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Chronic amphetamine enhances visual input to and suppresses visual output from the superior colliculus in withdrawal

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Heightened distractibility is a core symptom of Attention Deficit Hyperactivity Disorder (ADHD). Effective treatment is normally with chronic orally administered psychostimulants including amphetamine. Treatment prevents worsening of symptoms but the site of therapeutic processes, and their nature, is unknown. Mounting evidence suggests that the superior colliculus (SC) is a key substrate in distractibility and a therapeutic target, so we assessed whether therapeutically-relevant changes are induced in this structure by chronic oral amphetamine. We hypothesized that amphetamine would alter visual responses and morphological measures. Six-week old healthy male rats were treated with oral amphetamine (2, 5 or 10 mg/kg) or a vehicle for one month after which local field potential and multiunit recordings were made from the superficial layers of the SC in response to whole-field light flashes in withdrawal. Rapid Golgi staining was also used to assess dendritic spines, and synaptophysin staining was used to assess synaptic integrity. Chronic amphetamine increased local field potential responses at higher doses, and increased synaptophysin expression, suggesting enhanced visual input involving presynaptic remodelling. No comparable increases in multiunit activity were found suggesting amphetamine suppresses collicular output activity, counterbalancing the increased input. We also report, for the first time, five different dendritic spine types in the superficial layers and show these to be unaffected by amphetamine, indicating that suppression does not involve gross postsynaptic structural alterations. In conclusion, we suggest that amphetamine produces changes at the collicular level that potentially stabilise the structure and may prevent the worsening of symptoms in disorders like ADHD.

1. Introduction

Distractibility is defined as an attentional deficit where orientation toward irrelevant targets cannot be inhibited (Gaymard et al., 2003). Heightened levels of distractibility are found within a variety of psychiatric conditions, including Attention Deficit Hyperactivity Disorder (ADHD) (Douglas, 1983; Thorley, 1984) and schizophrenia (Grillon et al., 1990). It is also found within healthy ageing (Gaymard et al., 2003; Mishra et al., 2014) where it is believed to underpin a decline in various cognitive functions, therefore impacting negatively on quality of life in otherwise healthy people (Kim et al., 2007). Despite the high prevalence of heightened distractibility, limited attempts have been made to understand its basis within the brain, with focus almost exclusively on the prefrontal cortex and associated cortical networks (Campbell et al., 2012; Chadick et al., 2014). However, converging evidence implicates the superior colliculus (SC) as a key neural substrate for distractibility. The colliculus is responsible for orienting head and eye movements (Grantyn et al., 2004) and covert attention toward sensory stimuli (Rizzolatti et al., 1987). It is highly conserved across species and work in a range of species shows that collicular lesions cause decreased distractibility (Goodale et al., 1978; Milner et al., 1978; Sprague and Meikle, 1965), while removal of prefrontal cortex inhibitory control of the colliculus, leading to heightened activity in the structure, results in increased distractibility in humans (Gaymard et al., 2003). Additionally, there is evidence that the colliculus may play a role in ADHD, a core symptom of which is heightened distractibility (Brace et al., 2015a, b; Dommett et al., 2009; Dommett and Rostron, 2011; Klein et al., 2003; Milner et al., 1978; O'Driscoll et al., 2005; Swanson et al., 1991).

A key role for the colliculus in distractibility is possible because of intricate connections to the basal ganglia, a series of nuclei believed to act as the central device for action selection

