd: χ^2 (4) = 4.53, p = 0.339, V = 0.178) indicating that amphetamine does not impact the occurrences of local field potential visual responses. Waveform averages of the filtered data were plotted in order to analyse the local field potentials. This data was also tested for normality using the methods stated above.

In order to determine what effect the light flash stimulus had on local field potentials in the superior colliculus following chronic amphetamine treatment, data collected from waveform averages was analysed using repeated measures ANOVAs, with STIMULUS INTENSITY as the within measures factor, and CHRONIC TREATMENT as the between measures factor. This analysis revealed there were no significant main effects on onset latency of either STIMULUS INTENSITY (F (2.87, 291.45) = 0.761, p = 0.511, η^2 = 0.01, observed power = 0.21) or CHRONIC TREATMENT (F (3, 102) = 0.475, p = 0.700, η^2 = 0.01, observed power = 0.14). There were also no interaction effects between the two (F (8.57, 291.45) = 0.67, p = 0.728, η^2 = 0.02, observed power = 0.32), and these results can be seen in Figure 4.7(A).

Further analysis found that there was no main effect of STIMULUS INTENSITY on peak latency (F (2.66, 326.63) = 1.63, p = 0.187, η^2 = 0.01, observed power = 0.40), but there was a significant main effect of CHRONIC TREATMENT (F (3, 123) = 4.16, p = 0.008, η^2 = 0.09, observed power = 0.84), with post hoc Tukey tests revealing that the 2 mg/Kg group had a significantly lower peak latency in comparison to the 5 mg/Kg group (p = 0.009) and the 10 mg/Kg group (p= 0.027). There were also a significant interaction effect between STIMULUS INTENSITY and CHRONIC TREATMENT groups (F (7.97, 326.63) = 2.24, p= 0.024, η^2 = 0.05, observed power = 0.87), with simple effects tests revealing this interaction effect was driven by significant differences between the 2 mg/Kg group and all other

groups at the first (0 mg/Kg, p = 0.004; 5 mg/Kg, p = 0.026; 10 mg/Kg, p = 0.003) and second light intensities (0 mg/Kg, p = 0.037; 5 mg/Kg, p = 0.040; 10 mg/Kg, p = 0.009), and between the 2 mg/Kg and 10 mg/Kg groups at the third light intensity (p = 0.031). This effect can also be seen in Figure 4.7(B). The 5 mg/Kg groups also had a significantly longer latency at the fifth stimulus intensity in comparison to the other dose groups (0 mg/Kg, p = 0.006; 2 mg/Kg, p = 0.001; 10 mg/Kg, p = 0.015).

There was a significant main effect of STIMULUS INTENSITY (F (1.56, 191.90) = 11.18, p < 0.001, η^2 = 0.08, observed power = 0.98) on the maximum amplitude, but no main effect of CHRONIC TREATMENT (F (3, 123) = 1.93, p = 0.129, η^2 = 0.04, observed power = 0.49). Contrasts found significant differences between all except the first and second (p = 0.993) consecutive light intensities (p < 0.001) and from Figure 4.7(C) it can also be seen that for all chronic treatment groups that as the light intensity increases, the maximum amplitude of the response also increases. Although there was no main effect of CHRONIC TREATMENT, the 10 mg/Kg group appears to have a greater maximum amplitude across all stimulus intensities in comparison to the control group (Figure 4.7 (C)). There was also no significant interaction effect between STIMULUS INTENSITY and CHRONIC TREATMENT (F (4.68, 191.90) = 1.81, p = 0.117, η^2 = 0.04, observed power = 0.59).

Finally, for the area under the curve data there were significant main effects of STIMULUS INTENSITY (F (1.71, 210.77) = 115.83, p < 0.001, η^2 = 0.49, observed power = 1.00) but no main effect of CHRONIC TREATMENT (F (3, 123) = 2.01, p = 0.116, η^2 = 0.05, observed power = 0.51), but from Figure 4.7(D) it can be seen that the low 2 mg/Kg chronic treatment group appeared to have a greater area under the curve in comparison to both the higher dose groups, as well as the control group at each light intensity. It is also seen

that as light intensity increases, the area also increases for each dose group, and this observation is supported by within-subjects difference contrasts which found significant differences between each successive intensity pairing ($p < 0.001$). There was also a significant interaction effect between STIMULUS INTENSITY and CHRONIC TREATMENT ($F_{(5.14, 210.77)} = 2.57$, $p = 0.027$, $\eta^2 = 0.06$, observed power = 0.80). This interaction was driven by the significantly higher magnitude of response (area) of the 2 mg/Kg dose group in comparison to all other groups at the first light intensity (0 mg/Kg, $p = 0.020$; 5 mg/Kg, $p = 0.027$; 10 mg/Kg, $p = 0.039$) to the 0 mg/Kg and 5 mg/Kg groups at the second (0 mg/Kg, $p = 0.014$; 5 mg/Kg, $p = 0.030$) and third light intensities (0 mg/Kg, $p = 0.028$; 5 mg/Kg, $p = 0.034$), and to just the 0 mg/Kg group ($p = 0.021$) at the fourth stimulus intensity.
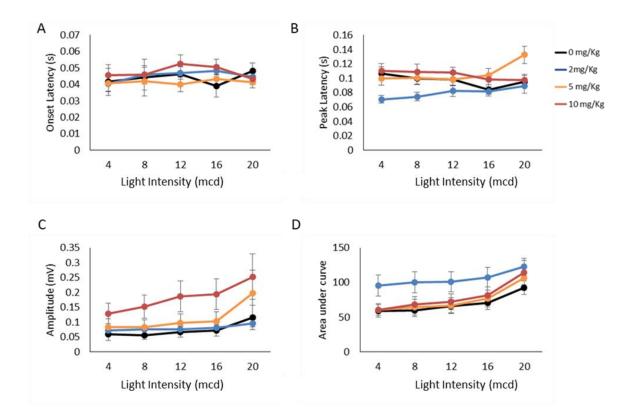
Figure 4.7 The A) onset latency, B) peak latency, C) maximum amplitude and D) area under the curve (mean ± SEM) of waveform averages calculated from local field potentials in the superior colliculus in response to increasing light intensity of a flashing stimulus, following chronic amphetamine treatment (key shows chronic amphetamine dose groups). Only recordings with responses to all stimuli were included in this analysis (0 mg/Kg, N = 32; 2 mg/Kg, N = 32; 5 mg/Kg, N = 31; 10 mg/Kg, N = 20).

4.3.3 ACUTE AMPHETAMINE CHALLENGE

Multiunit activity

In addition to measuring the animals' stimulus response, five cumulatively increasing acute doses of amphetamine were administered intravenously, and multiunit activity was recorded and analysed from the SC following a light flash stimulus as described above. Visual responses were found in multiunit activity following at least one acute dose of amphetamine in 90.8% of recordings (N = 98). There were no significant associations between chronic dose and whether a visual response was recorded at any acute dose strength (Baseline: χ^2 (4) = 1.72, p = 0.786, V = 0.133; 0.5 mg/Kg: χ^2 (4) = 2.18, p = 0.702, V = 0.149; 1 mg/Kg: χ^2 (4) = 5.02, p = 0.285, V = 0.226; 2 mg/Kg: χ^2 (4) = 7.07, p = 0.132, V = 0.269; 4 mg/Kg: χ^2 (4) = 2.47, p = 0.651, V = 0.159; 8 mg/Kg: χ^2 (4) = 1.48, p = 0.830, V = 0.123) indicating that chronic amphetamine treatment has not impacted the occurrences of multiunit activity visual responses. Data was collected from PSTHs, as seen in Figure 4.8, and assessed for normality.
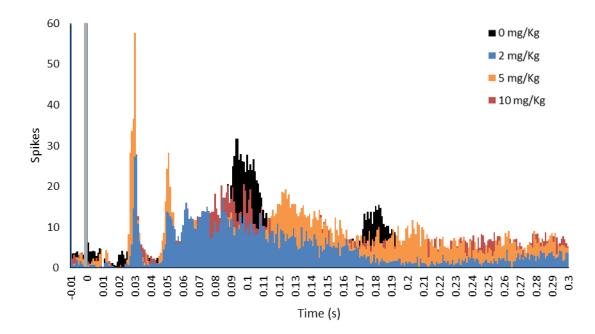
Figure 4.8 Average PSTHs of multiunit activity in response to midrange visual stimuli for each chronic treatment group prior to acute amphetamine administration. The grey line indicates onset of visual stimuli.

Repeated measures ANOVAs were then run using CHRONIC TREATMENT as the between measures factor and ACUTE DOSE as the within measures factor. There was a significant main effect of ACUTE DOSE on the onset latency ($F_{(3.560, 299.07)}$ = 2.80, p =0.032, η^2 = 0.03, observed power = 0.73), difference contrasts found significant differences between the third and fourth (p = 0.035), and the fourth and fifth consecutive acute dose pairs (p = 0.015), furthermore as observed in Figure 4.9(A) the trend of the data shows that onset latency decreased as the acute dose of amphetamine increased. There was no main effect of CHRONIC TREATMENT on onset latency ($F_{(3, 84)}$ = 0.55, p = 0.648, η^2 = 0.02, observed power = 0.16) and no interaction effect ($F_{(10.68, 299.07)}$ = 1.13, p = 0.337, η^2 = 0.04, observed power = 0.61).

There was also a significant main effect of ACUTE DOSE on the peak latency ($F_{(4.25, 357.31)} = 3.04$, $p = 0.015$, $\eta^2 = 0.04$, observed power = 0.82), with contrasts showing a significant difference in latency from the fourth acute dose to the fifth ($p = 0.014$) and as observed in Figure 4.9(B), as the acute dose of amphetamine increases, the peak latency also increases. There were no significant main effects of CHRONIC TREATMENT on the peak latency ($F_{(3, 84)} = 1.88$, $p = 0.140$, $\eta^2 = 0.06$, observed power = 0.47) and no interaction effect ($F_{(12.76, 357.74)} = 0.74$, $p = 0.723$, $\eta^2 = 0.03$, observed power = 0.45).

ACUTE DOSE also had a significant main effect on the normalised maximum amplitude ($F_{(3.60, 298.43)} = 11.70$, $p < 0.001$, $\eta^2 = 0.12$, observed power = 1.00), with contrasts showing a significant increase in the peak amplitude following administration of the first dose of amphetamine when compared to baseline activity ($p < 0.001$), then a subsequent reduction in amplitude between the first and second dose of amphetamine ($p = 0.025$), and a final further reduction in amplitude measured between the fourth and fifth acute dose ($p < 0.001$) this effect can be observed in fig. 4.6(C). Again there were no significant main effects of CHRONIC TREATMENT ($F_{(3, 83)} = 0.32$, $p = 0.812$, $\eta^2 = 0.01$, observed power = 0.11) and no interaction effect ($F_{(10.79, 298.43)} = 0.98$, $p = 0.469$, $\eta^2 = 0.03$, observed power = 0.54).

Finally, there was a significant main effect of ACUTE DOSE on area under the curve ($F_{(3.25, 273.10)} = 23.27$, $p < 0.001$, $\eta^2 = 0.22$, observed power = 1.00). Difference contrasts showed that there was a significant increase in response magnitude from baseline to the first dose of amphetamine administered ($p < 0.001$) and again between the first and second acute administration of amphetamine ($p = 0.001$), then the response magnitude decreased significantly from the third to fourth dose ($p = 0.001$) and there was a further

reduction from the fourth to the fifth dose ($p < 0.001$) which can be observed in Figure 4.9(D). CHRONIC TREATMENT did not have a significant main effect on the area under the curve ($F_{(3, 84)} = 0.34$, $p = 0.793$, $\eta^2 = 0.01$, observed power = 0.12) and there was no significant interaction effect ($F_{(9.75, 273.10)} = 1.33$, $p = 0.215$, $\eta^2 = 0.05$, observed power = 0.67).
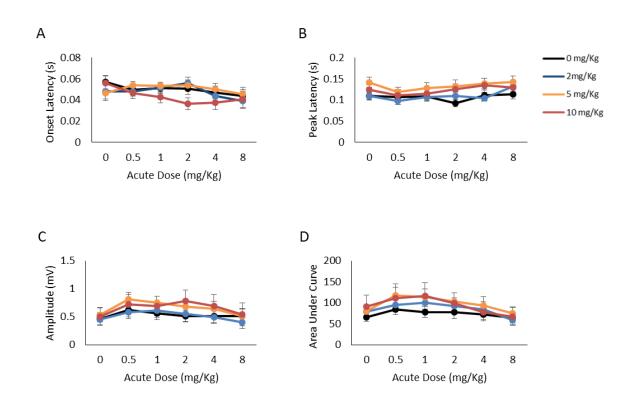
Figure 4.9 The A) onset latency, B) peak latency, C) maximum amplitude (normalised) and D) area under the curve (mean ± SEM) of PSTHs calculated from multiunit activity in response to increasing cumulative acute doses of amphetamine, following chronic amphetamine treatment (key shows chronic amphetamine dose groups). Only recordings with responses to all stimuli were included in this analysis (0 mg/Kg, N = 22; 2 mg/Kg, N = 24; 5 mg/Kg, N = 22; 10 mg/Kg, N = 20).

Local field potentials

In addition to investigating the multiunit activity, data was also filtered in order to analyse the LFPs in response to an acute amphetamine challenge. Visual responses were found in local field potentials following at least one acute dose of amphetamine in 98.8% of recordings (N = 84). There were no significant associations between chronic dose and whether a visual response was recorded at any acute dose strength (Baseline: χ^2 (4) = 2.86, p = 0.582, V = 0.184; 0.5 mg/Kg: χ^2 (4) = 4.64, p = 0.326, V = 0.235; 1 mg/Kg: χ^2 (4) = 7.49, p = 0.112, V = 0.299; 2 mg/Kg: χ^2 (4) = 10.96, p = 0.112, V = 0.299; 4 mg/Kg: χ^2 (4) = 6.39, p = 0.172, V = 0.276; 8 mg/Kg: χ^2 (4) = 2.82, p = 0.588, V = 0.183) indicating that chronic amphetamine treatment has not impacted the occurrences of visual responses in local field potentials. The normality of the data was assessed as above and repeated measures ANOVAs were used to analyse the data, using ACUTE DOSE as the within measures factor, and CHRONIC TREATMENT as the between measures factor. ACUTE DOSE had a significant main effect (F (3.29, 230.48) = 4.00, p = 0.007, η^2 = 0.05, observed power = 0.86) on the onset latency of the response with difference contrasts showing a significant decrease in latency from the baseline activity to the first acute dose (p < 0.001) and a further decrease from the first to second acute dose (p = 0.026) as seen in Figure 4.10(A). There were no significant main effects of CHRONIC TREATMENT (F (3, 70) = 0.41, p = 0.745, η^2 = 0.02, observed power = 0.13) and no interaction effect (F (9.88, 230.48) = 1.41, p = 0.179, η^2 = 0.06, observed power = 0.70). There were also no significant main effects of ACUTE DOSE (F (4.13, 288.80) = 2.00, p = 0.093, η^2 = 0.03, observed power = 0.61) or CHRONIC TREATMENT (F (3, 70) = 2.00, p = 0.122, η^2 = 0.08, observed power = 0.49) on the peak latency, and no interaction effect (F (12.38, 288.80) = 1.03, p = 0.426, η^2 = 0.04, observed power = 0. 61), as seen in Figure 4.10(B).

There was, however, a significant main effect of ACUTE DOSE ($F_{(3.28, 229.76)} = 3.41$, $p = 0.015$, $\eta^2 = 0.05$, observed power = 0.79) on the maximum peak to peak amplitude of the waveform average, but no main effect of CHRONIC TREATMENT ($F_{(3, 70)} = 0.57$, $p = 0.638$, $\eta^2 = 0.02$, observed power = 0.16) and no interaction effect ($F_{(9.85, 229.76)} = 0.42$, $p = 0.932$, $\eta^2 = 0.02$, observed power = 0.22). Contrasts revealed the amplitude decreased significantly from the third to the fourth dose ($p = 0.048$), and from the fourth to the fifth dose ($p = 0.001$), this effect is also apparent from Figure 4.10(C). There was also a significant main effect of ACUTE DOSE ($F_{(3.45, 241.48)} = 9.50$, $p < 0.001$, $\eta^2 = 0.12$, observed power = 1.00) on the area under the curve, but again no main effect of CHRONIC TREATMENT ($F_{(3, 70)} = 2.32$, $p = 0.083$, $\eta^2 = 0.09$, observed power = 0.56) and no interaction effect ($F_{(10.35, 241.48)} = 1.47$, $p = 0.146$, $\eta^2 = 0.06$, observed power = 0.74). Contrasts show that the area increased significantly from baseline to the first acute dose ($p = 0.006$) and increased again from the first to second dose ($p < 0.001$), the area then decreased significantly between the fourth and fifth dose ($p = 0.001$), as seen in Figure 4.10(D).
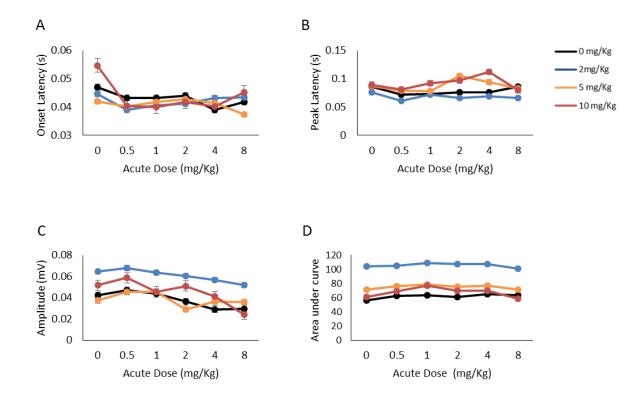
Figure 4.10 The A) onset latency, B) peak latency, C) maximum amplitude and D) area under the curve (mean ± SEM) of waveform averages calculated from local field potentials in the SC in response to increasing cumulative acute doses of amphetamine, following chronic amphetamine treatment (key shows chronic amphetamine dose groups). Only recordings with responses to all stimuli were included in this analysis (0 mg/Kg, N = 20; 2 mg/Kg, N = 24; 5 mg/Kg, N = 18; 10 mg/Kg, N = 12).

4.3.4 VOLUME MATCHED SALINE

Multiunit activity

As a control for the acute amphetamine challenge, volume matched quantities of saline were administered intravenously. To do so the collicular response to a light flash stimulus was recorded for each dose of 0.9% saline using the same method applied during the acute amphetamine challenge. Visual responses were found in multiunit activity following

at least one saline administration in 92.2% of recordings (N = 90). There were no significant associations between chronic dose and whether a visual response was recorded at any dose of saline (Baseline: χ^2 (4) = 5.61, p = 0.230, V = 0.250; dose 1: χ^2 (4) = 8.64, p = 0.071, V = 0.310; dose 2: χ^2 (4) = 4.10, p = 0.393, V = 0.213; dose 3: χ^2 (4) = 6.01, p = 0.198, V = 0.258; dose 4: χ^2 (4) = 6.95, p = 0.139, V = 0.278; dose 5: χ^2 (4) = 6.57, p = 0.161, V = 0.270) indicating that chronic amphetamine treatment has not impacted on the occurrences of visual responses. The data collected was first tested for normality as described above. Data was then analysed using the same methods utilised in the analysis of the acute amphetamine challenge, by using repeated measures ANOVAs with CHRONIC TREATMENT as a between measures factor and ACUTE DOSE as a within measures factor. No significant main effects of ACUTE DOSE (F (4.26, 289.62) = 0.97, p = 0.434, η^2 = 0.01, observed power = 0.35) or CHRONIC TREATMENT (F (3, 68) = 0.52, p = 0.669, η^2 = 0.02, observed power = 0.15) were found on onset latency, and no interaction effects between ACUTE DOSE and CHRONIC TREATMENT (F (12.78, 289.62) = 1.07, p = 0.384, η^2 = 0.05, observed power = 0.64). There were also no significant main effects of either the ACUTE DOSE (F (4.02, 269.22) = 0.68, p = 0.609, η^2 = 0.01, observed power = 0.22) or CHRONIC TREATMENT (F (3, 68) = 0.94, p = 0.426, η^2 = 0.04, observed power = 0.25) on the peak latency, and no interaction effects (F (12.05, 269.22) = 0.87, p = 0.579, η^2 = 0.04, observed power = 0.51). No significant main effects of ACUTE DOSE (F (3.98, 266.40) = 0.38, p = 0.838, η^2 = 0.01, observed power = 0.13) or CHRONIC TREATMENT (F (3, 67) = 0.87, p = 0.459, η^2 = 0.04, observed power = 0.23) on the maximum amplitude were found, and there were also no interaction effects (F (11.93, 266.40) = 0.99, p = 0.457, η^2 = 0.04, observed power = 0.57). Lastly, for the area under the curve, there were no significant main effects of ACUTE DOSE (F (2.15, 143.70) = 0.70, p = 0.510, η^2 = 0.01, observed power

= 0.17) or CHRONIC TREATMENT (F (3, 67) = 0.89, p = 0.453, η^2 = 0.04, observed power = 0.23), and no interaction effects (F (6.44, 143.70) = 1.21, p = 0.301, η^2 = 0.05, observed power = 0.49). These results are displayed in Figure 4.11.
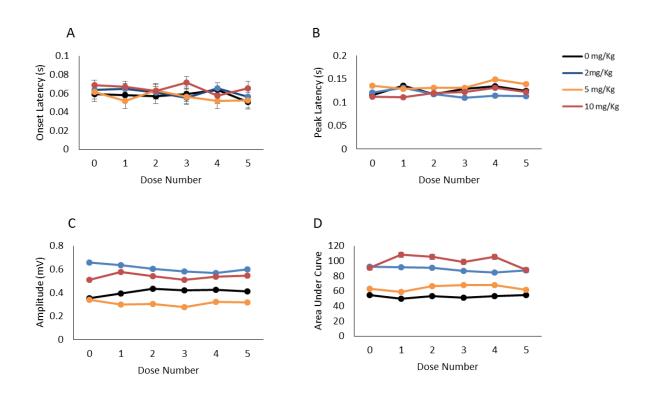
Figure 4.11 The A) onset latency, B) peak latency, C) maximum amplitude (normalised) and D) area under the curve (mean ± SEM) of PSTHs calculated from multiunit activity in response to intravenous (i.v.) injections of 0.9 % saline, volume matched to the acute amphetamine doses, following chronic amphetamine treatment (key shows chronic amphetamine dose groups). Only recordings with responses to all stimuli were included in this analysis (0 mg/Kg, N = 18; 2 mg/Kg, N = 21; 5 mg/Kg, N = 20; 10 mg/Kg, N = 15).

Local field potentials

As with the multi-unit activity above, the local field potentials for the acute saline control group were analysed using the same methods used to analyse the acute amphetamine challenge. Visual responses were found following at least one saline administration in 98.7% of recordings (N = 78). There were no significant associations between chronic dose and whether a visual response was recorded at any dose of saline (Baseline: χ^2 (4) = 2.51, p = 0.642, V = 0.179; dose 1: χ^2 (4) = 6.64, p = 0.156, V = 0.292; dose 2: χ^2 (4) = 4.49, p = 0.344, V = 0.240; dose 3: χ^2 (4) = 4.49, p = 0.344, V = 0.240; dose 4: χ^2 (4) = 4.90, p = 0.298, V = 0.251; dose 5: χ^2 (4) = 1.62, p = 0.806, V = 0.144) which indicates that chronic amphetamine treatment has not impacted on the occurrences of visual responses. When comparing the chronically treated groups it was found that there were no significant main effects of the ACUTE DOSE of saline on the onset latency of the waveform averages (F (3.23, 187.58) = 0.92, p = 0.438, η^2 = 0.02, observed power = 0.26) and also no main effect of CHRONIC TREATMENT (F (3, 58) = 1.06, p = 0.374, η^2 = 0.05, observed power = 0.27). There was also no interaction effect between ACUTE DOSE and CHRONIC TREATMENT (F (9.70, 187.58) = 1.10, p = 0.368, η^2 = 0.05, observed power = 0.56). There were no significant main effects of ACUTE DOSE (F (2.97, 145.73) = 1.05, p = 0.374, η^2 = 0.02, observed power = 0.28) or CHRONIC TREATMENT (F (3, 49) = 2.61, p = 0.062, η^2 = 0.14, observed power = 0.60) on peak latency, and no interaction effects (F (8.92, 145.73) = 1.31, p = 0.238, η^2 = 0.07, observed power = 0.62). The maximum amplitude of the waveform averages had no significant differences in ACUTE DOSE (F (2.93, 152.40) = 0.46, p = 0.703, η^2 = 0.01, observed power = 0.14) or CHRONIC TREATMENT (F (3, 52) = 1.23, p = 0.307, η^2 = 0.07, observed power = 0.31), and no interaction effect (F (8.79, 152.40) = 1.63, p = 0.112, η^2 = 0.09, observed power = 0.73). Finally, there were no significant main

effects on area under the curve observed for ACUTE DOSE ($F_{(1.66, 79.50)} = 0.09$, $p = 0.881$, $\eta^2 = 0.002$, observed power = 0.06) or CHRONIC TREATMENT ($F_{(3, 48)} = 1.329$, $p = 0.276$, $\eta^2 = 0.08$, observed power = 0.33), and no interaction effects ($F_{(5.00, 79.50)} = .54$, $p = 0.745$, $\eta^2 = 0.03$, observed power = 0.19). The results are summarised Figure 4.12.
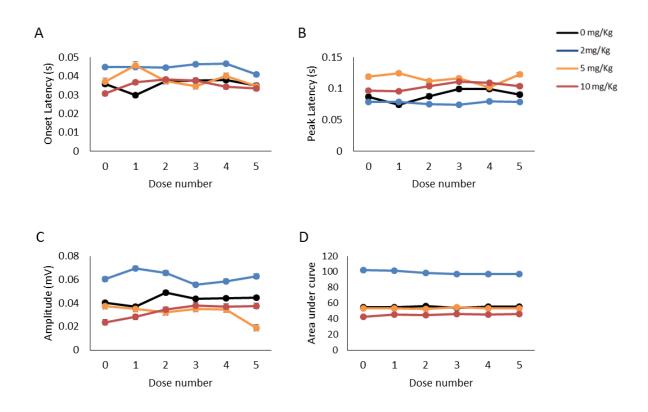
Figure 4.12 The A) onset latency, B) peak latency, C) maximum amplitude and D) area under the curve (mean ± SEM) of waveform averages calculated from local field potentials in response to i.v. injections of 0.9 % saline, volume matched to the acute amphetamine doses, following chronic amphetamine treatment (key shows chronic amphetamine dose groups). Only recordings with responses to all stimuli were included in this analysis (0 mg/Kg, N = 14; 2 mg/Kg, N = 16; 5 mg/Kg, N = 14; 10 mg/Kg, N = 9).

4.3.5 BIPHASIC MULTIUNIT ACTIVITY

Stimulus response curve

As it has been observed that the collicular response to a light flash stimulus is multiphasic, further analysis into the multiunit activity was performed, separating that data into an initial phase which encompassed the activity following the direct retinal input, and a second phase which included activity in the SC following general cortical input. A biphasic response was identified in 74% of recordings to at least one stimulus intensity, however analysis was only performed when a biphasic response was found at every intensity (N = 26). There were no significant associations between chronic dose and whether a biphasic response was recorded at any stimulus intensity (4 mcd: χ^2 (3) = 3.24, p = 0.356, V = 0.347; 8 mcd: χ^2 (3) = 3.36, p = 0.339, V = 0.353; 12 mcd: χ^2 (3) = 4.92, p = 0.178, V = 0.427; 16 mcd: χ^2 (3) = 7.20, p = 0.066, V = 0.517; 20 mcd: χ^2 (3) = 5.73, p = 0.125, V = 0.461). From this filtered data PSTHs have been plotted, and the maximum amplitude and area under the curve (normalised to the length of the phase) for each phase were calculated. For this analysis only the main effect of PHASE and any interaction effects involving PHASE are reported as the effects of CHRONIC TREATMENT, STIMULUS INTENSITY and ACUTE DOSE have already been investigated above.

When comparing the chronically treated groups, it was found that there was a significant main effect of PHASE (F (1, 23) = 22.36, p < 0.001, η^2 = 0.49, observed power = 1.00) on the area under the curve. Simple contrasts found that the area under the curve was significantly larger in phase 1 in comparison to phase 2 (p < 0.001), as can be seen from Figure 4.13(A and B). There was also a significant interaction effect between STIMULUS INTENSITY and PHASE (F (2.62, 60.32) = 10.06, p < 0.001, η^2 = 0.30, observed power =

0.99), with simple effects tests revealing that this interaction was driven by significant differences between the phases at all stimulus intensities except for the fourth stimulus intensity (first, p = 0.002; second, p = 0.030; third, 0.001; fifth, p < 0.001). There were no significant interactions between CHRONIC TREATMENT and PHASE ($F_{(3, 23)}$ = 1.688, p = 0.197, η^2 = 0.18, observed power = 0.38) or between CHRONIC TREATMENT, STIMULUS INTENSITY, and PHASE ($F_{(7.87, 60.32)}$ = 1.71, p = 0.116, η^2 = 0.18, observed power = 0.68).

There was a significant main effect of PHASE on maximum amplitude ($F_{(1, 17)}$ = 4.72, p = 0.044, η^2 = 0.22, observed power = 0.54), with contrasts revealing the amplitude was significantly larger in the first phase in comparison to the second (p = 0.044), these results can be seen in Figure 4.13 (C & D). There was a significant interaction effect between PHASE and CHRONIC TREATMENT ($F_{(3, 17)}$ = 3.53, p = 0.38, η^2 = 0.38, observed power = 0.68), with simple effects tests revealing the 2 mg/Kg group had a significantly greater amplitude in comparison to the 0 mg/Kg group (p = 0.012) which only occurred during the first phase, and during the second phase the peak amplitude of the 10 mg/Kg group was significantly larger than the 2 mg/Kg group. These results can be seen in Figure 4.13 (C & D). There were no significant interactions between PHASE and STIMULUS INTENSITY ($F_{(1.35, 22.95)}$ = 0.73, p = 0.441, η^2 = 0.04, observed power = 0.14), or between CHRONIC TREATMENT, STIMULUS INTENSITY, and PHASE ($F_{(4.05, 22.95)}$ = 0.95, p = 0.456, η^2 = 0.14, observed power = 0.25).
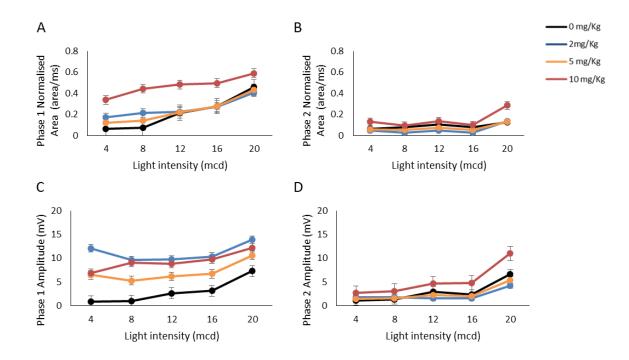
Figure 4.13 The A) first phase and B) second phase normalised area under the curve, and the C) first phase and D) second phase maximum amplitude calculated from PSTHs of multiunit activity in response to a stimulus response curve (mean ± SEM, key shows chronic amphetamine dose groups; 0 mg/Kg, N = 7; 2 mg/Kg, N = 11; 5 mg/Kg, N = 2; 10 mg/Kg, N = 6).

Acute Amphetamine Challenge

A biphasic response was identified in 68.8% of recordings to at least one acute dose of amphetamine, however analysis was only performed when a biphasic response was found at every acute dose (N = 24). There were no significant associations between chronic dose and whether a biphasic response was recorded for any acute dose (Baseline: χ^2 (3) = 2.55, p = 0.467, V = 0.326; 0.5 mg/Kg: χ^2 (3) = 3.45, p = 0.327, V = 0.379; 1 mg/Kg: χ^2 (3) = 3.45, p = 0.327, V = 0.379; 2 mg/Kg: χ^2 (3) = 4.03, p = 0.259, V = 0.410; 4 mg/Kg: χ^2 (3) = 0.23, p = 0.973, V = 0.097; 8 mg/Kg: χ^2 (3) = 0.23, p = 0.973, V = 0.097). For the area under the curve data there was a significant main effect of PHASE (F (1, 20) = 35.39, p <

0.001, η^2 = 0.64, observed power = 1.00) where the area of the first phase was significantly greater than that of the second phase, as can be observed in Figure 4.14 (A & B). There were no significant interaction effects found between PHASE and either ACUTE DOSE (F (2.40, 47.92) = 2.00, p = 0.138, η^2 = 0.09, observed power = 0.43) or CHRONIC TREATMENT (F (3, 20) = 1.11, p = 0.368, η^2 = 0.14, observed power = 0.25), and no interaction between PHASE, CHRONIC TREATMENT and ACUTE DOSE (F (7.19, 47.92) = 1.32, p = 0.262, η^2 = 0.17, observed power = 0.51). Analysis of the maximum amplitude data revealed no significant main effects of PHASE (F (1, 12) = 1.34, p = 0.270, η^2 = 0.10, observed power = 0.19), in addition to no interaction effects between any of the parameters (PHASE and CHRONIC TREATMENT, F (3, 12) = 0.35, p = 0.788, η^2 = 0.08, observed power = 0.10; PHASE and ACUTE DOSE, F (3.05, 36.54) = 0.86, p = 0.470, η^2 = 0.07, observed power = 0.22; PHASE, CHRONIC TREATMENT and ACUTE DOSE, F (9.13, 36.54) = 0.56, p = 0.819, η^2 = 0.12, observed power = 0.23), as seen in Figure 4.11 (C & D).
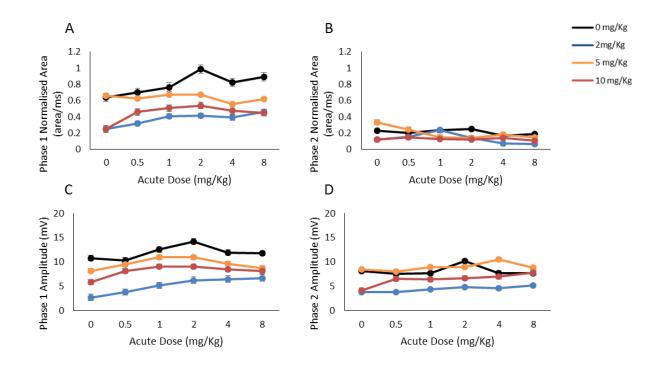
Figure 4.14 The A) first phase and B) second phase normalised area under the curve, and the C) first phase and D) second phase peak amplitude calculated from PSTHs of multiunit activity in response to an acute amphetamine challenge (mean ± SEM, key shows chronic amphetamine dose groups; 0 mg/Kg, N = 6; 2 mg/Kg, N = 4; 5 mg/Kg, N = 5; 10 mg/Kg, N = 9).

Volume matched Saline

A biphasic response was identified in 77.2% of recordings to at least one dose of saline, however analysis was only performed when a biphasic response was found at every acute dose (N = 30). There were no significant associations between chronic dose and whether a biphasic response was recorded at any saline dose (Baseline: χ^2 (3) = 6.56, p = 0.087, V = 0.468; dose 1: χ^2 (3) = 2.45, p = 0.485, V = 0.286; dose 2: χ^2 (3) = 2.49, p = 0.477, V = 0.288; dose 3: χ^2 (3) = 0.87, p = 0.833, V = 0.170; dose 4: χ^2 (3) = 0.45, p = 0.929, V = 0.123; dose 5: χ^2 (3) = 5.17, p = 0.160, V = 0.415). When investigating the effects of the saline trial on the biphasic response it was found that there was a significant main effects of PHASE on

area under the curve ($F_{(1, 7)} = 5.83$, $p = 0.046$, $\eta^2 = 0.45$, observed power = 0.55), where the area of the first phase was significantly greater than that of the second phase, as seen in Figure 4.15 (A & B), this effect is similar to that found following acute amphetamine administration above. There were no significant interaction effects found (PHASE and CHRONIC TREATMENT, $F_{(3, 7)} = 0.06$, $p = 0.982$, $\eta^2 = 0.02$, observed power = 0.06; PHASE and ACUTE DOSE, $F_{(2.00, 14.03)} = 1.80$, $p = 0.201$, $\eta^2 = 0.21$, observed power = 0.31; PHASE, CHRONIC TREATMENT and ACUTE DOSE, $F_{(6.01, 14.03)} = 1.02$, $p = 0.452$, $\eta^2 = 0.30$, observed power = 0.28). Furthermore, analysis of the maximum amplitude found no significant main effects of PHASE ($F_{(1, 6)} = 5.70$, $p = 0.054$, $\eta^2 = 0.49$, observed power = 0.52), and no interaction effects (PHASE and CHRONIC TREATMENT, $F_{(3, 6)} = 1.16$, $p = 0.399$, $\eta^2 = 0.37$, observed power = 0.19; PHASE and ACUTE DOSE, $F_{(247, 14.81)} = 0.27$, $p = 0.807$, $\eta^2 = 0.04$, observed power = 0.09; PHASE, CHRONIC TREATMENT and ACUTE DOSE, $F_{(7.41, 14.81)} = 0.29$, $p = 0.953$, $\eta^2 = 0.13$, observed power = 0.11). These results can be seen in Figure 4.15 (C & D).
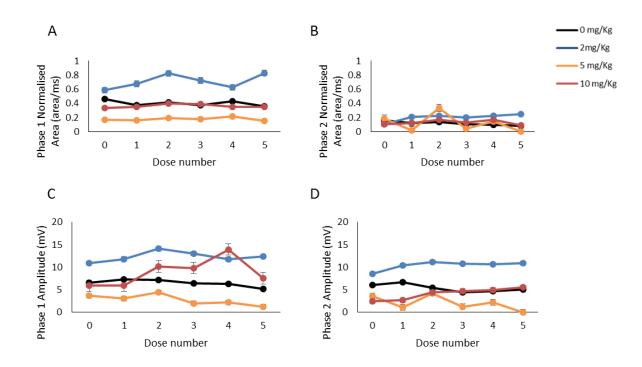
Figure 4.15 The A) first phase and B) second phase normalised area under the curve, and the C) first phase and D) second phase peak amplitude calculated from PSTHs of multiunit activity in response to a volume matched Saline control (mean ± SEM, key shows chronic amphetamine dose groups; 0 mg/Kg, N = 7; 2 mg/Kg, N = 7; 5 mg/Kg, N = 8; 10 mg/Kg, N = 8).