(Gurney et al., 2001; Redgrave et al., 1999). The colliculus makes direct connections to dopaminergic neurons in substantia nigra pars compacta and the ventral tegmental area (Comoli et al., 2003; Dommett et al., 2005; Takakuwa et al., 2017). In turn the structure also receives dopaminergic input (Bolton et al., 2015; Perez-Fernandez et al., 2017; Rolland et al., 2013). As well as this dopaminergic input, the colliculus receives extensive GABAergic input from the substantia nigra pars reticulata (Hikosaka, 2007; Kaneda et al., 2008). Recent research suggests that when this GABAergic input is reduced, the effects of dopamine on the colliculus are revealed such that reduced dopamine activity is associated with enhanced visual responses (Rolland et al., 2013). As well as these connections with the basal ganglia both the superficial and deep layers of the SC have direct ascending connections to the thalamus. In the case of the superficial layers of the SC, which process visual information, this is to the lateral posterior nucleus and the pulvinar and then forward to the neostriatum (McHaffie et al., 2005), or via a link in the deep layers of the SC (Lee et al., 1997), which project to the rostral intralaminar regions of the thalamus on route to the neostriatum (McHaffie et al., 2005). Where collicular activity is enhanced, via these connections the structure can make a stronger bid for behavioural expression and is therefore more likely to win against competitors resulting in an increased probability of orienting eye and head movements (and covert attentional shifts) which can manifest as 'distraction'. Conversely, by depressing responses in the SC, the probability of orienting movements and attentional shifts would be reduced (Dommett et al., 2009).

Increased distractibility is not always treated, but amphetamine has been found to be effective in reducing distractibility in ADHD (Brown and Cooke, 1994; Spencer et al., 2001) and in healthy subjects (Agmo et al., 1997; Halliday et al., 1990). Although the psychostimulant is efficacious, it's therapeutic mechanism of action is still unknown.

However, there is now mounting evidence that the colliculus could be a key site of action. For example, acute amphetamine has been shown to suppress activity in the visually responsive superficial layers of the SC in healthy animals (Dommett et al., 2009; Gowan et al., 2008) and in rodent models of ADHD (Clements et al., 2014). In addition, we have recently demonstrated that chronic treatment with orally-administered amphetamine selectively alters collicular-dependent behaviour in a manner consistent with suppression of activity in the area (Turner et al., 2018a). Furthermore, amphetamine is known to act on dopaminergic neurons to increase synaptic dopamine levels, and based on previous research (Rolland et al., 2013) we can infer that this should suppress visual responses in the colliculus. Evidence suggests that chronic treatment with amphetamine (or methylphenidate) in patients prevents the worsening of ADHD symptoms that occurs on a short time scale in a proportion of placebo-administered controls (Faraone et al., 2002), hence chronic drug treatment for ADHD clearly leads to changes that assist the therapeutic process. However, the nature of those changes and their location is currently unknown.

To assess whether therapeutically-relevant changes occur at the level of the SC following chronic psychostimulant administration, we chronically administered amphetamine orally to rats at a range of doses and examined the impact of the drug on visual processing and structural characteristics in the two-week period immediately after drug treatment, which we considered to be when the animal is in withdrawal. Research has consistently shown that chronic amphetamine treatment impacts on spine density and dendritic remodelling elsewhere in the brain (Robinson and Kolb, 1997, 1999; Selemon et al., 2007) and that these changes can be detected within 2 weeks and persist for several months (Acerbo et al., 2005; Kolb et al., 2003; Li et al., 2003; Robinson and Kolb, 1997, 1999). In line with this, there is evidence of altered expression of synaptophysin, a synaptic protein involved in regulation of

vesicular exocytosis (Bergmann et al., 1993; Grabs et al., 1994), following amphetamine administration in several structures (Bisagno et al., 2004; Rademacher et al., 2006; Rademacher et al., 2007; Stroemer et al., 1998). Based on this previous research, we hypothesized that chronic treatment with amphetamine would alter visual responses in the colliculus and dendritic and synaptic measures within the structure.

2. Methods and Materials

All experiments were approved by the Institutional Ethical Review Committee at the Open University, where work took place (The Animal Welfare and Ethics Board). Work was also conducted with the authority of the appropriate UK Home Office Licenses and adhered to guidelines set out in the Animals [Scientific Procedures] Act (1986), EU Directive 86/609/EEC, and the "Guide for the care and use of Laboratory Animals" (Council, 2010).

2.1 Subjects

Male Hooded Lister rats, bred in-house and housed in standard conditions as previously described (see Supplementary Material 1 (Turner et al., 2018a, b) were used in all experiments. All procedures were carried out in the dark phase and, therefore, when rats are most active.