4.3.6 RECONSTRUCTION OF RECORDING SITE

Reconstructions were performed to establish the location of the recording sites used for electrophysiology. There were no significant differences found between chronic treatment groups for collicular layer (χ^2 (8) = 2.71, p = 0.951, V = 0.113) or distance from bregma (χ^2 (8) = 10.39, p = 0.239, V = 0.221). Reconstructions of the control groups can be seen in Figure 4.16, and amphetamine treated groups are presented in Figure 4.17. In both cases sites overlap so total sample size is provided in the figure legend.

Interaural 2.96 mm* Bregma -6.04 mm

Interaural 2.70 mm Bregma -6.30 mm

Interaural 2.28 mm Bregma -6.72 mm
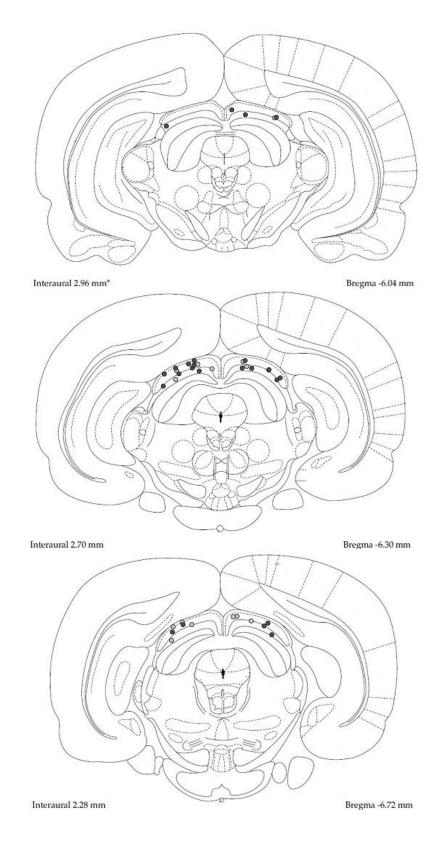
Figure 4.16. Reconstructions of electrophysiology recording sites in control groups (black: Vehicle, N = 48; grey: untreated, N = 34). All recordings took place in the Zo, SuG, or Op layer of the superficial superior colliculus between -6.04mm and -6.72mm from bregma. Adapted from Paxinos and Watson (1998).
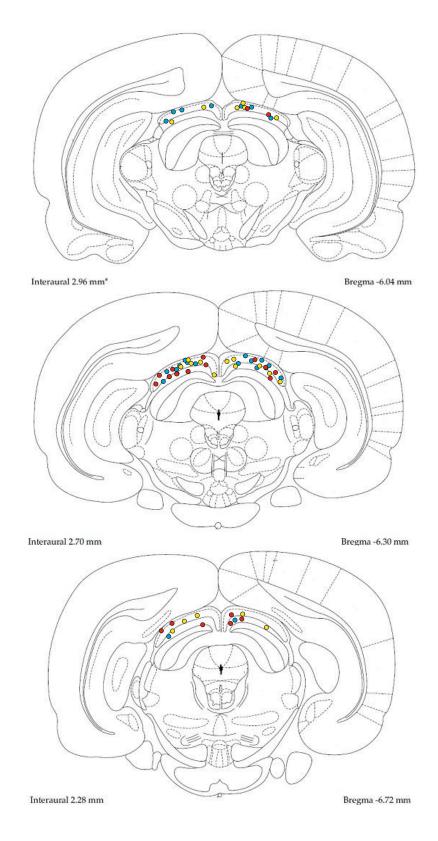
Figure 4.17. Reconstructions of electrophysiology recording sites in amphetamine treated groups (blue: 2 mg/Kg, N = 32; yellow: 5 mg/Kg, N = 37; red: 10 mg/Kg, N = 26). All recordings took place in the Zo, SuG, or Op layer of the superficial superior colliculus between -6.04mm and -6.72mm from bregma. Adapted from Paxinos and Watson (1998).

4.4 Discussion

When analysing the multiunit activity in the superior colliculus in response to a light stimulus it was observed that increasing the light intensity resulted in reduced onset and peak latencies. The maximum amplitude and magnitude of the response, gauged from the area under the curve, both increased as the light intensity was increased, up to an intensity of 16 mcd, the maximum amplitude then decreased at 20 mcd, however the response magnitude was still larger at 20 mcd in comparison to 16 mcd due to a prolonged "ringing" effect seen in the response as it returns to base line. An example of this effect can be seen in Figure 4.18.
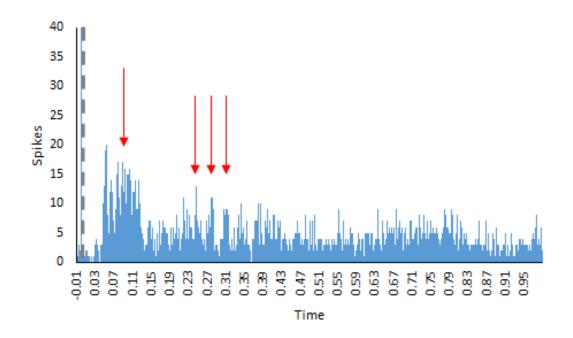
Figure 4.18 A PSTH of multiunit data recorded in response to a 20 mcd light flash stimuli. Red arrows indicate peaks in the data, with a ringing effect of smaller peaks following the initial response to the stimuli onset (grey dashed line) as activity returns to a baseline level.

This response to increasing light intensity was anticipated as it is the typical response found in a stimulus response curve (Stein et al., 1976, Altman and Malis, 1962, Hetherington et al., 2017). The only significant main effect of chronic treatment found was on the onset latencies; it could be observed the onset latencies of the 5 mg/Kg and 10 mg/Kg groups appeared to be longer than the control and 2 mg/Kg groups, however despite their being a main effect of chronic treatment there were no significant differences found between groups when post hoc Tukey tests were performed. The observed power of this analysis was 0.68, which is slightly below the target value so it is possible that the significant result may be an artefact of the analysis performed, rather than a genuine effect of chronic treatment. However, an impact on the response latencies would be expected at high doses if chronic amphetamine treatment was suppressing of activity in the SC, as has been found in previous research (Dommett et al., 2009, Clements et al., 2014).

When analysing the local field potential data in response to the light flash, in order to assess the potential differences in incoming data to the superior colliculus, it was found that light intensity did not affect the onset or peak latency, but there was a significant main effect of light intensity on the maximum amplitude and the magnitude of the response (as measured by the area under the curve), with both increasing as the light intensity was increased. There were also significant main effects of chronic treatment on the peak latency. From post hoc Tukey tests it was observed that the peak latency of the 2 mg/Kg group was significantly lower than both the 5 and 10 mg/Kg groups. This shows a low therapeutic dose caused a faster peak response to a stimulus when compared to the higher therapeutic dose and abuse level dose and indicates that low therapeutic doses could have a greater effect long term, but since this effect was found only in the local

field potential data and not in the multiunit activity it is indicative of incoming information arriving faster, as opposed to faster processing within the SC. It can therefore be concluded that the signal in response to the light flash arrives at the SC faster when using a therapeutic dose of amphetamine, but the signal does not leave the SC any quicker since there were no differences found in multiunit latency data, this could suggest suppression of activity in the SC. This trend may also be seen in the response magnitude data, since in the plots of responses the 2 mg/Kg group appeared to have the highest response magnitude, and from investigating interaction effects it was seen that at lower stimulus intensities the 2 mg/Kg group was significantly higher than the other dose groups, however there was no overall significant main effect of chronic treatment on response magnitude. If this is a genuine effect of chronic amphetamine treatment and not an artefact of the analysis, this could again be representative of suppression of activity, with activity being suppressed in the two highest dose groups in comparison to the 2 mg/Kg group as evident from their reduced responses to the stimuli. There may also be evidence of suppression within the SC in the 2 mg/Kg group, since the signal of the 2 mg/Kg group had a greater magnitude in comparison to the control when it arrived in the SC, but since there were no significant differences found in the MUA, the magnitude of the responses within the SC have been suppressed.

The analysis of multiunit activity in response to the acute amphetamine challenge revealed that that i.v. amphetamine administration decreased both the onset latency and the peak latency, with the general trend being the higher the dose, the shorter the latency. Acute administration of amphetamine also had a significant effect on the maximum amplitude and the magnitude of the response. It was observed that acute doses initially increased the amplitude and magnitude of the response, however as the

cumulative dose increased the amplitude and magnitude of the responses steadily decreased. These findings are in line with previous observations which showed that visual responses in the superior colliculus were dose dependently depressed following i.v. administration of amphetamine (Gowan et al., 2008, Clements et al., 2014). Similarly, there were significant main effects of acute amphetamine treatment on the maximum amplitude and response magnitude of the local field potentials, and it was observed that acute amphetamine appeared to depress visual responses at the highest doses. There was no significant main effect of acute amphetamine on the onset or peak latency of the local field potentials, and also no significant main effects of chronic amphetamine treatment in response to the acute amphetamine challenge for either the multiunit activity or local field potentials, and no significant interaction effects. Volume matched saline administration had no effects on multiunit activity or local field potentials, and therefore the depression of visual responses observed above can be confidently attributed to the effects of the amphetamine administration, as opposed to any general effect of i.v. injections.

When multiunit activity was divided into initial (retinal) response, and secondary (cortical) response, it was found that in addition to the effects of stimulus intensity previously observed, the first phase had a significantly higher maximum amplitude and area under the curve in comparison to the second phase. Similar significant differences in the main effects of phase on the visual responses were observed for the acute amphetamine and volume matched saline trials, but there were no significant interactions found between chronic or acute amphetamine treatment and response phase. There was a significant interaction found between chronic treatment and phase for the maximum amplitude of the response in the stimulus response data; during the first phase the 2 mg/Kg group had

a significantly greater amplitude in comparison to the 0 mg/Kg group, then during the second phase the peak amplitude of the 10 mg/Kg group was significantly larger than the 2 mg/Kg group. This indicates that the collicular response to primary retinal input was greatest following treatment with therapeutic doses of amphetamine, but the response to secondary cortical input was greatest following chronic treatment with the highest doses of amphetamine. It was observed from the overall analysis of MUA that there were no significant differences between the chronic treatment groups, however from the biphasic analysis it can be seen that the maximum amplitude of the MUA was significantly larger than the control group following initial retinal input, but was lost in the response following cortical input. Therefore suppression of activity by amphetamine within the SC may occur following input from other areas of the brain. It should be noted also that although there were never any significant differences found between the treated and untreated control groups in any analysis, there was also consistently low power observed for these comparisons, which may have influenced the significance, giving a higher chance of Type II errors.

From these results it is clear that acute amphetamine treatment results in depression of visual activity at high doses, as supported by findings in previous studies (Gowan et al., 2008). There was also a heightened response to the light stimulus in the signal arriving in the SC following chronic treatment which was only present for the lower dose, indicating that this could be a therapeutic effect. Since there was no difference in the subsequent MUA there is also indication that chronic amphetamine treatment is suppressing visual responsiveness in the superficial layers of the SC.

CHAPTER 5. MORPHOLOGICAL CHANGES IN THE SUPERIOR COLLICULUS FOLLOWING CHRONIC AMPHETAMINE TREATMENT

5.1 Introduction

Chronic amphetamine treatment has been linked to a number of structural changes within the brain, in both animals and in people receiving treatment for ADHD. Previous studies have found that chronic amphetamine treatment results in changes to the dendritic branching and spine density within the nucleus accumbens and prefrontal cortex (PFC) in the rat (Robinson and Kolb, 1999) and in the PFC of non-human primates (Selemon et al., 2007). There is evidence that shows repeated treatments with amphetamine affects dendritic spine morphology in the PFC (Robinson and Kolb, 1999, Selemon et al., 2007), but there does not appear to be consensus on the effects of this treatment. The study investigating the effects of repeated treatment on rats found an increase in the density of dendritic spine on pyramidal cells within the medial PFC (Robinson and Kolb, 1999), whereas Selemon et al. (2007) found that the peak spine density of pyramidal cells in the PFC was reduced following the induction of amphetamine sensitization in macaques. There is also evidence to suggest dendritic spine type can be affected by psychostimulant drugs, such as cocaine with some studies finding increases in the densities of specific spine types, for example, thin spines or mushroom spines, in the nucleus accumbens following drug treatment (Lee et al., 2006), however so far no studies have been performed in order to investigate the dendritic spine densities and spine types within the superior colliculus. Furthermore, previous research has focused on treatment

regimens designed to induce sensitization, as opposed to clinically relevant doses and methods of drug administration which were found to induce tolerance, as discussed in chapter 2.4. Additionally, several meta-analyses have been performed on magnetic resonance imaging (MRI) data which was collected from people diagnosed with ADHD. These studies found evidence to suggest that long term treatment of ADHD with psychostimulant drugs resulted in a reduction in the severity of structural abnormalities commonly associated with ADHD (Valera et al., 2007, Spencer et al., 2013). Structural abnormalities found in untreated children and adolescents with ADHD include reduction in the total cerebral volume (Castellanos et al., 2002, Castellanos et al., 1996, Valera et al., 2007). Specifically smaller volumes have been identified in the PFC (Mostofsky et al., 2002) and basal ganglia (Aylward et al., 1996), areas known to be involved in the regulation of distractibility. Since reductions in brain volumes have previously been observed in areas of the brain relating to distractibility in treatment naïve children and adolescents with ADHD, and treatment with amphetamine was found to attenuate this volume deficit, chronic amphetamine treatment may also have an affect the volume or cell density of the SC as another neural correlate of distractibility.

Previous studies have found abnormalities in the morphology of SC when investigating spontaneous hypertensive rats as a rodent model of ADHD (Brace et al., 2015a). Specifically it was found that there was a reduction in the glia: neuron ratio in the ADHD model rats. This reduction in the ratio is believed to be linked to a possible reduction in neuronal size, as other research has indicated that an increase in the glia: neuron ratio is correlated with an increase in the size of neurons (Herculano-Houzel, 2014). ADHD has also been linked to abnormal or reduced function in astrocytes (Killeen et al., 2013, Todd and Botteron, 2001) which reduces the energy available to neurons. Furthermore

treatment with psychostimulants has been found to increase astrocyte activation (Narita et al., 2009). Since there is previous evidence to suggest chronic psychostimulant treatment in humans can reduce structural abnormalities caused by ADHD in other areas of the brain, it is hypothesised that chronic amphetamine treatment in rats may also affect the glia: neuron ratio within the superior colliculus.

In addition to potential gross morphological differences in cell densities and glia: neuron ratios, and changes to dendritic spines, it is possible that psychostimulant drug treatment could have an effect on synaptic integrity. A study investigating whether stress alters the effects of amphetamine in rats found that chronic amphetamine treatment reduced expression of synaptophysin, an essential synaptic vesicle protein, in the CA1 region of the hippocampus, and increased expression of synaptophysin in the caudate putamen (Bisagno et al., 2004). It has also been found that the induction of behavioural sensitization in rats using repeated administration of amphetamine resulted in a reduction in the levels of synaptophysin found in the nucleus accumbens (Subramaniam et al., 2001).

HYPOTHESES

It was hypothesised that:

- Chronic amphetamine treatment would cause changes to dendritic spine densities and spine types;
- Presynaptic changes would occur following chronic treatment, as expressed by the levels of synaptophysin.
- There would be a significant effect on the gross morphology and glia/neuron ratio within the SC following chronic amphetamine treatment.

5.2 Methods

5.2.1 GROSS ANATOMICAL MEASURES

Animals (Untreated N=3; Vehicle N= 4; 2 mg/Kg N= 3; 5 mg/Kg N=6; 10 mg/Kg N=4) within this group were sacrificed by i.p. injection of pentobarbatone (Animalcare, York, UK), perfused with 0.9 % saline and fixed with 4 % paraformaldehyde in 0.1 M PB, pH 7.4 within 2 weeks of the final dose of the chronic treatment, as described in section 4.2. The brain was then removed and stored in fixative for at least 24 hours, then transferred to 20 % sucrose for at least 36 hours. As with the reconstruction processing described above brains were frozen to -20 °C in isopentane (VWR International, Lutterworth, UK) and coronal sections were sliced using a cryostat (CM1900, Leica, Milton Keynes, UK) at a thickness of 50 µm with one in five sections retained beginning at a random starting point within the first five sections. For volume analysis the whole brain was sectioned, whilst for cell counts only the SC was sectioned in this way.

Volume analysis

For the volume analysis of the superficial layers of the SC as defined by (Paxinos and Watson, 1998), the Cavalieri principle was used. This principle states that the volume of an object can be estimated by calculating the product of the distance between planes (T) and the sum of areas on systematic-random sections through the object (Mandarim-de-Lacerda, 2003) as shown in Figure 5.1.
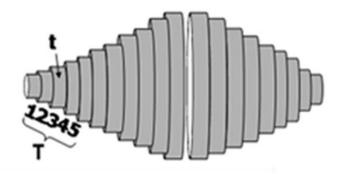
Figure 5.1. The Cavalieri principle that the volume of an object can be calculated as the product of a constant interval of length (T i.e. 250 - five consecutive 50 μm sections) and the total area in μm^2 (ΣA) of all sections through the structure at interval t beginning at a random starting point between 1 and 5. Adapted from Mandarim-de-Lacerda (2003).