2.2 Chronic drug treatment

Oral administration of amphetamine (Sigma Aldrich, UK) or vehicle in apple juice was conducted as previously described (Turner et al., 2018a, b; Wheeler et al., 2007) and is therefore detailed in the Supplementary Material 1. Drugs were administered daily for 4 weeks (excluding weekends) for a total of 20 days (Kuczenski and Segal, 2002). All treatment took place in the holding room, after daily weighing of the rats (to determine dose and monitor health status), at the start of the dark phase. Three doses of amphetamine were selected (10 mg/kg, 5 mg/kg, and 2 mg/Kg) to ensure some clinical relevance and included those that have previously been shown to impact on collicular-dependent behaviours (Turner et al., 2018a). The final day of treatment fell on a Friday and in all cases subsequent procedures were conducted between 3 and 14 days after treatment end are summarised in Figure 1. Previous work has demonstrated that some effects associated with amphetamine

withdrawal can be found as within 24 hours after treatment cessation (Barr et al., 2010; Barr et al., 2013) and can persist for over 14 days (Hitzemann et al., 1977; Onn and Grace, 2000; Renard et al., 2014; Robinson and Kolb, 1997). The exact duration of any effect is likely to depend on the exact treatment regime and effects on some measures may persist for longer than others. The 3-14 day period selected here was chosen because existing literature suggested effects of withdrawal would be present for this whole period and it allowed sufficiently-sized cohorts to be used, bearing in mind the time consuming nature of some of the techniques, especially the *in vivo* electrophysiology.

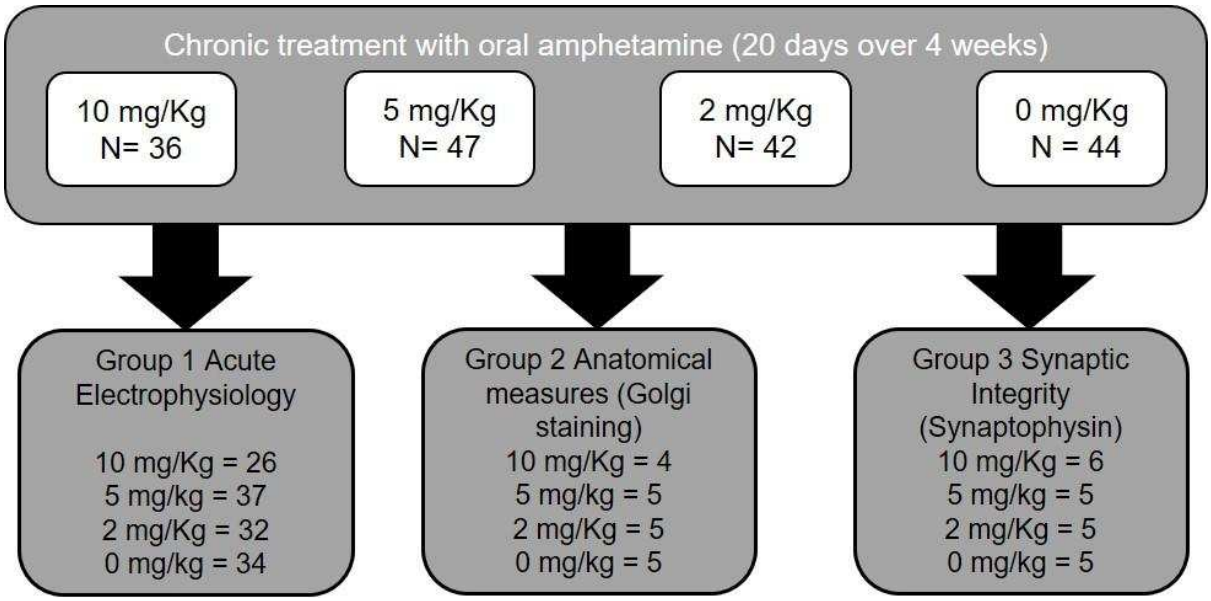

Figure 1: An overview of the experimental paradigm with sample sizes for each technique and drug treatment.