Images of the sections were captured using a Microfibre digital camera attached to a Nikon Eclipse 80i microscope (Nikon UK LTD, Kingston-upon-Thames, UK) at a magnification of x1 (Nikon Plan UW, 1x/0.04, WD 3.2). The whole brain and the superficial layers of the SC were then traced throughout the slices using Stereo-Investigator Software (MBF Biosciences, Magdeburg, Germany) and a Cavalieri probe was then applied to each of the traced contours. The whole brain was required because factors such as the physical size of the animal influence the maximum brain size (Raz et al., 1998) and it has therefore been suggested that comparing solely volumes of intracranial structures between groups would not provide reliable data (Knutson et al., 2001). As such, the volume fraction of the superficial SC within the reference volume (the whole brain) was calculated, to give a proportion of the structure (i.e. superficial layers) within the whole brain structure:

$$Volume\ fraction = \frac{Volume\ of\ superficial\ SC}{Volume\ of\ whole\ brain}$$

These data were confirmed as having a normal distribution using either the Kolmogorov–Smirnov or Shapiro-Wilk and measures of skewness and kurtosis as appropriate, before analysis was conducted using a One-Way ANOVA (SPSS, IBM Corp., Armonk, NY, USA) to analyse differences between the chronic treatment groups.

Cell counts

As above images of the sections were captured using a Microfibre digital camera attached to a Nikon Eclipse 80i microscope (Nikon UK LTD, Kingston-upon-Thames, UK). Contours were drawn at low magnification (x1; Nikon Plan UW, 1x/0.04, WD 3.2) around the superficial layers of the SC to identify the region of interest. The stereologically unbiased Optical Fractionator method within Stereo-Investigator Software (MBF Biosciences, Magdeburg, Germany) was used to obtain an estimate of the total number of cells in the superficial SC. The fractionator principle states that if you take as a random sample a known fraction of a population (West et al., 1991), then the unbiased estimate for the population is the value from the sample divided by the fraction sampled; using the following formula.

$$Number\ of\ cells = \sum Q \times \frac{1}{Fraction\ sampled}$$

Where Q is the number of nuclei counted, and the fraction sampled consists of the multiplication of three components. The first component is the section sampling fraction (ssf) which was a constant at 1 in 5 because every fifth section was sampled. The second component is the area sampling fraction (asf) which is the counting frame area divided the gird size area. The superficial SC area traced on each slice served as the grid size area.

The counting frame area was X: 25 µm and Y: 25 µm and constant throughout the experiment for all animals. The final component is the height sampling fraction (hsf) which is the height of the Optical Dissector divided by the mean tissue thickness. Although all slices were cut at 50 µm, shrinkage can occur during alcohol dehydration and therefore the thickness of the slice at each counting frame was measured (the distance between the point where the top of slice is in focus until the bottom of the slice is in focus). Within this area specific rules were used for counting cells in the frame and are demonstrated in Figure 5.2.
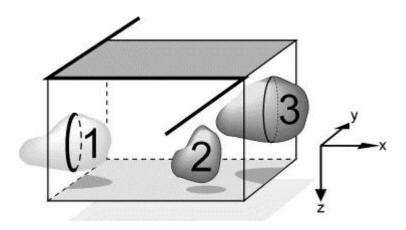
Figure 5.2. An example of counting frame inclusion and exclusion criteria. Any cell in contact with either the top, left, or bottom of the counting frame would be excluded. In this example cell 1 would be excluded as it crosses the border on the left, however cell 3 would be counted. (Keuker et al., 2001)

For a cell to be counted the nucleus had to fall within the counting frame without coming into contact with one of the exclusion lines. In order to quantify the cells, the nucleus of each cell was used to both identify the cell type (whether it was a neuron or glial cell) as well as assess which cells fell within the counting frame. Nuclei from different cell types were differentiated based on morphological criteria of shape and relative size (Figure

5.3). Neurons were identified by their larger shape (generally) and non-spherical outline, as well as a pale and uniformly Nissl-stained cytoplasm with a well-marked nucleolus. Glial nuclei were identified by being generally smaller in size, ovoid shape with the absence of stained cytoplasm, the presence of a thicker nuclear membrane, and more heterogeneous chromatin within the nucleus (Cotter et al., 2002). Although it is not possible to definitively distinguish between glial types, on the basis of their appearance it is highly probable that the large majority are astrocytes.
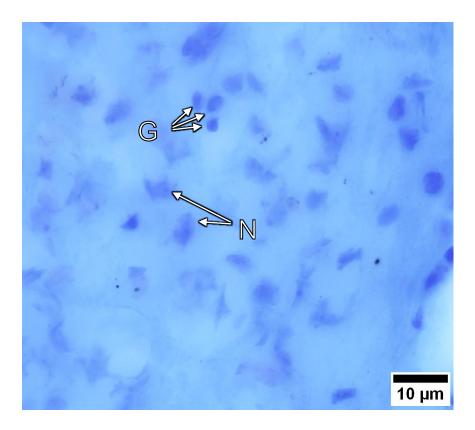
Figure 5.3. Examples of cresyl stained neuronal and glial cells in the SC N: neuron; G: glia.

The counts for neurons and glia were confirmed as having a normal distribution using the same methods as above before analysis was conducted using a One-Way ANOVA to analyse differences between the chronic treatment groups. In addition, the density of each type of cell, calculated using the volume measures was analysed with a One-Way

ANOVA as above. Finally, the glia: neuron ratio was calculated and a One-Way ANOVA also used to determine any differences between the chronic treatment groups.

5.2.2 FINE ANATOMICAL MEASURES
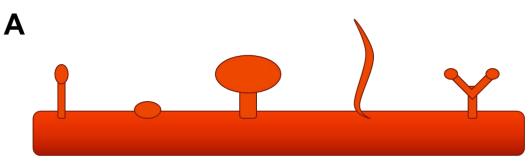
Cell microstructure

Animals (Untreated N=2; Vehicle N= 5; 2 mg/Kg N= 5; 5 mg/Kg N=5; 10 mg/Kg N=4) used for Golgi stain were sacrificed by i.p. injection of pentobarbatone (Animalcare, York, UK), perfused with 0.9 % saline and fixed with 1 % glutaraldehyde and 2 % paraformaldehyde in 0.1 M PB within 2 weeks of the final dose of the chronic treatment. The animal's brain was removed and was stored in 2.5 % glutaraldehyde in 0.1 M PB for at least 24 hours before being switched to 4 % paraformaldehyde until ready for staining.

For the Golgi staining, brains were placed in slicing moulds and sections approximately 3 mm thick were collected through the superior colliculus. These sections were then rinsed briefly in 0.1 M PB and treated in 1 % osmium tetroxide for at least 7 hours or overnight, then transferred to 3.5 % potassium dichromate and left at 4 °C for 4 days, with agitation of the sample on day 2. Sections were then immersed in 1.5 % silver nitrate and the precipitate was gently cleared with a paint brush. The sections were then kept in silver nitrate for 4 days at 4 °C. Following staining the sections were rinsed in 70 % ethanol, the precipitate was cleared and the section was mounted in agar to prevent cracking. Sections were sliced using a vibrating microtome (VT1000, Leica, Milton Keynes, UK) using a high frequency and low speed. The sections were preserved by dehydration in ethanol, and cover slipping using DPX (VWR International, Lutterworth, UK).

Dendrites were located in the SC using random sampling across all superficial layers ensuring suitable coverage of the structure. Dendritic spine density and branching was measured using Neurolucida neuron tracing software (MBF Bioscience, Williston, VT, USA) by creating an image stack through the z-axis, tracing the length of the dendrites and marking each spine occurrence and the spine length to the dendrite surface. There were 227 dendrites analysed in total (mean ± SEM 11.95 ± 1.23 dendrites per animal), and there were no significant differences in the number of dendrites sampled per animal across treatment groups ($F_{(3, 15)}$ = 0.294, p = 0.829). The average length of dendrites measured was 95.13 ± 5.0 μm (mean ± SEM), with a minimum dendritic length of 30.10 μm. The length of the dendrites sampled also did not differ significantly ($F_{(3, 223)}$ = 1.23, p = 0.398) Spine type was also recorded, with differing spine types characterised by morphological criteria as defined previously by Risher et al. (2014) and illustrated in Figure 5.4.

The spine densities and lengths were then analysed using a One-Way ANOVA, following Kolmogorov-Smirnov tests of normality and appropriate measures of skewness and kurtosis, to identify any significant differences between chronic treatment groups. The spine type data was analysed initially with a chi-squared test, then by using a repeated measures ANOVA, using CHRONIC TREATMENT as the between measures factor and SPINE TYPE as the within measures factor.
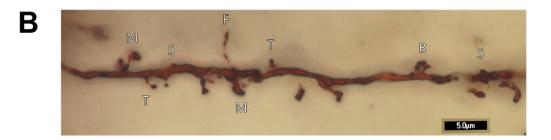
Figure 5.4 A: Spine types found on dendrites within the superior colliculus included: thin spines which were shorter than 2 μm; stubby spines which had a length to width ratio of less than 1; Mushroom spines which were greater than 0.6 μm wide; filopodia which were longer than 2 μm and generally did not have a clearly defined head; and branched spines which had multiple heads (Risher et al., 2014). Detached spines, where the spine head appeared separate to the spine neck also occurred infrequently. B: Golgi stained spines on a typical dendrite within the SC.

Synaptic integrity

Animals (Untreated N=6; Vehicle N= 5; 2 mg/Kg N= 5; 5 mg/Kg N=5; 10 mg/Kg N=6) within this group were sacrificed by i.p. injection of pentobarbatone (Animalcare, York, UK), perfused with 0.9 % saline and fixed with 4 % paraformaldehyde in 0.1 M PB, pH 7.4 within 2 weeks of the final dose of the chronic treatment. The brain was then removed

and stored in fixative for at least 24 hours, then transferred to 20 % sucrose for at least 36 hours. As previously described brains were frozen to -20 °C in isopentane (VWR International, Lutterworth, UK) and coronal sections were sliced using a cryostat (CM1900, Leica, Milton Keynes, UK) at a thickness of 50 μm. Sections were collected through superior colliculus and stored free floating in cryoprotectant storage solution until staining. Sections were warmed to room temperature and four sections per animal were chosen, each approximately 6.3 mm posterior to bregma. Sections were rinsed in 0.1 M phosphate buffer, then placed in 1% Sodium Borohydrate for 30 minutes. Sections were subsequently rinsed in phosphate buffer until the cessation of effervescence. Sections were then placed in 3% hydrogen peroxide for 15 min before being rinsed again in phosphate buffer. Sections were then left in incubation buffer for one hour, then incubated in 1:1000 dilution of mouse anti-synaptophysin (Sigma-Aldrich, Gillingham, UK) antibody overnight. The sections were then rinsed in phosphate buffer and incubated in a 1:200 dilution of biotinylated donkey anti-mouse IgG (Jackson ImmunoResearch, Pennsylvania, USA) for 1 hour, then rinsed in phosphate and tris buffers. A peroxidase ABC kit was used to stain the tissue and DAB was then used to develop the stain. After rinsing in tris buffer sections were mounted on glass slides and air dried for two days before being dehydrated in ethanol, cleared in xylene and cover slipped with DPX.

Each slide was then photographed using a Microfibre digital camera attached to a Nikon Eclipse 80i microscope (Nikon UK LTD, Kingston-upon-Thames, UK). Image stacks through the z axis were obtained in 10 random locations across the superior colliculus for each section. Images were then analysed using ImageJ. First, stacks were then merged through the z axis, the background was subtracted and the image converted to 16-bit before

thresholding and watersheding images to display just the synaptophysin puncta, as seen in Figure 5.5. Imaging and thresholding parameters were kept uniform for each image.
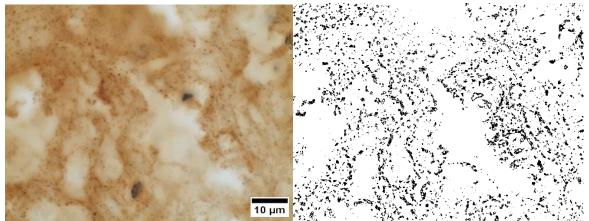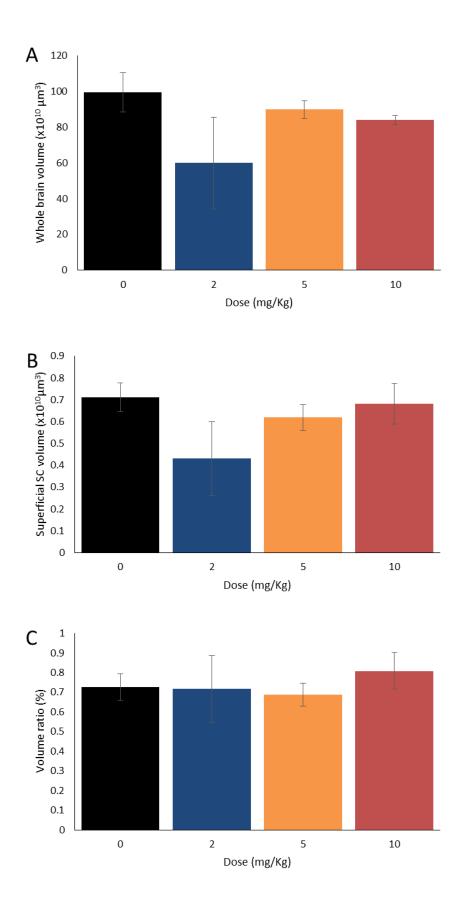
Figure 5.5 A: an example of stained tissue, merged through the z-axis. B: Image A following thresholding to display just the stained synaptophysin puncta.
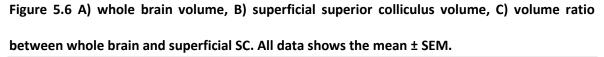
Analysis was performed using the ImageJ analyse particles function to determine the total number of puncta, and the percentage area covered by puncta staining. These Figures, along with the total area (μm^2) of the image, were then used to calculate the size and density of the puncta. Statistical analysis of the data involved Kolmogorov–Smirnov tests of normality, followed by One-Way ANOVAs for each of the 4 parameters (number, area, size, and density).

5.3 Results

5.3.1 VOLUMES AND RATIO

In order to check for any gross changes to the morphology of the superior colliculus following chronic amphetamine treatment, the volumes of the superficial SC and whole brain were estimated, and the volume fraction between the superficial SC and whole brain was also calculated. The data was determined to be normally distributed using a Shapiro-Wilk test. It was also determined using T-tests that there was no significant difference between the treated (0 mg/Kg) and untreated control groups for the volume of the superficial SC ($t_{(5)}$ = -1.720, p = 0.146), the volume of the whole brain ($t_{(5)}$ = -2.291, p = 0.071) or the volume fraction $t_{(5)}$ = -0.57, p = 0.957), therefore only the treated control was used during the main analysis. One-Way ANOVAs revealed that there were no significant differences in either whole brain volume ($F_{(3, 13)}$ = 2.244, p = 0.132, η^2 = 0.34, observed power = 0.44) or superficial SC volume ($F_{(3, 13)}$ = 1.046, p = 0.554, η^2 = 0.14, observed power = 0.16), and no significant difference in the volume fraction ($F_{(3, 13)}$ = 0.531, p = 0.669, η^2 = 0.11, observed power = 0.13). These results can be seen in Figure 5.6.

Figure 5.6 A) whole brain volume, B) superficial superior colliculus volume, C) volume ratio between whole brain and superficial SC. All data shows the mean ± SEM.
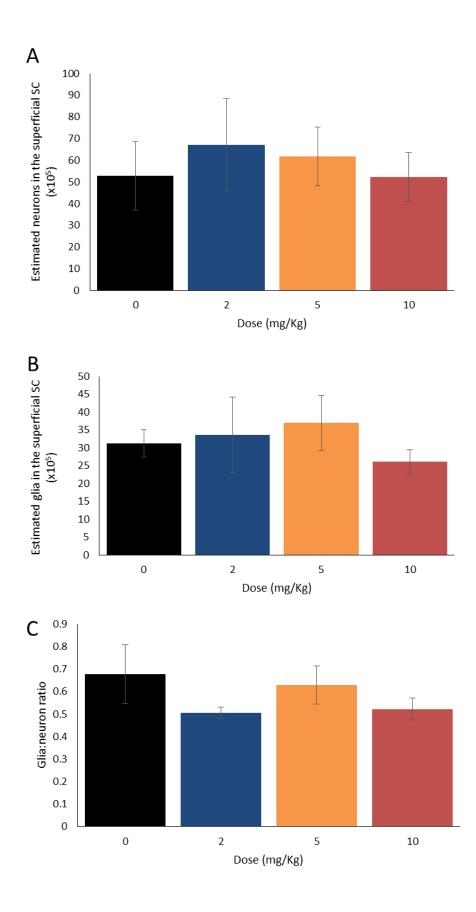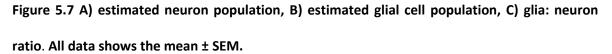
5.3.2 CELL COUNTS AND DENSITIES

To further explore the potential effects of chronic amphetamine treatment on the structure of the SC, estimates of both neuronal and glial cell counts were obtained, and the glia: neuron ratio calculated. This data was then also used in combination with the volume data obtained above in order to estimate the average cell densities of both cell types with the superficial SC. Firstly, all data was determined to be normally distributed using a Shapiro-Wilk test, and there was also found to be no significant differences between the treated and untreated controls for the neuron counts ($t_{(5)}$ = -1.00, p = 0.362), glia counts ($t_{(5)}$ = -1.91, p = 0.114), neuron density ($t_{(5)}$ = 0.21, p = 0.840), glia density ($t_{(5)}$ = 0.26, p = 0.808), and the glia: neuron ratio ($t_{(2.47)}$ = 0.429, p = 0.702), and so only the treated control group has been reported for the main analysis.

With the use of One-Way ANOVAs it was determined there were no significant differences between the chronic treatment groups for the estimated number of neurons ($F_{(3, 11)}$ = 0.317, p = 0.813, η^2 = 0.08, observed power = 0.09), the estimated number of glial cells ($F_{(3, 11)}$ = 0.839, p = 0.500, η^2 = 0.19, observed power = 0.18), or the ratio between them ($F_{(3, 11)}$ = 1.081, p = 0.397, η^2 = 0.23, observed power = 0.22), and these results are shown in Figure 5.7 below.

Figure 5.7 A) estimated neuron population, B) estimated glial cell population, C) glia: neuron ratio. All data shows the mean ± SEM.

The analysis of the cell densities similarly found no significant differences in the density of neurons ($F_{(3, 11)}$ = 0.651, p = 0.599, η^2 = 0.15, observed power = 0.15) or glial cells ($F_{(3, 11)}$ = 0.838, p = 0.501, η^2 = 0.19, observed power = 0.18) between chronic treatment groups. The average densities for each cell type can be seen in Figure 5.8.
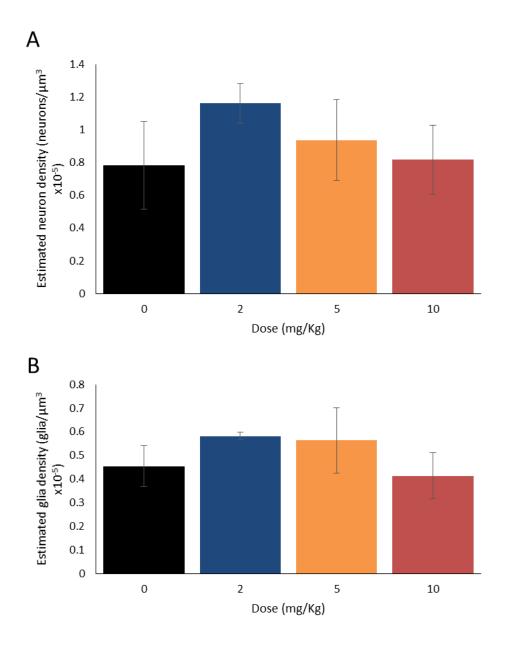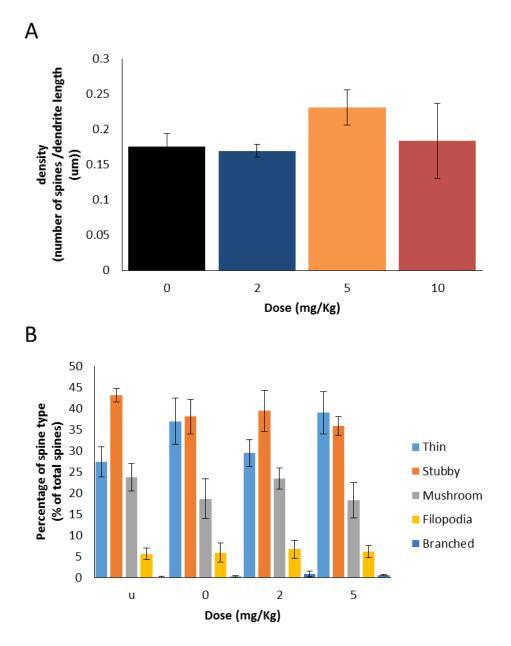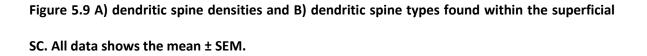
Figure 5.8 A) Neuronal and B) glial cell densities in the superficial SC. All data shows the mean ± SEM.

5.3.3 DENDRITIC SPINE DENSITIES AND TYPES

A Kolmogorov-Smirnov test found that the spine density of all chronic treatment groups except 2 mg/Kg (p = 0.033) were normally distributed, however, since non-parametric testing produced the same results when run, only parametric test results have been reported. The untreated group was not included in this analysis as there were no significant differences between the treated and untreated controls (t (5) = 0.15, p = 0.886). A One-Way ANOVA found no significant differences between the four chronic treatment groups (F (3, 15) = 1.046, p = 0.401, η^2 = 0.17, observed power = 0.23) for total spine density as can be seen in Figure 5.9 (A).
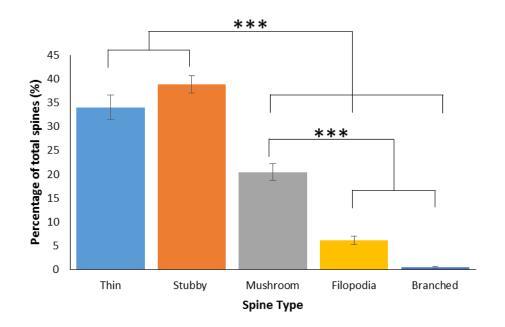
The spine types were also investigated, the results of the initial Chi-square test found no significant differences in spine type between groups (χ^2 (9) = 3.265, p = 0.953). Further investigation was performed using a repeated measures ANOVA to investigate the differences in the percentage of spines of each type as a within measures factor. For this analysis the branched spine data was excluded as this spine type occurred infrequently, and thus the group size was below the minimum needed for a repeated measures ANOVA. This analysis found that although there were no significant main effects of CHRONIC TREATMENT (F (3, 15) = 0.470, p = 0.708, η^2 = 0.09, observed power = 0.12), and no interaction between CHRONIC TREATMENT and SPINE TYPE (F (5.66, 28.28) = 0.396, p = 0.866, η^2 = 0.07, observed power = 0.14) there was a significant main effect of SPINE TYPE (F (1.89, 28.28) = 36.744, p < 0.001, η^2 = 0.71, observed power = 1.00). Contrasts revealed there was no significant differences between the percentage of thin and stubby spines (p = 0.380) but there were significantly more thin and stubby spines than mushroom (p < 0.001) and significantly more mushroom spines than filopodia (p < 0.001). This data is shown in Figure 5.9 (B).

A

B

Figure 5.9 A) dendritic spine densities and B) dendritic spine types found within the superficial SC. All data shows the mean ± SEM.
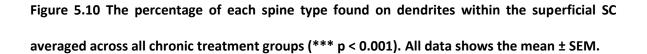
Following the repeated measures ANOVA, a further One-Way ANOVA was run to investigate the relative prevalence of each spine type, including branched spines, found within the superficial superior colliculus averaged across all chronic treatment groups, since there were no significant differences found between groups. Here it can be seen there is a significant difference in spine type averaged across all groups ($F_{(4, 100)}$ =
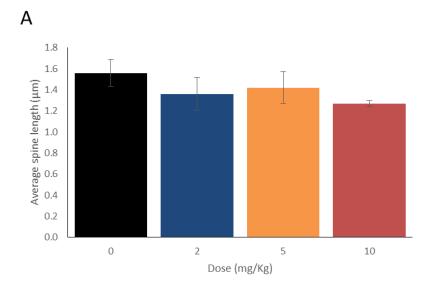
104.612, p < 0.001, η^2 = 0.81, observed power = 1.00). Post hoc Tukey HSD tests revealed there was no significant difference between the percentages of thin and stubby spines (p = 0.249), but both of these groups were significantly more prevalent than the mushroom, filopodia and branched spines (p < 0.001). There were also significantly more mushroom spines than filopodia and branched spines (p < 0.001). These results can be seen in Figure 5.10.

Figure 5.10 The percentage of each spine type found on dendrites within the superficial SC averaged across all chronic treatment groups (* p < 0.001). All data shows the mean ± SEM.**
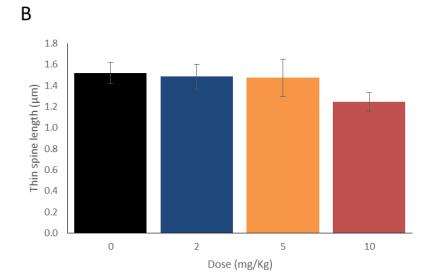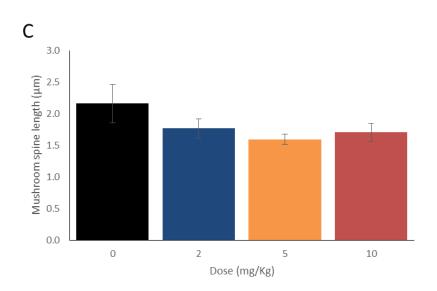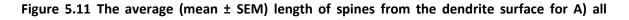
Further investigation into dendritic spine morphology was performed by investigating the average length of dendritic spines using a One-Way ANOVA. This analysis found no significant differences in total average spine length between chronic treatment groups ($F_{(3, 17)}$ = 0.74, p = 0.549, η^2 = 0.15, observed power = 0.17). The length of thin and mushroom spines were also assessed individually as these were the most abundant spine

types, aside from stubby spines, however the length of stubby spines was often negligible and difficult to measure accurately. There were no significant differences found between chronic treatment groups for either thin ($F_{(3, 17)}$ = 0.67, p = 0.584, η^2 = 0.13, observed power = 0.15) or mushroom spines ($F_{(3, 17)}$ = 1.69, p = 0.235, η^2 = 0.27, observed power = 0.33). Although there were no significant main effects of CHRONIC TREATMENT identified, from Figure 5.11 it can be seen that groups treated with amphetamine consistently appeared to have slightly shorter spines in comparison to the vehicle treated group.

Figure 5.11 The average (mean ± SEM) length of spines from the dendrite surface for A) all spines, B) thin spines, C) mushroom spines.

5.3.4 SYNAPTIC INTEGRITY

Following immunohistochemical staining and data collection as described above, it was determined using Kolmogorov-Smirnov tests that all the data was normally distributed and there were no significant differences between the untreated and treated control groups for the number of synaptic puncta (t (9) = 0.28, p = 0.783), the percentage area covered by puncta (t (4.538) = -0.09, p = 0.929), the average size of individual synaptic puncta (t (9) = -0.55, p = 0.598), or the density of the puncta (t (9) = 0.634, p = 0.542). Since there were no differences between these different control groups only the treated control has been reported in the main analysis.

One-Way ANOVAs revealed there were no significant differences in the number of synaptic puncta (F (3, 17) = 0.752, p = 0.536, η^2 = 0.12, observed power = 0.18), the percentage area covered by puncta (F (3, 17) = 1.067, p = 0.389, η^2 = 0.16, observed power = 0.24) or the density of the puncta (F (3, 17) = 0.858, p = 0.482, η^2 = 0.13, observed power = 0.20) between the four chronic treatment groups. These results can be seen in Figure 5.12. There was however a significant difference in the average size of the puncta between groups (F (3, 17) = 10.451, p < 0.001, η^2 = 0.65, observed power = 0.99), and this result can be seen in Figure 5.12(c). Post hoc Tukey tests revealed that the control group had significantly smaller puncta in comparison to the 10 mg/Kg group (p= 0.024), and the puncta of the 2 mg/Kg group were significantly smaller than both the 10 mg/Kg (p < 0.001) and the 5 mg/Kg groups (p = 0.009).
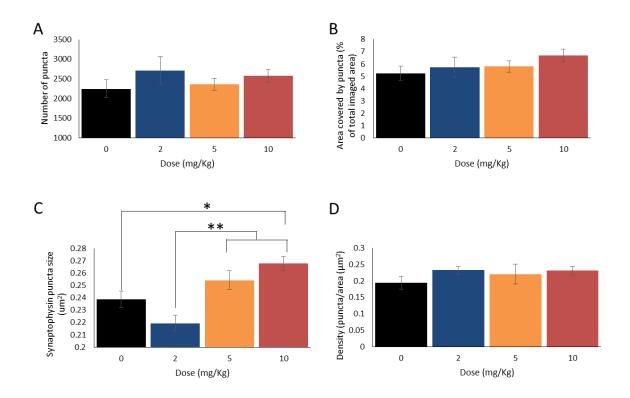
Figure 5.12 A) The number of puncta, B) percentage area covered by puncta, C) average puncta size, D) average puncta densities within the superficial SC (* p < 0.05, ** p < 0.01)). All data shows the mean ± SEM.

5.4 Discussion

Following chronic amphetamine treatment there were no significant differences found between chronic treatment groups in the volume of the superficial SC or the volume fraction between the superficial SC and the whole brain. Studies which had found that psychostimulant treatment did have an effect on brain volume were investigating volume deficits specific to ADHD, where treatment was to reduce the volume deficit (Spencer et al., 2013, Valera et al., 2007). Therefore a change in volume following amphetamine treatment in a healthy brain may not necessarily be expected, as previously reported effects on brain volume may occur in a targeted manner, and affect only specific regions of the brain (Spencer et al., 2013). It should also be noted that the sample size used for assessing volume, despite being in line with sample sizes used in previous investigations, was small and thus the observed power for the analysis was also very small. Similarly there were no significant differences found in the cell counts and densities of either neurons or glia, or in the ratio between glial cells and neurons, but again a small sample size was used for this analysis, resulting in a low level of observed power for the analysis, therefore it is possible that chronic amphetamine treatment may affect these measures, however a larger sample size would be needed in order to make an accurate assessment.

There were no significant differences between chronic treatment groups in the dendritic spine density of neurons within the superficial layers of the SC, and there were also no differences found in the expression of different spine types between chronic treatment groups. Previous studies which found that amphetamine did effect dendritic spine densities were focused on the effects of amphetamine following the induction of sensitization (Robinson and Kolb, 1999, Selemon et al., 2007), however as previously

discussed in chapter two, oral chronic amphetamine treatment has been found to induce behavioural tolerance which may be a contributing factor in the maintenance of spine density following amphetamine administration. These studies also found changes in spine density in localised regions, and the findings have not been replicated in all regions of the brain (Selemon et al., 2007) and so this effect of amphetamine may be specific to the PFC and nucleus accumbens. A slight decrease in spine length was also observed following amphetamine treatment, although this effect did not reach significance. In previous research longer spines have been observed in the striatum following i.p. administration of methylphenidate (Kim et al., 2009). This was believed to be linked to long term potentiation and may contribute to the effects of behavioural sensitisation found following i.p. psychostimulant administration (Kim et al., 2009, Kalivas and O'Brien, 2008). For this reason shorter spine lengths may reasonably be linked to behavioural tolerance observed following oral administration of amphetamine.

Since the investigation of the superior colliculus using this adapted rapid Golgi method is a novel approach, further analysis was conducted in order to assess some of the morphological characteristics of neurons within the SC. It was found that the most prevalent spine types within the superficial SC were the more immature spines, with significantly more stubby and thin spines than mushroom spines. This could indicate that much of this region may be in the early stages of synaptogenesis (Yuste and Bonhoeffer, 2004). Further evidence of this is seen by the presence of a significant number of dendritic filopodia, which are often believed to be precursors of dendritic spines (Morest, 1969, Ziv and Smith, 1996), and previous studies have found that filopodia can be found abundantly during periods of rapid synaptogenesis, but as the dendrite matures the populations of filopodia are replaced by more spine like structures (Dailey and Smith,

1996, Ziv and Smith, 1996). The exact role of filopodia, however, are not yet fully understood, and there is also evidence from other research that indicates development and maturation of dendritic spines does not occur along a single directional pathway (Hering and Sheng, 2001) therefore further research into the timeline of spine development within the SC would be needed in order for this interpretation to be proved.

Finally, synaptic integrity was also explored, and it was found that although there were no significant differences in the number of synaptic puncta, there was a significant difference in the size of the puncta between groups, with the control group having significantly smaller puncta than the highest (10 mg/Kg) dose group, and the lowest (2 mg/Kg) dose group having significantly smaller puncta that both the 5 and 10 mg/Kg groups. An increase in the size of synaptic puncta could be indicative of an increase in synaptic efficacy, and synaptic scaling in this manner shows the SC may be sensitive to amphetamine induced synaptic remodelling (Bisagno et al., 2004). Investigations into synaptophsin expression have previously linked an increase in puncta size with synaptic enhancement; there is indication that when a presynaptic terminal contains enlarged synaptophysin puncta, the post synaptic spines were also found to be enlarged and neurons contacted by presynaptic terminals with a larger synaptophysin content were more likely to display increased miniature excitatory postsynaptic currents (mEPSC) frequency (Misra et al., 2010), which is indicative of changes in postsynaptic receptor expression.

As mentioned in chapter 3.4, there is evidence that chronic amphetamine treatment may suppress activity in the SC, and this is one potential mechanism of action utilised in the treatment of conditions such as ADHD (Brace et al., 2015a, Clements et al., 2014,

Hetherington et al., 2017, Overton, 2008). If chronic amphetamine treatment is suppressing collicular activity, the synaptic scaling and upregulation of synaptophysin following high doses of chronic amphetamine treatment could be a result of neuroadaptation mediating the impact amphetamine treatment has on collicular output (Turrigiano, 2008). A compensatory balance of this nature may explain previous findings that amphetamine has sustained efficacy as a treatment for ADHD without evidence of improved efficacy over time (Fredriksen et al., 2014). However, synaptophysin changes have also been demonstrated following induction of behavioural sensitization as a response to withdrawal from amphetamine treatment (Subramaniam et al., 2001), Therefore it is also possible that changes in synaptophysin puncta displayed in our study may have been an acute response to withdrawal.

CHAPTER 6. DISCUSSION

6.1 Principal findings

This thesis aimed to investigate whether lasting neuroadaptations occurred within the SC following chronic amphetamine treatment using a paradigm designed to mimic the administration of drugs used to treat heightened distractibility in humans, such as in the case of ADHD. This investigation was performed by determining whether chronic amphetamine treatment resulted in changes to i) locomotor activity; ii) collicular dependent behaviours; iii) the physiological responsiveness of the SC in response to visual stimuli; iv) the physiological responsiveness of the SC following an acute amphetamine challenge; iv) the morphology of the superficial layers of the SC.

6.1.1 CHANGES IN LOCOMOTOR ACTIVITY

Following an acute oral dose of amphetamine the groups treated with either 5 mg/Kg or 10 mg/Kg exhibited continual high levels of activity throughout the monitoring period, recorded during peak psychostimulant activity (Kuczenski and Segal, 2002, Martínez-Clemente et al., 2013, Sakai et al., 1983), whereas there was a significant reduction in activity levels over time observed in the control groups and group treated with 2 mg/Kg, in line with anticipated habituation to a novel environment. There were large significant differences observed between the high dose groups (5 mg/Kg and 10 mg/Kg) and the low dose (2 mg/Kg) and control groups. These differences and high levels of locomotor activity seen in the high dose groups are likely due to the fact that amphetamine is a

psychomotor stimulant, therefore causing increased levels of activity when large doses were administered (Casanova et al., 2013, Morales-Mulia et al., 2007).

Following chronic treatment there was a significant reduction in the level of locomotor activity for both the 5 mg/Kg and 10 mg/Kg groups in comparison to the data obtained in response to an acute dose. Decreases in the activity of the groups treated with higher doses of amphetamine indicates that chronic oral amphetamine treatment resulted in the development of a drug tolerance, rather than sensitization. This effect may be due to the long half-life of psychostimulant drugs when ingested in this manner (Kuczenski and Segal, 2002, Davidson et al., 2005), since tolerance to psychostimulants has been similarly observed in studies using methods of constant drug delivery (Davidson et al., 2005, Zimmer et al., 2014). The presence of behavioural tolerance is evidence that neuroadaptations have occurred in response to chronic treatment.