2.3 Acute Electrophysiology

2.3.1 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 for surgery was assessed by loss of the pedal and eye blink reflexes. Both eyes were sutured open and liquid tear gel (Viscotears®, Novartis

Pharmaceuticals Ltd., Surry, UK) applied to prevent desiccation. The animal was then placed in a stereotaxic frame (Kopf Instruments, Tujunga, USA) in the skull flat position. Body temperature was measured throughout the experiment using a rectal thermometer connected to a thermostatically-controlled heating blanket (Harvard Apparatus Ltd, Edenbridge, UK) to maintain temperature at 36-38 ˚C. Following application of local anaesthetic (Ethyl Chloride BP, Cryogesic ®, Acorus Therapeutics Ltd., Chester, UK), scalp retraction, bilaterial craniotomy and durotomy were performed, creating two 3 mm Ø burr holes exposing the cortex above the SC (right:-6.3 mm AP to Bregma, and +2 mm ML to the midline; left: -6.3 mm AP to Bregma; and +3.5 mm ML to the midline) to allow for simultaneous recordings from both SCs. In addition, two trepanned holes (1 mm Ø) were created anterior to the SC burr holes at specific stereotaxic co-ordinates for electroencephalographic (EEG) recordings (+1 mm anterior, +2 mm lateral; and -4mm posterior, +3mm lateral, relative to Bregma, (Devonshire et al., 2009). Differential and active EEG electrodes (loop-tipped silver wire, 0.2 mm Ø; Intracel) were placed ~1 mm subcranially into the rostral and caudal trepanned holes, respectively, to obtain continuous EEG information. Finally, respiration rate was recorded using a three-axis accelerometer IC (ADXL330KCPZ, Analog Devices, Norwood, MA, USA) device attached to the animal's lateral abdomen (Devonshire et al., 2009). Both EEG and respiration rate were used to monitor the animal during the recordings and used offline to confirm there were no differences in anaesthetic depth between the groups.

2.3.2 Recording procedure

Tungsten electrodes (Parylene-C-insulated; 2 MΩ, A-M Systems Inc., Carlsborg, WA, USA) were positioned directly above the superficial layers of the SC at the coordinates stated above at a depth of – 2.0 mm from the brain surface. The electrodes were then gradually lowered

during presentation of a light stimulus (green LED flashing at 0.5 Hz, 10 ms duration, 20 mcd positioned 5 mm anterior to the contralateral eye) until a strong light response was detected in both the audio feed from the recording (NL120, The Neurolog System, Digitimer, Hertfordshire, UK) and visual feed via Spike2 (CED, Cambridge, UK). Once the electrodes were positioned in the superficial layers, the animal remained in the dark for a further 25 min to adapt to the darkness before actual recordings began. Visual responses from 150 stimulations were then recorded at five different stimulus intensities (from minimum to maximum light: 4, 8, 12, 16 and 20 mcd). For all recordings, extracellular low frequency (local field potential; LFP) and high-frequency (multiunit activity) signals were amplified (gain 10,000 and 1000, respectively), band-pass filtered (LFP: 0.1–500 Hz, multiunit activity: 500–10 kHz) (Logothetis, 2008), digitized at 11 kHz and recorded to PC using a 1401+ data acquisition system (Cambridge Electronic Design Systems, Cambridge, UK), running Spike2 software (Cambridge Electronic Design, Cambridge, UK) and saved for offline analysis.

2.3.3 Reconstruction Histology

Following all recordings, a direct current of 10 µA was passed through the each of the electrodes for 5 s (Constant Voltage Isolated Stimulator DS2A MK2, Digitimer, Welwyn Garden City, UK) to mark the recording site. The animals were then transcardially perfused with physiological saline followed by 4% paraformaldehyde in phosphate buffered fixative. The brains were then placed in fixative for 24 h before being transferred to 20% sucrose for a further 36 h. They were then frozen to -18°C in isopentane (WWR International, Lutterworth, UK) and cut into 50 µm coronal sections using a cryostat (CM1900, Leica, Milton Keynes, UK) with the cutting chamber held at -20°C. The slices were dehydrated with alcohol and Nissl stained with cresyl violet (0.5%) (Sigma Aldrich, Gillingham, UK), before cover-slipping for histological verification of recording sites, which were subsequently plotted onto

reconstructed sections from Paxinos and Watson (Paxinos and Watson, 1998) to confirm location of recording in the superficial layers of the colliculus.