6.1.2 COLLICULAR-DEPENDENT BEHAVIOUR

When investigating the response to a test of visual distractibility following chronic treatment (0 mg/Kg, 2 mg/Kg, 5 mg/Kg, and 10 mg/Kg) there was a general trend observed which showed a dose-dependent decrease in the number of stimuli responded to following the initial response. This observation is in line with previous studies which showed a decrease in collicular activity in the presence of amphetamine (Dommett et al., 2009, Gowan et al., 2008, Clements et al., 2014). However, this effect did not reach statistical significance. It should be noted, however, that the statistical analysis of this effect had an observed power of 0.36, and low power denotes a higher probability of type II errors.

In an investigation into height dependent air righting, as a measure of collicular dependent activity in rats, there was a significant difference found between dose groups (0 mg/Kg, 2 mg/Kg, 5 mg/Kg, and 10 mg/Kg) when exploring the difference in righting latency between the 10 cm and 50 cm drops. The vehicle treated control group exhibited behaviour which would be expected in a healthy rat with a normal SC, with a large difference in righting latency found between drops from 10 cm and 50 cm. Similar results were found for the 2 mg/Kg group and no significant difference was found in comparison to the control group. The 10 mg/Kg group, however, had a significantly reduced ability to modulate righting latency according to height. A reduction in the ability to modulate righting latency in a height dependent manner could indicate reduced functionality in the SC following chronic amphetamine treatment at high doses (Pellis et al., 1989), indicating potential suppression of activity in the SC (Clements et al., 2014, Gowan et al., 2008). The 5 mg/Kg group also showed a slight reduction in ability to height dependently control righting latency, however this reduction was not significantly different to the control group. Since there were no significant effects observed for the 2 mg/Kg dose, which is the most closely associated with typical therapeutic doses of amphetamine, this could suggest that the action of amphetamine reported here is unlikely to underlie the therapeutic effects of amphetamine. However, given the difficulty of extrapolating between doses, it should not be ruled out.

6.1.3 VISUAL RESPONSIVENESS IN THE SUPERIOR COLLICULUS

In the investigation of visual responsiveness, it was observed that as the intensity of light stimulus was increased there was a subsequent significant reduction in the onset and

peak latency of responses measured in multiunit activity in the SC, and a consistent significant increase in the magnitude of the response. This faster and larger response is as expected with increasing stimulus intensity (Stein et al., 1976, Altman and Malis, 1962). Regarding the effects of chronic amphetamine treatment on the SC response to a light flash stimulus, a significant difference in the onset latency of the response between treatment groups (0 mg/Kg, 2 mg/Kg, 5 mg/Kg, and 10 mg/Kg) was identified, however post hoc testing revealed no further differences between individual dose pairings. Although post hoc testing revealed no significant differences between groups, from observation of the data it appeared that the 5 mg/Kg and 10 mg/Kg dose groups had slightly longer latencies in comparison to the 0 mg/Kg and 2 mg/Kg dose groups, which may be indicative of suppression of activity in the SC following high doses of chronic amphetamine treatment.

Increasing the stimulus intensity also had a significant effect on the LFP data; the maximum amplitude and the magnitude of the response both increased as the light intensity increased. Chronic treatment also had a significant effect on the peak latency, with the peak response occurring faster in the 2 mg/Kg group in comparison to the two highest dose groups. Since the effect was found in the LFP data, which represents peri-synaptic activity including any postsynaptic activity (Ekstrom, 2010, Logothetis, 2008), but was not found in the multiunit spiking activity, this may indicate that incoming information is arriving at the SC faster following chronic treatment with a low therapeutic dose of amphetamine. However, as indicated from the multiunit activity, subsequent processing within the SC was not any quicker. At lower stimulus intensities, the 2 mg/Kg group also had a greater magnitude of response to the stimulus when compared to all the other dose groups (0 mg/Kg, 5 mg/Kg, and 10 mg/Kg). There was also evidence of

suppressed activity following amphetamine treatment both for the 5 and 10 mg/Kg treatment groups, since their responses were significantly slower and smaller than the 2 mg/Kg group. Furthermore since it was observed that the 2 mg/Kg groups had a faster peak latency and greater response magnitude in the incoming signal, but no increases in these parameters in the MUA, this is evidence that activity is being suppressed within the SC following chronic amphetamine treatment.

There was also a significant interaction effect found between the chronic treatment groups and the phase of the response in the multiunit activity, in which the light response of the 2 mg/Kg group had a significantly larger maximum amplitude when compared to the control group during the first phase. This effect, however, was lost by the second phase of the response, where the 2 mg/Kg group had a significantly smaller amplitude compared to the 10 mg/Kg group. So, the response within the SC was increased following treatment with a 2 mg/Kg therapeutic dose of amphetamine following retinal input, but this response was lost following secondary input, therefore the suppression of activity may occur within the SC after initial retinal input, following input of signals from other areas of the brain.

6.1.4 RESPONSES FOLLOWING AN ACUTE AMPHETAMINE CHALLENGE

From the MUA, it was found that acute amphetamine administration significantly decreased both the onset latency and the peak latency of the response to the visual stimulus. There was also an initial increase in the amplitude and magnitude of the response, however as the cumulative dose increased the amplitude and magnitude of the responses steadily decreased. This decrease in activity following amphetamine

administration is in line with previous observations made by Clements et al. (2014) and Gowan et al. (2008) that acute i.v. administration of amphetamine resulted in dose dependent depression of visual responses in the SC. Similar effects of acute amphetamine treatment on the maximum amplitude and response magnitude were found in the LFP data, where acute amphetamine appeared to depress visual responses at the highest doses, however there was no effect on the onset or peak latency of the LFPs. There were also no significant effects of chronic amphetamine treatment or interaction effects in response to the acute amphetamine challenge for either the multiunit activity or local field potentials.

6.1.5 EFFECTS OF CHRONIC TREATMENT ON COLLICULAR MORPHOLOGY

No significant differences in gross morphological measures were observed following chronic amphetamine treatment. This includes no changes in the volume ratio between the superficial SC and the whole brain, and no change in the densities of neurons or glial cells or in their ratio. Although changes in measures of gross morphology have been detected in previous research following amphetamine treatment, in these cases the effect of treatment was to reduce abnormalities specific to ADHD (Spencer et al., 2013, Valera et al., 2007), and therefore amphetamine might not affect a healthy brain in the same manner.

From the analysis of dendritic spines in the superficial superior colliculus, there were no significant differences found in the dendritic spine density or spine length although a general trend was observed where the dendritic spines of amphetamine treated animals appeared to be shorter than those of the control group, which may be linked to

behavioural tolerance in response to chronic treatment (Kim et al., 2009, Kalivas and O'Brien, 2008). Amphetamine treatment has previously been associated with an increase in spine density (Robinson and Kolb, 1997, Robinson and Kolb, 1999), however the increase was due to an increase in the prevalence of branched spines. Since it has been observed that branched spines appeared very infrequently in the SC this may explain why no increase in spine density was observed following chronic amphetamine treatment. Furthermore the increases in spine densities found previously were located in the prefrontal cortex and nucleus accumbens, and this effect has not been replicated in all other areas (Selemon et al., 2007). It may therefore be localised to specific regions of the brain and not a common action of amphetamine. Given the absence of impact of chronic amphetamine treatment on dendritic spines in the SC, this suggests that the neuroadaptations which underlie the suppressive effects of amphetamine on collicular activity are not mediated by changes in postsynaptic structure.

Additional analysis was conducted in order to further investigate the morphological characteristics of neurons within the SC, and since these structures were not affected by chronic amphetamine treatment this analysis was conducted across all groups (untreated, 0 mg/Kg, 2 mg/Kg, 5 mg/Kg, and 10 mg/Kg). It was discovered that the spines found in the superficial visual layers of the SC were typically immature, with thin and stubby spines being significantly more prevalent than mushroom spines, which could indicate that this region is in the early stages of synaptogenesis (Yuste and Bonhoeffer, 2004). A significant number of filopodia were also present on dendrites within the SC. Filopodia have previously been observed to initiate physical contact with nearby axons and this contact could then lead to the development of a presynaptic bouton (Ziv and Smith, 1996). The proposed role of filopodia as precursor of more typical dendritic spines (Morest, 1969, Ziv

and Smith, 1996) would support the suggestion that the SC is in the early stages of synaptogenesis, however the exact role of filopodia is not yet fully understood, and there is also research to suggest that the maturation of dendritic spines does not occur along a set, single directional pathway (Hering and Sheng, 2001).

To further examine the effects of chronic amphetamine treatment on synapses within the SC, synaptophysin puncta were investigated as a measure of synaptic integrity. A significant difference in the size of the puncta was found between the chronically treated groups, with the highest (10 mg/Kg) dose group having significantly larger puncta than the control group, and the lowest (2 mg/Kg) dose group having significantly smaller puncta that both the 5 and 10 mg/Kg groups. An increase in the size of synaptic puncta found following amphetamine treatment may be indicative of a rise in synaptic efficacy. Synaptic scaling in this manner could show that the SC is susceptible to synaptic remodelling induced by amphetamine treatment (Bisagno et al., 2004). An increase in puncta size has also been associated with other measures of synaptic enhancement in previous research, including enlargement of postsynaptic spines and indication of changes in the expression of postsynaptic receptors in synapses containing enlarged synaptophysin puncta (Misra et al., 2010). The upregulation of synaptophysin following chronic administration of high doses of amphetamine is potentially indicative of neuroadaptations occurring to mediate the impact amphetamine treatment has on collicular activity (Turrigiano, 2008).

6.2 Implications

The indication that chronic amphetamine treatment with the two highest doses (5 mg/Kg and 10 mg/Kg) is suppressing activity in the SC was observed both from behaviour, by testing the righting latencies of animals in order to assess collicular function, and from assessing physiology in response to a light flash. There was further suggestion of suppression seen from the non-significant findings that amphetamine treated animals appeared to habituate to a light stimulus sooner than the control group, and the length of dendritic spines in the SC of amphetamine treated animals was generally shorter than those in the control groups, which could suggest spines were less mature following amphetamine administration (Kim et al., 2009). These findings give evidence that at high doses there are long lasting, activity suppressing effects of chronic oral amphetamine treatment which specifically affect the SC, supporting the theory that the SC is one of the areas therapeutically targeted by amphetamine within the brain (Dommett et al., 2009, Gowan et al., 2008). These effects, however, were found in the two highest dose groups, which were also the only groups to exhibit behavioural tolerance following chronic treatment, but significant effects were not found in the 2 mg/Kg group. As discussed in Section 2.2.2 the 2 mg/Kg group most closely represents a typical therapeutic dose used to treat increased distractibility, and so for these reasons the suppression of collicular activity seen in the above examples may not be representative of therapeutic effects of amphetamine when used for the long term treatment of increased distractibility.

Significant effects of chronic amphetamine treatment with a dose of 2 mg/Kg on the colliculus were observed from the analysis of visual responsiveness. From the analysis of the LFPs it was discovered that the peak latency of the response to the light flash was

significantly shorter in the 2 mg/Kg group compared to any other group, however there were no significant differences found between the 2 mg/Kg group and the control in the peak latency of the response for the MUA. A similar pattern is also seen in the magnitude of response, where the analysis of LFPs found that the 2 mg/Kg group had an initially greater response magnitude to the whole field light stimulus in comparison to all other groups, but there were no significant differences in response magnitude between the chronically treated groups for the analysis of MUA. As such this is evidence of suppression of activity within the SC following chronic treatment with 2 mg/Kg amphetamine, since the signal arriving in the SC had a faster peak response and greater magnitude when compared to the control, but there were no significant differences found between the control and 2 mg/Kg group in the outgoing signal, as represented by the MUA, therefore the speed and magnitude of the responses within the SC have been suppressed.

Further evidence of suppression of activity following chronic amphetamine treatment could be seen when the MUA was split into two distinct phases. In the first phase, which represents the response to direct retinal input to the SC, the amplitude of response of the 2 mg/Kg treated group was the largest, which is comparable to the effects found previously in the magnitude of the response in the LFPs of the 2 mg/Kg group, where there was a potential enhancement in the strength of the response when compared to the control. This effect was then lost by the second phase, in which the maximum amplitude of the response observed in the 2 mg/Kg group was significantly smaller than the amplitude of the 10 mg/Kg group. So the analysis of the entire response period determined that there were no significant differences in response amplitude between the chronic treatment groups. However, from the biphasic analysis it can be seen that in the 2 mg/Kg group the maximum amplitude of the MUA recorded was significantly larger than

the control group following initial retinal input. This effect was then lost during the second phase, in the response following cortical input. Therefore, these findings may indicate that suppression of activity by amphetamine within the SC occurs following input from other areas of the brain.

From these findings it may be suggested that although chronic treatment with amphetamine resulted in stronger signals arriving in the SC, it also resulted in the suppression of the impact of those signals on collicular activity, potentially through neuroadaptation within the SC, causing inconsistency between the effect on LFP and MUA. One potential mechanism that could underlie this discrepancy is that amphetamine may decrease the number of transient voltage-gated sodium channels in the SC, consequently increasing the threshold for action potential generation in the colliculus (Peterson et al., 2006). A similar effect of amphetamine has been previously identified for the PFC following chronic treatment, although in this case drugs were administered via i.p. injection (Peterson et al., 2006). Suppression of activity in the SC would also be consistent with the behavioural findings which suggest that in the 5 mg/Kg and 10 mg/Kg groups chronic amphetamine treatment reduces activity in the colliculus and suppresses collicular dependent air-righting behaviour.

Given this evidence, the observation of enlarged synaptic puncta following chronic treatment with 5 mg/Kg and 10 mg/Kg doses of amphetamine, an indication of synaptic enhancement (Misra et al., 2010), may also be linked to the suppression of activity in the SC, in that neuroadaptations which affect synaptophysin expression may have a role in mediating the impact amphetamine treatment has on collicular output as a form of autoregulation (Turrigiano, 2008). Previous research has found that neurons in other

brain regions which are in contact with presynaptic terminals containing enhanced synaptophysin puncta were associated with an increase in mEPSC frequency, and this may also be indicative of changes in postsynaptic receptor expression (Misra et al., 2010). Increased mEPSCs may also be consistent with the findings from electrophysiology data, in which there appeared to be an increase in the maximum amplitude of the response in LFPs following treatment with the highest dose of amphetamine, although this effect did not reach significance. It is not clear which changes occurred first: whether treatment with amphetamine initially caused a reduction in the number of transient voltage-gated sodium channels, and there was a subsequent increase in the size of synaptophysin puncta to compensate for the increased threshold for action potential generation; or whether a reduction in the number of sodium channels followed the amphetamine mediated increase in puncta size, hence preserving the strength of the SC signal output. However since it has also been observed, both in previous research and in this investigation, that acute amphetamine administration reduces responses in the MUA (Gowan et al., 2008), it may be theorised that this was the initial effect of chronic amphetamine treatment. A compensatory balance influencing the MUA may also support the evidence from previous research which found that amphetamine has continued efficacy as a treatment for ADHD but there was no evidence that efficacy improved over time (Fredriksen et al., 2014). These effects are also supported by previous investigation which has found that a reduction in dopamine causes an increase in visual responses (Rolland et al., 2013), and therefore by increasing dopamine levels, amphetamine treatment would result in suppression of activity.

The immediate effects of amphetamine on the levels of extracellular monoamine neurotransmitters are already well known (Easton et al., 2007, Kuczenski and Segal,

1989), but this research suggests a more complex pattern of changes following chronic treatment. Going forward, gaining a better understanding of the effects of long-term amphetamine treatment should be prioritised, ideally so that is possible to establish order of any neuroadaptations. As outlined above, levels of monoamine neurotransmitters may be influenced by the neuroadaptations occurring as a result of chronic amphetamine treatment. Treatment of several other psychiatric conditions, such as depression, also relies on altering the levels of monoamine neurotransmitters, and therefore the results presented here may be of interest. Additionally, several conditions linked to altered monoamine functioning, including depression, are known to be comorbid with ADHD (Biederman et al., 2008). Based on this research, it is possible to speculate that the neuroadaptations could influence the comorbid condition as well as ADHD and that interactions between the treatment effects on both conditions should be considered.

The present investigation also shows, for the first time that the rapid Golgi method can be used to investigate the dendritic spines within the SC, and provides the first characterisation of the colliculus at this level. Although further investigation into the dendritic structure of the superficial SC is required, the novel method used and the results obtained, particularly with regard to the stages of synaptogenesis, form a basis from which the directions of future investigations into the morphology of the SC could be determined.

6.3 Limitations

A number of potential limitations of the above investigation should be recognised and addressed in future investigations. One such limitation identified is that the observed power of a number of analyses was below the target figure of 0.8. Low power was particularly an issue in the investigation of collicular morphology, although sample sizes were typical for this type of study, with the analyses for neuron (observed power = 0.15) and glia densities (observed power = 0.18), spine density (observed power = 0.23), and spine type (observed power = 0.12) among those affected. Low power was also an issue in the investigation of distractible behaviour (stimulations to habituation, observed power = 0.36; main effect of chronic treatment on duration of response, observed power = 0.12). Low statistical power means there is a greater risk of type II errors, and therefore the acceptance of the null hypothesis, that chronic treatment had no effect on any of the above listed measures, may have been incorrect, and the effects of treatment may have been underestimated as a result. Power could be increased in these investigations by increasing the sample sizes used, in order to conclusively determine whether the null hypothesis is correctly accepted.

Although the chronic treatment paradigm used in this investigation was designed to closely mimic the methods of administration used by humans and effort was made to ensure that the doses selected were therapeutically relevant, the blood plasma levels of amphetamine were not measured following administration to ensure the expected concentrations were achieved. Further investigation should consider measuring the blood plasma levels as additional validation of the doses used.