2.3.4 Data Processing

Waveforms were filtered using a low pass filter to give local field potential (LFP) data and high pass filter to give multiunit activity. The local field potential data was used to produce a waveform average in Spike2 (1 s duration, 0.1 s offset) for each stimulus intensity. This was exported into Matlab (Mathworks, Massachusetts, United States) to extract three response parameters: onset latency, peak latency and peak-to-peak amplitude. A response was deemed to have occurred if the voltage trace exceeded a pre-determined threshold after stimulus onset, but not before 20 ms post stimulus. The latter requirement was used to avoid any stimulus-related artifacts; collicular LFP responses to light flash stimuli in dark-adapted rats have been reported to have an average onset latency more than 27 ms (Dyer and Annau, 1977; Gowan et al., 2008). The threshold for change was set at ±1.96 standard deviations (i.e. within 95% confidence levels) from the mean activity during the pre-stimulus baseline, that is the 100 ms prior to the light flash. For the multiunit responses, PSTHs were produced in Spike2 (1-ms bins, 1 s duration, 0.1 s offset) and the data was exported into Matlab for extraction of response parameters: onset latency, peak latency and amplitude. As with the LFP data, a response was deemed to have occurred if the trace exceeded a threshold of ±1.96 standard deviations from the mean activity in the 100 ms pre-stimulus baseline period.

2.3.5 Statistical analysis

All statistical analysis was completed in SPSS. Chi-square tests of independence were used to assess whether treatment with amphetamine affected the likelihood of an LFP or multiunit response at each stimulus intensity. Where responses were found, parameter measures (i.e.

latencies and amplitude) were confirmed as suitable for use in parametric tests using the Kolmogorov-Smirnov test and measures of kurtosis and skewness. Repeated measures ANOVAs were then used with CHRONIC DOSE as the between-measures factor and STIMULUS INTENSITY as the within-measures factor. Significant main effects were further analysed with within-subject contrasts (simple and difference) and post-hoc Tukey tests for the within-measures and between-measures factors respectively. Where there was a significant interaction effect, tests of simple effects were run. These tests allow examination of the effects of one variable at the individual levels of the other variable and are an accepted method to investigate significant interactions (Field, 2009). If the assumption of sphericity was violated, the Greenhouse-Geisser results were reported (Greenhouse and Geisser, 1959). In all cases effect sizes are reported for statistical tests, with partial eta-squared (η_p^2) reported for ANOVAs and Cramer's V reported for chi-square analyses (Kim, 2017; Richardson, 2011).

To check that the depth of anaesthesia was comparable in the different chronic treatment groups during testing, the dominant EEG frequency was obtained using a power spectrum analysis (Spike2) for the full 300 s of each recording (i.e. 150 stimulations). The respiration rate per min was calculated during the first and last 30 s of this period and then used to calculate an average rate per min over the whole recording period. Based on the EEG frequency bands, all animals were found to be in stage III-3 or III-4 (Guedel, 1920). There were no significant differences in the dominant EEG frequency between the chronic treatment groups ($F(3, 60) = 0.22$, $p = 0.881$, $\eta_p^2 = 0.01$). They were also found to have comparable respiration rates ($F(3, 44) = 2.34$, $p = 0.087$, $\eta_p^2 = 0.15$).

2.4 Anatomical measures - Golgi Staining

2.4.1 Histological methods

Animals 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. The brain was removed and stored in 2.5 % glutaraldehyde in 0.1 M PB for at least 24 h before being switched to 4 % paraformaldehyde until staining begun. Brains were placed in slicing moulds and sections approximately 3 mm thick were collected through the SC. These sections were then rinsed briefly in 0.1 M PB and treated in 1 % osmium tetroxide for at least 7 h or overnight, then transferred to 3.5 % potassium dichromate and left at 4 °C for 4 days, with agitation of the sample on day two. Sections were then immersed in 1.5 % silver nitrate and the precipitate was gently cleared with a paint brush. The sections were 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).

Within the SC, we randomly sampled neurons and dendrites for analysis. This random sampling method was applied across all superficial layers ensuring suitable coverage of the structure. In total 227 dendrites were analysed (mean ± SEM 11.95 ± 1.23 dendrites per animal), with no significant differences in the number sampled per animal across treatment groups ($F(3, 15) = 0.294$, $p = 0.0829$). Data were collected from different order dendrites, with the majority (43.6%) being first order, followed by second order (37%) with the remain sample consisting of third (15%), fourth (3.5%) and fifth (0.9%) order, with the order of dendrites

sampled comparable across groups ($F(3, 23) = 1.85$, $p = 0.140$). The minimum dendritic length analysed was 30.10 µm with an average length of 95.13 ± 5.0 µm (SEM). The length of the dendrites sampled was also comparable across groups ($F(3, 223) = 1.23$, $p = 0.398$). The average thickness of traced dendrites was 1.11 ± 0.17 µm, also not significant differing between groups ($F(3, 223) = 0.936$, 0.424) Spine type was recorded, using morphological criteria previously defined (Risher et al., 2014). Briefly, a spine was defined as: thin if its length exceeded its width; stubby if the length is equal to or less than its width; mushroom if it has a long or short spine neck with large head (diameter > 0.6 µm); filopodia if its length is at least twice the width and there are no bulbous head regions and finally; branched if there is more than one head region on one spine neck. Spine density 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. Density was calculated by dividing the number of spines through by the length of the dendrite (Risher et al., 2014).

2.4.2 Statistical analysis of dendritic measures

All analyses were completed within SPSS where data was first confirmed as suitable for use in parametric tests using the Kolmogorov-Smirnov test and measures of kurtosis and skewness. A repeated measures ANOVA, using CHRONIC DOSE as a between-measures factor and SPINE TYPE as a within measures factor was used to analyse the percentage of each spine type. Spine density data was analysed using a One-Way ANOVA to identify any significant differences between dose groups. As with the electrophysiology data, partial eta-squared is reported as an effect size, along with the p-value.

2.5 Synaptic integrity - synaptophysin

2.5.1 Immunohistochemical methods

Animals 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. The brain was then removed and stored in fixative for at least 24 h, then transferred to 20 % sucrose for at least 36 h. 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 SC and stored free floating in cryoprotectant storage solution until staining. Sections were warmed to room temperature and 4 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 min. 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 1 h, 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 antibody (Jackson, Pennsylvania, USA) for 1 h, 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 SC for each section. Images were then analysed using ImageJ. First, stacks were

merged through the z axis, then the background was subtracted, and the image converted to 16-bit before thresholding and water-shedding images to display just the synaptophysin puncta. Imaging and thresholding parameters were kept uniform for each image.

2.5.2 Analysis of synaptophysin

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 was conducted in SPSS where it was first confirmed as suitable for use in parametric tests using the Kolmogorov-Smirnov test and measures of kurtosis and skewness. A One-Way ANOVA was then conducted for each of the four parameters (number, area, size, and density) and reported with both p-values and effect size.

3. Results

3.1 Chronic amphetamine affects local field potentials more than multiunit activity

Complex local field potential visual responses were found to at least one stimulus intensity in 98% of recordings (N=127) (Figure 2A). The responses in the vehicle-treated control group across all intensities showed an average onset latency of 29.0 ± 4.0 ms (mean ± SEM) and a peak latency of 95 ± 6.0 ms. The average peak-to-peak amplitude in this group was 127.00 ± 18.4 µV. Analysis of the responses found of all animals indicated that for local field potential responses, there was no significant association between chronic dose and whether a visual response was found at any stimulus intensity (4 mcd: χ^2 (3) = 0.91, p = 0.882, V = 0.084; 8 mcd χ^2 (3) = 1.01, p = 0.798, V = 0.089, 12 mcd χ^2 (3) = 0.99, p = 0.804, V = 0.088, 16 mcd χ^2 (3) = 4.81, p = 0.187, V = 0.193 and 20 mcd χ^2 (3) = 2.51, p = 0.474, V = 0.139), indicating that chronic treatment with amphetamine did not impact on the likelihood of a local field potential visual