It should also be considered that, although the SC is a highly conserved structure (Krauzlis et al., 2013), there are differences in the processing of sensory information between rodents and humans which may also impact processing involved in attention and distractibility. The above investigation focussed on assessing the effects of amphetamine on the superficial visual layers of the SC. Rats, however, do not typically rely on vision and instead preferentially use their whiskers in order to explore their environment (Castro-Alamancos and Favero, 2016, Lee et al., 2016), and furthermore the rat's visual system is fundamentally different to humans, in that they lack a fovea (Jacobs et al., 2001). Despite this, previous investigations have shown that rats are able to use their visual systems effectively to perform attentional tasks following training (Lee et al., 2016). It is also known that the intermediate layers of the SC contribute to the processing of other sensory inputs, including input from auditory stimuli and the whiskers in rats (Castro-Alamancos and Favero, 2016, Brace et al., 2015b), but the assumption cannot be made that amphetamine would have the same long term effects in these layers as it does in the visual layers. Psychostimulant drug treatment has previously been found to modify responses in the barrel cortex in response to whisker stimulation (Bekavac and Waterhouse, 1995). For this reason it may be of interest to further investigate the effects of chronic oral amphetamine treatment on collicular processing of whisker stimulation, as it is the primary method utilised by rats to explore their environment (Castro-Alamancos and Favero, 2016, Lee et al., 2016), in order to determine whether effects correspond to those found in the visual layers.

It may also be argued that the behavioural test of visual distractibility used is really a simple measure of visual attention because there was no other task to be distracted from – just simple exploratory behaviour. Certainly, the two constructs of attention and

distractibility are strongly related. There is evidence that an increase in distractibility, such as in the case of ADHD, is known to reduce attentional control during certain tasks (Friedman-Hill et al., 2010), and both attention and distractibility have been previously found to be linked to similar regions within the brain, including the SC and the PFC (Chao and Knight, 1995). The proposed circuitry for attentional processing includes both bottom up and top down control mechanisms (Gazzaley et al., 2005; Miller and Buschman, 2013) with the PFC implicated in both types and the colliculus implicated in endogenous attention (Mueller et al., 2017), meaning it is possible that the effects seen here stem from alterations in endogenous attention. For distractibility there is still debate as to whether control is influenced by a "distractibility network" within the brain, or by a number of separate areas which may all have different influences on the levels of distractibility, however previous evidence has been found that disruption of the pathways between areas associated with distractibility also influences distractibility (Gaymard et al., 2003b), implying there is coordination in the control of distractibility between different areas within the brain, including the SC and the PFC, which is critical for attention. In addition to this, there is also some evidence from the results of biphasic response analysis above that suppression of activity in the SC occurs following input from other areas of the brain, although this warrants further investigation as to where this influence may originate. However, it must be acknowledged that despite the two being related, distractibility is just one factor that may influence levels of attention, and attentional control (Bari and Robbins, 2013). Therefore, whilst the test performed was very useful in determining broadly whether collicular-dependant functioning was influenced by the chronic amphetamine treatment, additional investigation with the introduction of trained

tasks such as Continuous Performance Tasks (e.g. Ding et al. (2018)) may be helpful in further differentiating attention from distractibility.

Finally, since chronic treatment was administered throughout the adolescent period in rats, and all subsequent tests for long term effects were performed on adult rats, this limits how applicable these results are to children with ADHD. There is evidence, however, that ADHD affects adults as well as children, and each cohort is considered to be distinct (Moffitt et al., 2015). Therefore, the findings presented still have relevance to the clinical population.

6.4 Future Research

In addition to testing blood plasma levels following amphetamine administration to ensure therapeutic relevance and measuring the animals' response to whisker stimulation following amphetamine treatment as discussed above, it may also be beneficial to investigate the response of the animals during the chronic treatment phase in future research into the effects of chronic amphetamine treatment. During the current investigation all experimental measures were conducted in the 2 weeks immediately following the cessation of treatment, and therefore the responses were obtained during drug withdrawal. Previous investigation has found that continuous chronic treatment with amphetamine resulted in increased activity while in withdrawal immediately following treatment with a high (10 mg/Kg/day) dose of amphetamine, but found that a lower dose (5 mg/Kg/day) resulted in increased "depressive-like" behaviour during the withdrawal phase (Cryan et al., 2003). Therefore, in order to differentiate whether effects found are as a result of chronic amphetamine treatment or due to withdrawal from chronic treatment, measures should be recorded during both of these phases.

Future studies may also consider conducting further analysis of spine densities and types present in the SC at multiple time points or, potentially coupling brain slice electrophysiology with two-photon microscopy, in order to better understand these characteristics of collicular morphology. This would give further insight into the maturation of spines in this region, and may especially provide greater understanding of the role of filopodia in the SC, as there is currently contention over the process of maturation of dendritic spines (Hering and Sheng, 2001) and whether filopodia truly are a precursor to typical dendritic spines (Ziv and Smith, 1996, Morest, 1969). Future

directions for further investigation may also involve the use of two photon imaging or in vitro electrophysiology to investigate sodium currents in order to better understand the cellular mechanisms and determine what long lasting effects amphetamine treatment is having on voltage gated sodium channels found within the SC, as has been investigated previously in the PFC (Peterson et al., 2006).

Since previous investigations, which found behavioural sensitization in response to chronic i.p. administration of amphetamine, also discovered previous amphetamine exposure increased drug self-administration (Lorrain et al., 2000, Chiodo et al., 2008); it may, therefore, be interesting to investigate the impact of chronic oral amphetamine administration on either self-administration of drugs or the response to other stimulant drugs such as cocaine. Since chronic oral amphetamine administration was found to induce tolerance, it may be expected that self-administration would be reduced following a chronic orally administered treatment paradigm, since previous studies have also found that mini-pump administration of amphetamine reduced the rates of self-administration of cocaine (Chiodo and Roberts, 2009). This would also be in line with previous findings that treatment of ADHD with psychostimulant drugs reduced the liability of developing a SUD in comparison to a cohort with untreated ADHD (Biederman et al., 1999).

Interest in the chronic effects of amphetamine treatment is due to its use in the treatment of heightened distractibility in humans, primarily in the case of ADHD (Himelstein et al., 2000, Overton, 2008). School age children with ADHD are often treated to reduce distractibility while learning (Accardo et al., 1999, Teicher et al., 2000), however there is evidence that repeated exposure to psychostimulants may affect experience-dependent plasticity within the brain (Kolb et al., 2003). Previous investigations have used

i.p. administration of amphetamine, therefore it may be interesting investigate the effects of an oral treatment paradigm on measures of the brain's structural plasticity, such as dendritic spine density, following the introduction of environmental enrichment to simulate a learning environment.

6.5 Conclusions

The aim of this thesis was to investigate the effects of long term therapeutically relevant amphetamine treatment on the SC, due to the fact that the SC has been previously identified as a neural substrate for distractibility (Wurtz and Albano, 1980, Goodale et al., 1978, Overton, 2008) as well as a potential target for the therapeutic effects of amphetamine in ADHD (Dommett et al., 2009, Gowan et al., 2008). However, no previous investigations had explored the long lasting effects of chronic therapeutic amphetamine treatment on the SC. Consistent indications that chronic amphetamine treatment resulted in suppression of activity in the SC during drug withdrawal were discovered from behavioural and electrophysiological findings. It is theorised that the mechanism of action of this suppression may be due to a reduction in the number of transient voltage gated sodium ion channels, increasing the threshold required for the generation of action potentials (Peterson et al., 2006). As amphetamine is also known to increase extracellular levels of monoamines (Easton et al., 2007, Kuczenski and Segal, 1989), the suppression of visual activity is also in line with previous investigations which have found that a reduction in dopamine resulted in increased visual responses (Rolland et al., 2013). There was also evidence of a compensatory mechanism mediated by presynaptic reorganisation involving synaptophysin, potentially resulting in an enhancement of the signal arriving in the SC. The techniques developed in this thesis also allowed the structure and types of dendritic spines prevalent in neurons in the superficial SC to be described for the first time, and also found that gross postsynaptic structures were unaffected by treatment with amphetamine.

The therapeutic effects of amphetamine in reducing distractibility in conditions such as ADHD may be due in part to the suppression of activity in the SC, and the compensatory effect observed may contribute to findings that the efficacy of amphetamine in the treatment of ADHD is sustained, but doesn't improve, over time (Fredriksen et al., 2014). Future research should now focus on determining whether these effects are present throughout chronic treatment, or only in withdrawal. It may also be valuable to conduct further research into the dendritic structure of the SC over time, and to classify the types of neurons present in the SC, for systematic analysis of neurites and their development in response to amphetamine treatment. Finally, future research should investigate cellular mechanism following chronic amphetamine treatment to further explore the mechanisms of action employed by amphetamine, and determine if treatment does result in a reduction of voltage gated sodium channels.

APPENDIX A. RESTRICTED ANOVAS PERFORMED FOR THE ANALYSIS OF LOCOMOTOR ACTIVITY

A.1 ACUTE EFFECTS OF AMPHETAMINE

Activity	Comparison	ANOVA
Horizontal activity	untreated & 0 mg/Kg	$F_{(5.73, 171.77)} = 2.34$, $p = 0.036$
	untreated & 2 mg/Kg	$F_{(5.71, 171.43)} = 1.99$, $p = 0.074$
	untreated & 5 mg/Kg	$F_{(4.58, 146.61)} = 3.14$, $p = 0.012$
	untreated & 10 mg/Kg	$F_{(3.42, 102.64)} = 1.46$, $p = 0.227$
	0 mg/Kg & 2 mg/Kg	$F_{(4.86, 145.71)} = 2.66$, $p = 0.003$
	0 mg/Kg & 5 mg/Kg	$F_{(4.39, 140.34)} = 6.53$, $p < 0.001$
	0 mg/Kg & 10 mg/Kg	$F_{(3.28, 98.45)} = 2.19$, $p = 0.089$
	2 mg/Kg & 5 mg/Kg	$F_{(5.00, 160.12)} = 5.53$, $p < 0.001$
	2 mg/Kg & 10 mg/Kg	$F_{(3.56, 106.92)} = 3.23$, $p = 0.019$
	5 mg/Kg & 10 mg/Kg	$F_{(3.64, 116.49)} = 0.71$, $p = 0.577$
Vertical activity	untreated & 0 mg/Kg	$F_{(6.33, 190.02)} = 1.08$, $p = 0.378$
	untreated & 2 mg/Kg	$F_{(5.27, 158.05)} = 1.05$, $p = 0.390$
	untreated & 5 mg/Kg	$F_{(4.43, 141.79)} = 2.51$, $p = 0.039$
	untreated & 10 mg/Kg	$F_{(2.71, 81.22)} = 3.06$, $p = 0.038$
	0 mg/Kg & 2 mg/Kg	$F_{(6.99, 209.76)} = 1.29$, $p = 0.257$
	0 mg/Kg & 5 mg/Kg	$F_{(6.59, 210.79)} = 1.38$, $p = 0.218$
	0 mg/Kg & 10 mg/Kg	$F_{(3.40, 102.07)} = 2.44$, $p = 0.061$
	2 mg/Kg & 5 mg/Kg	$F_{(5.55, 177.45)} = 2.73$, $p = 0.017$
	2 mg/Kg & 10 mg/Kg	$F_{(3.83, 84.79)} = 3.37$, $p = 0.025$
	5 mg/Kg & 10 mg/Kg	$F_{(2.97, 95.07)} = 1.65$, $p = 0.184$
Stereotypic activity	untreated & 0 mg/Kg	$F_{(4.91, 147.42)} = 2.90$, $p = 0.016$
	untreated & 2 mg/Kg	$F_{(5.27, 158.33)} = 0.86$, $p = 0.516$
	untreated & 5 mg/Kg	$F_{(4.25, 136.08)} = 3.19$, $p = 0.014$
	untreated & 10 mg/Kg	$F_{(4.67, 139.97)} = 4.03$, $p = 0.002$
	0 mg/Kg & 2 mg/Kg	$F_{(6.44, 193.18)} = 4.05$, $p = 0.001$
	0 mg/Kg & 5 mg/Kg	$F_{(3.35, 107.15)} = 4.93$, $p = 0.002$
	0 mg/Kg & 10 mg/Kg	$F_{(5.48, 164.46)} = 4.59$, $p < 0.001$
	2 mg/Kg & 5 mg/Kg	$F_{(4.18, 133.84)} = 4.59$, $p = 0.001$
	2 mg/Kg & 10 mg/Kg	$F_{(4.67, 170.22)} = 6.95$, $p < 0.001$
	5 mg/Kg & 10 mg/Kg	$F_{(2.69, 86.19)} = 1.27$, $p = 0.289$
Jumps	untreated & 0 mg/Kg	$F_{(6.86, 205.93)} = 1.59$, $p = 0.143$
	untreated & 2 mg/Kg	$F_{(7.22, 216.62)} = 1.04$, $p = 0.407$
	untreated & 5 mg/Kg	$F_{(7.83, 250.56)} = 2.91$, $p = 0.004$
	untreated & 10 mg/Kg	$F_{(2.82, 84.54)} = 3.68$, $p = 0.017$
	0 mg/Kg & 2 mg/Kg	$F_{(6.86, 205.90)} = 1.97$, $p = 0.062$
	0 mg/Kg & 5 mg/Kg	$F_{(8.00, 256.08)} = 3.65$, $p < 0.001$

Activity	Comparison	ANOVA
	0 mg/Kg & 10 mg/Kg	$F_{(2.87, 86.00)} = 3.80$, $p = 0.014$
	2 mg/Kg & 5 mg/Kg	$F_{(7.02, 224.59)} = 1.90$, $p = 0.070$
	2 mg/Kg & 10 mg/Kg	$F_{(3.26, 97.68)} = 3.20$, $p = 0.023$
	5 mg/Kg & 10 mg/Kg	$F_{(3.10, 99.11)} = 1.86$, $p = 0.140$
Distance travelled	untreated & 0 mg/Kg	$F_{(5.57, 167.11)} = 3.23$, $p = 0.006$
	untreated & 2 mg/Kg	$F_{(5.76, 172.82)} = 1.98$, $p = 0.074$
	untreated & 5 mg/Kg	$F_{(5.00, 159.96)} = 3.20$, $p = 0.009$
	untreated & 10 mg/Kg	$F_{(3.57, 107.11)} = 1.09$, $p = 0.361$
	0 mg/Kg & 2 mg/Kg	$F_{(5.61, 168.15)} = 1.94$, $p = 0.083$
	0 mg/Kg & 5 mg/Kg	$F_{(5.17, 165.30)} = 8.21$, $p < 0.001$
	0 mg/Kg & 10 mg/Kg	$F_{(3.71, 111.43)} = 2.58$, $p = 0.045$
	2 mg/Kg & 5 mg/Kg	$F_{(5.23, 167.43)} = 5.25$, $p < 0.001$
	2 mg/Kg & 10 mg/Kg	$F_{(3.73, 111.99)} = 2.49$, $p = 0.051$
	5 mg/Kg & 10 mg/Kg	$F_{(3.81, 122.03)} = 0.81$, $p = 0.515$

Table A.1 All interaction effects found between CHRONIC TREATMENT and TIME using restricted ANOVAs run using each combination of treatment group pairs. Data from locomotor activity recorded on day one, following acute administration of amphetamine.

A.2 CHRONIC EFFECTS OF AMPHETAMINE

Activity	Comparison	ANOVA
Horizontal activity	untreated & 0 mg/Kg	$F_{(6.72, 201.45)} = 1.37$, $p = 0.221$
	untreated & 2 mg/Kg	$F_{(5.49, 164.63)} = 4.25$, $p = 0.001$
	untreated & 5 mg/Kg	$F_{(7.21, 230.64)} = 0.88$, $p = 0.524$
	untreated & 10 mg/Kg	$F_{(4.22, 126.59)} = 1.58$, $p = 0.181$
	0 mg/Kg & 2 mg/Kg	$F_{(5.52, 165.55)} = 3.86$, $p = 0.002$
	0 mg/Kg & 5 mg/Kg	$F_{(7.27, 232.61)} = 1.54$, $p = 0.152$
	0 mg/Kg & 10 mg/Kg	$F_{(4.36, 130.74)} = 1.09$, $p = 0.365$
	2 mg/Kg & 5 mg/Kg	$F_{(6.16, 197.16)} = 3.56$, $p = 0.002$
	2 mg/Kg & 10 mg/Kg	$F_{(4.24, 127.26)} = 2.43$, $p = 0.048$
	5 mg/Kg & 10 mg/Kg	$F_{(5.21, 166.58)} = 0.79$, $p = 0.563$
Vertical activity	untreated & 0 mg/Kg	$F_{(6.29, 188.70)} = 2.20$, $p = 0.068$
	untreated & 2 mg/Kg	$F_{(5.68, 170.29)} = 2.09$, $p = 0.061$
	untreated & 5 mg/Kg	$F_{(6.30, 201.66)} = 1.01$, $p = 0.421$
	untreated & 10 mg/Kg	$F_{(5.24, 157.29)} = 1.74$, $p = 0.126$
	0 mg/Kg & 2 mg/Kg	$F_{(6.39, 191.92)} = 2.44$, $p = 0.024$
	0 mg/Kg & 5 mg/Kg	$F_{(6.81, 217.93)} = 2.43$, $p = 0.022$
	0 mg/Kg & 10 mg/Kg	$F_{(6.19, 185.80)} = 1.07$, $p = 0.386$
	2 mg/Kg & 5 mg/Kg	$F_{(6.72, 215.18)} = 3.57$, $p = 0.001$
	2 mg/Kg & 10 mg/Kg	$F_{(5.42, 162.68)} = 1.89$, $p = 0.093$
	5 mg/Kg & 10 mg/Kg	$F_{(5.71, 182.66)} = 1.20$, $p = 0.307$
Stereotypic activity	untreated & 0 mg/Kg	$F_{(6.23, 186.78)} = 1.07$, $p = 0.383$
	untreated & 2 mg/Kg	$F_{(5.83, 174.90)} = 2.56$, $p = 0.022$
	untreated & 5 mg/Kg	$F_{(6.43, 205.71)} = 0.83$, $p = 0.556$
	untreated & 10 mg/Kg	$F_{(5.74, 172.19)} = 1.28$, $p = 0.269$
	0 mg/Kg & 2 mg/Kg	$F_{(5.00, 150.02)} = 1.98$, $p = 0.062$
	0 mg/Kg & 5 mg/Kg	$F_{(6.51, 208.18)} = 2.23$, $p = 0.037$
	0 mg/Kg & 10 mg/Kg	$F_{(5.63, 168.99)} = 1.14$, $p = 0.344$
	2 mg/Kg & 5 mg/Kg	$F_{(5.98, 191.23)} = 5.41$, $p < 0.001$
	2 mg/Kg & 10 mg/Kg	$F_{(5.47, 164.21)} = 4.03$, $p = 0.001$
	5 mg/Kg & 10 mg/Kg	$F_{(6.35, 203.09)} = 1.85$, $p = 0.086$
Distance travelled	untreated & 0 mg/Kg	$F_{(6.72, 201.64)} = 2.13$, $p = 0.045$
	untreated & 2 mg/Kg	$F_{(6.03, 180.74)} = 3.89$, $p = 0.001$
	untreated & 5 mg/Kg	$F_{(6.75, 215.87)} = 1.15$, $p = 0.332$
	untreated & 10 mg/Kg	$F_{(5.22, 156.57)} = 3.14$, $p = 0.009$
	0 mg/Kg & 2 mg/Kg	$F_{(6.01, 180.38)} = 1.64$, $p = 0.138$
	0 mg/Kg & 5 mg/Kg	$F_{(6.85, 219.24)} = 1.61$, $p = 0.136$
	0 mg/Kg & 10 mg/Kg	$F_{(5.46, 163.64)} = 1.41$, $p = 0.219$

Activity	Comparison	ANOVA
	2 mg/Kg & 5 mg/Kg	F (6.03, 193.07) = 2.47, p = 0.025
	2 mg/Kg & 10 mg/Kg	F (5.18, 155.45) = 1.97, p = 0.084
	5 mg/Kg & 10 mg/Kg	F (5.85, 187.09) = 1.30, p = 0.260

Table A.2 All interaction effects found between CHRONIC TREATMENT and TIME using restricted ANOVAs run using each combination of treatment group pairs. No interactions are reported for the number of jumps, as no significant interaction effect was found in the man analysis. Data from locomotor activity recorded on day 20, following chronic administration of amphetamine.