response. Repeated measures ANOVA revealed a main effect of STIMULUS INTENSITY on onset latency (Figure 2B, F (4.61, 271.17) = 13.76, $p < 0.001$, $\eta_p^2 = 0.12$). Within-subject contrasts revealed a significant decrease in onset latency, i.e. responses were faster at the highest two stimulation intensities relative to the first (i.e. 4 mcd < 16 mcd, 4mcd < 20 mcd) and to the one immediately prior (i.e. 12 mcd < 16 mcd, 16 mcd < 20 mcd). There was also a significant main effect of CHRONIC DOSE (F (3, 104) = 6.00, p = 0.001, $\eta_p^2 = 0.15$), with post-hoc Tukey tests showing the onset latency to be significantly increased in all dose groups relative to the vehicle group (2 mg/kg p = 0.022, 5 mg/kg p = 0.018, 10 mg/kg p = 0.001), although the drug groups did not differ from each other. There was no significant interaction (F (7.82, 271.17) = 0.82, p = 0.587, $\eta_p^2 = 0.02$). Unlike onset latency, there was no significant main effect of STIMULUS INTENSITY on peak latency (Figure 2C, F (2.50, 259.95) = 0.56, p = 0.695, $\eta_p^2 = 0.01$), although there was still a main effect of CHRONIC DOSE (F (3, 104) = 3.98, p = 0.010, $\eta_p^2 = 0.10$). However, on this occasion this main effect was related to latencies in the 2 mg/kg group being significantly shorter than the 5 mg/kg (p = 0.01) and the 10 mg/kg (p = 0.049) groups, with no difference relative to the control group. There was however, also a significant interaction between CHRONIC DOSE and STIMULUS INTENSITY (F (7.50, 259.95) = 2.47, p = 0.016, $\eta_p^2 = 0.07$). Tests of simple effects of STIMULUS INTENSITY within each CHRONIC DOSE level showed that peak latency changed significantly with stimulus intensity for only the 5 mg/kg dose only (F (4) = 5.02; p = 0.001); it remained unaffected by stimulus intensity for all other dose groups. The simple effects test of CHRONIC DOSE within each level of STIMULUS INTENSITY revealed there were significant differences between the different drug groups for the first (4 mcd) stimulus intensity (F (3) 4.28, p=0.007) and the final (20 mcd) stimulus intensity (F (3) = 5.78, p = 0.001). At the first stimulus intensity, the 2 mg/kg group had significant longer latency than the vehicle-treated (p=0.011) and 10 mg/kg group

(p=0.019), whilst at the final stimulus intensity, the 5 mg/kg group had a longer latency than the vehicle-treated group (p=0.005) and the 2 mg/kg group (p=0.001).

As may be expected there was a significant main effect of STIMULUS INTENSITY on response amplitude (Figure 2D, $F (1.67, 173.48) = 11.00$, $p < 0.001$, $\eta_p^2 = 0.10$), with significant increases in amplitude with each increase in stimulus intensity from 8 mcd to 20 mcd ($p < 0.01$). There was no significant main effect of CHRONIC DOSE ($F (3, 104) = 2.06$, $p = 0.110$, $\eta_p^2 = 0.06$). However, there was a significant interaction between STIMULUS INTENSITY and CHRONIC DOSE ($F (5.00, 173.48) = 2.37$, $p = 0.042$, $\eta_p^2 = 0.06$). Tests of simple effects of STIMULUS INTENSITY within each CHRONIC DOSE level showed that amplitude changed significantly with stimulus intensity for the 5 mg/kg ($F (4) = 6.41$; $p < 0.001$) and 10 mg/kg ($F (4) = 9.60$, $p < 0.001$), but remained unaffected by stimulus intensity for the other dose groups. The simple effects test of CHRONIC DOSE within each level of STIMULUS INTENSITY revealed there were no significant differences between the different drug groups for all but the final (20 mcd) stimulus intensity ($F (3) = 2.73$, $p = 0.048$), where the 10 mg/kg group had a significantly larger response than the 2 mg/kg group (p=0.049).