A.3 COMPARISON OF THE EFFECTS

Activity	Comparison	ANOVA (Day*Chronic treatment)
Horizontal activity	untreated & 0 mg/Kg	F (1, 30) = 3.85, p = 0.059
	untreated & 2 mg/Kg	F (1, 30) = 9.88, p = 0.004
	untreated & 5 mg/Kg	F (1, 32) = 7.31, p = 0.011
	untreated & 10 mg/Kg	F (1, 30) = 8.26, p = 0.007
	0 mg/Kg & 2 mg/Kg	F (1, 30) = 0.54, p = 0.470
	0 mg/Kg & 5 mg/Kg	F (1, 32) = 12.46, p = 0.001
	0 mg/Kg & 10 mg/Kg	F (1, 30) = 12.36, p = 0.001
	2 mg/Kg & 5 mg/Kg	F (1, 32) = 9.25, p = 0.005
	2 mg/Kg & 10 mg/Kg	F (1, 30) = 0.81, p = 0.374
	5 mg/Kg & 10 mg/Kg	F (1, 32) = 0.44, p = 0.514
Vertical activity	untreated & 0 mg/Kg	F (1, 30) = 4.93, p = 0.034
	untreated & 2 mg/Kg	F (1, 30) = 0.21, p = 0.651
	untreated & 5 mg/Kg	F (1, 32) = 7.89, p = 0.008
	untreated & 10 mg/Kg	F (1, 30) = 11.87, p = 0.002
	0 mg/Kg & 2 mg/Kg	F (1, 30) = 5.88, p = 0.022
	0 mg/Kg & 5 mg/Kg	F (1, 32) = 0.30, p = 0.588
	0 mg/Kg & 10 mg/Kg	F (1, 30) = 6.24, p = 0.018
	2 mg/Kg & 5 mg/Kg	F (1, 32) = 9.00, p = 0.005
	2 mg/Kg & 10 mg/Kg	F (1, 30) = 12.42, p = 0.001
	5 mg/Kg & 10 mg/Kg	F (1, 32) = 5.50, p = 0.025
Stereotypic activity	untreated & 0 mg/Kg	F (1, 30) = 6.44, p = 0.017
	untreated & 2 mg/Kg	F (1, 30) = 2.14, p = 0.154
	untreated & 5 mg/Kg	F (1, 32) = 0.86, p = 0.360
	untreated & 10 mg/Kg	F (1, 30) = 2.85, p = 0.102

Activity	Comparison	ANOVA (Day*Chronic treatment)
	0 mg/Kg & 2 mg/Kg	$F_{(1, 30)} = 2.80$, $p = 0.105$
	0 mg/Kg & 5 mg/Kg	$F_{(1, 32)} = 8.11$, $p = 0.008$
	0 mg/Kg & 10 mg/Kg	$F_{(1, 30)} = 14.52$, $p = 0.001$
	2 mg/Kg & 5 mg/Kg	$F_{(1, 32)} = 4.04$, $p = 0.053$
	2 mg/Kg & 10 mg/Kg	$F_{(1, 30)} = 9.43$, $p = 0.005$
	5 mg/Kg & 10 mg/Kg	$F_{(1, 32)} = 0.30$, $p = 0.588$
Jumps	untreated & 0 mg/Kg	$F_{(1, 30)} = 0.15$, $p = 0.699$
	untreated & 2 mg/Kg	$F_{(1, 30)} = 0.07$, $p = 0.789$
	untreated & 5 mg/Kg	$F_{(1, 32)} = 4.74$, $p = 0.037$
	untreated & 10 mg/Kg	$F_{(1, 30)} = 8.88$, $p = 0.006$
	0 mg/Kg & 2 mg/Kg	$F_{(1, 30)} = 6.02$, $p = 0.010$
	0 mg/Kg & 5 mg/Kg	$F_{(1, 32)} = 3.26$, $p = 0.080$
	0 mg/Kg & 10 mg/Kg	$F_{(1, 30)} = 8.36$, $p = 0.007$
	2 mg/Kg & 5 mg/Kg	$F_{(1, 32)} = 3.29$, $p = 0.079$
	2 mg/Kg & 10 mg/Kg	$F_{(1, 30)} = 8.45$, $p = 0.007$
	5 mg/Kg & 10 mg/Kg	$F_{(1, 32)} = 6.92$, $p = 0.013$
Distance travelled	untreated & 0 mg/Kg	$F_{(1, 30)} = 1.96$, $p = 0.172$
	untreated & 2 mg/Kg	$F_{(1, 30)} = 0.43$, $p = 0.517$
	untreated & 5 mg/Kg	$F_{(1, 32)} = 11.81$, $p = 0.002$
	untreated & 10 mg/Kg	$F_{(1, 30)} = 14.19$, $p = 0.001$
	0 mg/Kg & 2 mg/Kg	$F_{(1, 30)} = 0.39$, $p = 0.538$
	0 mg/Kg & 5 mg/Kg	$F_{(1, 32)} = 16.44$, $p < 0.001$
	0 mg/Kg & 10 mg/Kg	$F_{(1, 30)} = 17.65$, $p < 0.001$
	2 mg/Kg & 5 mg/Kg	$F_{(1, 32)} = 13.26$, $p = 0.001$
	2 mg/Kg & 10 mg/Kg	$F_{(1, 30)} = 15.40$, $p < 0.001$
	5 mg/Kg & 10 mg/Kg	$F_{(1, 32)} = 1.73$, $p = 0.198$

Table A.3 All interaction effects found between CHRONIC TREATMENT and DAY using restricted ANOVAs run using each combination of treatment group pairs. Data from locomotor activity recorded on the first and final day of drug administration.

Activity	Comparison	ANOVA (Day*Chronic Treatment*Time)
Horizontal activity	untreated & 0 mg/Kg	$F_{(6.65, 199.59)} = 1.49$, p = 0.178
	untreated & 2 mg/Kg	$F_{(4.56, 136.75)} = 1.34$, p = 0.254
	untreated & 5 mg/Kg	$F_{(5.98, 191.40)} = 1.98$, p = 0.071
	untreated & 10 mg/Kg	$F_{(4.00, 120.12)} = 1.75$, p = 0.144
	0 mg/Kg & 2 mg/Kg	$F_{(7.15, 214.63)} = 1.69$, p = 0.110
	0 mg/Kg & 5 mg/Kg	$F_{(5.86, 187.45)} = 4.43$, p < 0.001
	0 mg/Kg & 10 mg/Kg	$F_{(4.00, 120.06)} = 2.38$, p = 0.055
	2 mg/Kg & 5 mg/Kg	$F_{(6.62, 211.91)} = 2.09$, p = 0.049
	2 mg/Kg & 10 mg/Kg	$F_{(11, 330)} = 1.50$, p = 0.131
	5 mg/Kg & 10 mg/Kg	$F_{(4.14, 132.47)} = 0.82$, p = 0.520
Vertical activity	untreated & 0 mg/Kg	$F_{(6.85, 205.46)} = 1.28$, p = 0.263
	untreated & 2 mg/Kg	$F_{(6.70, 201.07)} = 1.62$, p = 0.134
	untreated & 5 mg/Kg	$F_{(5.26, 168.42)} = 1.20$, p = 0.313
	untreated & 10 mg/Kg	$F_{(3.30, 99.04)} = 2.88$, p = 0.035
	0 mg/Kg & 2 mg/Kg	$F_{(7.65, 229.56)} = 1.02$, p = 0.422
	0 mg/Kg & 5 mg/Kg	$F_{(6.94, 222.20)} = 0.74$, p = 0.642
	0 mg/Kg & 10 mg/Kg	$F_{(4.20, 125.91)} = 2.18$, p = 0.072
	2 mg/Kg & 5 mg/Kg	$F_{(6.45, 206.43)} = 1.17$, p = 0.321
	2 mg/Kg & 10 mg/Kg	$F_{(3.44, 103.23)} = 2.17$, p = 0.088
	5 mg/Kg & 10 mg/Kg	$F_{(3.46, 110.70)} = 2.10$, p = 0.095
Stereotypic activity	untreated & 0 mg/Kg	$F_{(5.69, 170.60)} = 2.34$, p = 0.037
	untreated & 2 mg/Kg	$F_{(6.60, 198.18)} = 2.11$, p = 0.047
	untreated & 5 mg/Kg	$F_{(5.60, 179.20)} = 1.48$, p = 0.192
	untreated & 10 mg/Kg	$F_{(5.74, 172.19)} = 3.03$, p = 0.009
	0 mg/Kg & 2 mg/Kg	$F_{(11, 330)} = 1.59$, p = 0.139
	0 mg/Kg & 5 mg/Kg	$F_{(5.51, 176.37)} = 2.52$, p = 0.027
	0 mg/Kg & 10 mg/Kg	$F_{(6.35, 190.58)} = 1.41$, p = 0.208
	2 mg/Kg & 5 mg/Kg	$F_{(11, 352)} = 2.62$, p = 0.003
	2 mg/Kg & 10 mg/Kg	$F_{(7.08, 212.40)} = 1.98$, p = 0.058
	5 mg/Kg & 10 mg/Kg	$F_{(4.83, 154.48)} = 2.33$, p = 0. 47
Jumps	untreated & 0 mg/Kg	$F_{(11, 330)} = 1.42$, p = 0.190
	untreated & 2 mg/Kg	$F_{(11, 330)} = 1.16$, p = 0.315
	untreated & 5 mg/Kg	$F_{(11, 352)} = 0.92$, p = 0.526
	untreated & 10 mg/Kg	$F_{(4.48, 134.42)} = 1.75$, p = 0.136
	0 mg/Kg & 2 mg/Kg	$F_{(7.89, 236.82)} = 1.69$, p = 0.102
	0 mg/Kg & 5 mg/Kg	$F_{(11, 352)} = 2.41$, p = 0.018
	0 mg/Kg & 10 mg/Kg	$F_{(4.17, 125.11)} = 2.65$, p = 0.034
	2 mg/Kg & 5 mg/Kg	$F_{(11, 352)} = 0.857$, p = 0.583
	2 mg/Kg & 10 mg/Kg	$F_{(4.69, 140.77)} = 1.45$, p = 0.215
	5 mg/Kg & 10 mg/Kg	$F_{(4.33, 138.58)} = 1.10$, p = 0.362

Activity	Comparison	ANOVA (Day*Chronic Treatment*Time)
Distance travelled	untreated & 0 mg/Kg	$F_{(6.66, 199.83)} = 1.54$, p = 0.158
	untreated & 2 mg/Kg	$F_{(11, 330)} = 1.55$, p = 0.145
	untreated & 5 mg/Kg	$F_{(6.04, 193.41)} = 2.38$, p = 0.030
	untreated & 10 mg/Kg	$F_{(4.35, 130.63)} = 2.27$, p = 0.060
	0 mg/Kg & 2 mg/Kg	$F_{(11, 330)} = 1.51$, p = 0.159
	0 mg/Kg & 5 mg/Kg	$F_{(6.26, 200.44)} = 4.58$, p < 0.001
	0 mg/Kg & 10 mg/Kg	$F_{(4.86, 145.91)} = 2.57$, p = 0.030
	2 mg/Kg & 5 mg/Kg	$F_{(11, 352)} = 2.65$, p = 0.013
	2 mg/Kg & 10 mg/Kg	$F_{(4.86, 145.64)} = 1.94$, p = 0.061
	5 mg/Kg & 10 mg/Kg	$F_{(4.49, 143.55)} = 1.37$, p = 0.242

Table A.4 All interaction effects found between CHRONIC TREATMENT, TIME, and DAY using restricted ANOVAs run using each combination of treatment group pairs. Data from locomotor activity recorded on the first and final day of drug administration.

APPENDIX B. ELECTROPHYSIOLOGICAL RESPONSES OF THE TREATED AND UNTREATED CONTROL GROUPS

B.1 STIMULUS RESPONSE MULTIUNIT ACTIVITY

No significant main effects of CONTROL GROUP in onset latency, ($F_{(1, 36)} = 0.147$, $p = 0.704$, $\eta^2 = 0.004$, observed power = 0.07), peak latency ($F_{(1, 37)} = 0.001$, $p = 0.970$, $\eta^2 > 0.01$, observed power = 0.05), maximum amplitude ($F_{(1, 37)} = 0.547$, $p = 0.464$, $\eta^2 = 0.02$, observed power = 0.11) or the area under the curve ($F_{(1, 37)} = 1.26$, $p = 0.269$, $\eta^2 = 0.03$, observed power = 0.19); no interaction effects between CONTROL GROUP and STIMULUS INTENSITY for any variable.

B.2 STIMULUS RESPONSE LOCAL FIELD POTENTIALS

No significant main effects of CONTROL GROUP on onset latency ($F_{(1, 24)} = 0.272$, $p = 0.607$, $\eta^2 = 0.01$, observed power = 0.08), peak latency ($F_{(1, 46)} = 0.16$, $p = 0.694$, $\eta^2 = 0.003$, observed power = 0.07), maximum amplitude ($F_{(1, 46)} = 0.50$, $p = 0.482$, $\eta^2 = 0.01$, observed power = 0.11), and area under the curve ($F_{(1, 46)} = 3.96$, $p = 0.053$, $\eta^2 = 0.08$, observed power = 0.50), and no significant interaction effects between the CHRONIC TREATMENT and STIMULUS INTENSITY.

B.3 ACUTE AMPHETAMINE TRIAL MUA

There was no significant main effects of CONTROL GROUP on onset latency ($F_{(1, 30)} = 0.85$, $p = 0.365$, $\eta^2 = 0.02$, observed power = 0.15) and no interaction effect ($p = 0.292$); peak latency ($F_{(1, 30)} = 0.55$, $p = 0.464$, $\eta^2 = 0.02$, observed power = 0.11) and no interaction effect ($p = 0.097$); maximum amplitude (normalised against the baseline firing rate) ($F_{(1, 30)} = 0.004$, $p = 0.952$, $\eta^2 < 0.01$, observed power = 0.05) and no interaction

effect (p =0.365) area under the curve (F (1, 30) = 0.05, p =0.820 η^2 = 0.002, observed power = 0.06), and no interaction effects (p = 0.133).

B.4 ACUTE AMPHETAMINE TRIAL LFP

There was no significant main effects of CONTROL GROUP on onset latency (F (1, 26) = 1.43, p =0.242, η^2 = 0.05, observed power = 0.21) and no interaction effect (p = 0.820); peak latency (F (1, 26) = 0.08, p =0.781, η^2 = 0.003, observed power = 0.06) and no interaction effect (p = 0.764); peak amplitude (F (1, 26) = 0.006, p =0.938, η^2 < 0.01, observed power = 0.05) and no interaction effect (p =0.365) area under the curve (F (1, 30) = 0.05, p =0.820 η^2 = 0.002, observed power = 0.06), and no interaction effects (p = 0.133).

B.5 SALINE CONTROL MUA

There were no significant differences in onset latency (F (1, 32) = 1.35, p = 0.254, η^2 = 0.04, observed power = 0.20) and no interaction effect (p = 0.493); peak latencies (F (1, 32) = 1.75, p = 0.196, η^2 = 0.05, observed power = 0.25) and no interactions (p = 0.289); maximum amplitudes (F (1, 29) = 3.04, p = 0.092, η^2 = 0.10, observed power = 0.39) and no interaction effect (p = 0.643); area under the curve (F (1, 26) = 0.01, p = 0.938, η^2 < 0.01, observed power = 0.05) and no interactions effects (p = 0.886).

B.6 SALINE CONTROL LFP

There were no significant main effects of CONTROL GROUP on onset latency (F (1, 30) = 1.20, p = 0.283, η^2 = 0.04, observed power = 0.19), peak latency (F (1, 30) = 0.29, p = 0.595, η^2 = 0.01, observed power = 0.08), maximum amplitude (F (1, 30) = 0.29, p = 0.596, η^2 = 0.01, observed power = 0.08) or area under the curve (F (1, 30) = 4.15, p = 0.051, η^2 =

0.12, observed power = 0.50). There were also no interaction effects between the CONTROL GROUP groups and the SALINE DOSE for any measure.

B.7 BIPHASIC SR MUA

There were no significant main effects of CONTROL GROUP on either the total area under the curve ($F_{(1, 5)} = 0.001$, $p = 0.971$, $\eta^2 = 0.001$, observed power = 0.050), or the maximum amplitude ($F_{(1, 5)} = 1.25$, $p = 0.314$, $\eta^2 = 0.20$, observed power = 0.15), and no interactions between CONTROL GROUP and PHASE.

B.8 BIPHASIC ACUTE MUA

There was no significant main effect of CONTROL GROUP on either the area under the curve ($F_{(1, 12)} = 0.89$, $p = 0.363$, $\eta^2 = 0.07$, observed power = 0.14), or the maximum amplitude ($F_{(1, 5)} = 0.18$, $p = 0.691$, $\eta^2 = 0.03$, observed power = 0.06), and no interactions between CONTROL GROUP and PHASE.

B.9 BIPHASIC SALINE MUA

There were no significant main effects of CONTROL GROUP for either area under the curve ($F_{(1, 6)} = 0.004$, $p = 0.954$, $\eta^2 = 0.001$, observed power = 0.05) or maximum amplitude ($F_{(1, 5)} = 0.08$, $p = 0.788$, $\eta^2 = 0.02$, observed power = 0.06), and no interactions between CONTROL GROUP and PHASE.

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