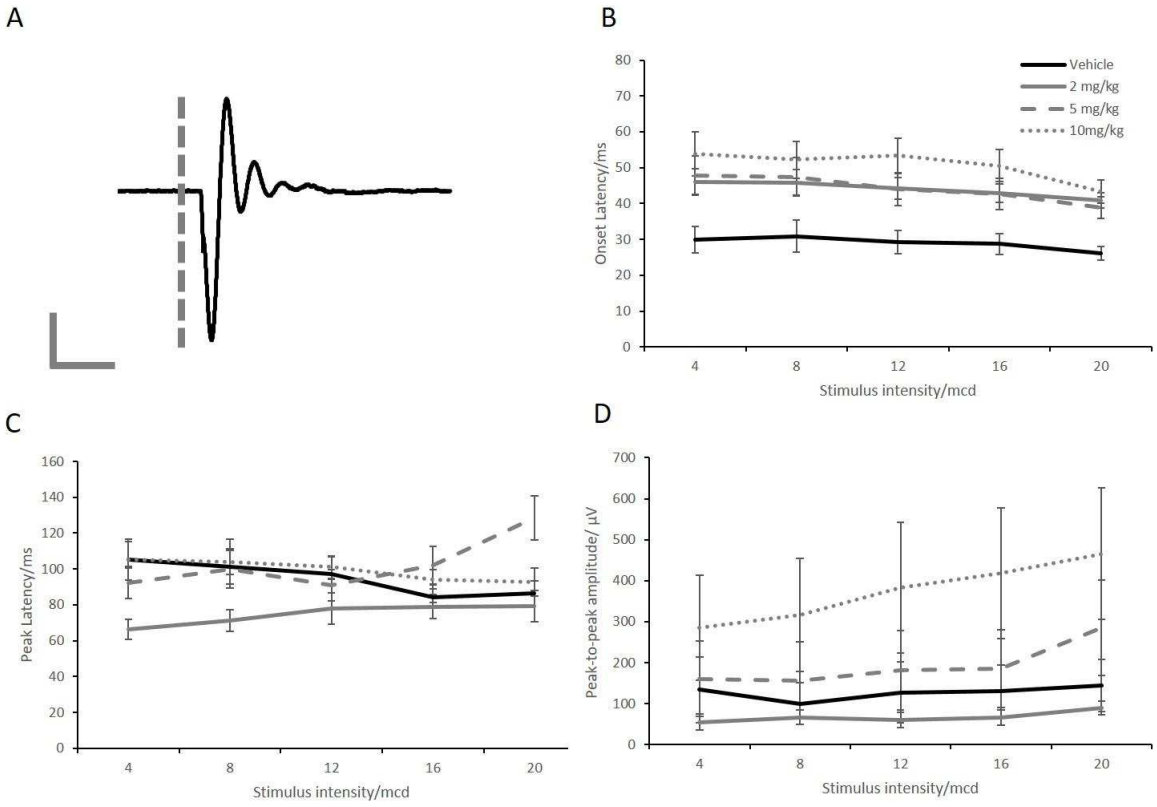

Figure 2: A typical local field potential response to a visual stimulus in the superficial layers of the colliculus in a vehicle-treated animal. Example at mid intensity (12 mcd) delivered at time shown by dashed line. Vertical scale bar = 400 µV; horizontal scale bar = 50 ms (A). Line graphs to show the relationship between stimulus intensity and onset latency (B), peak latency (C) and peak-to-peak amplitude (D) for the different chronic treatment groups. Analysis and data is shown for recordings only where responses were found at all intensities (Total sample N=108: Vehicle N=29, 2 mg/kg N=28, 5 mg/kg N=29 and 10 mg/Kg N=22). A representative key is shown in (B). In all cases numerical data presented is as the mean ± SEM.

Overall, visual responses were found to at least one stimulus intensity in 94% of the multiunit recordings made (N=121). A typical response is shown in Figure 3A**.** Responses in our vehicle-treated control group averaged across all stimulus intensities had an onset latency of 66.0 ± 2.0 ms and a peak latency of 114.0 ± 4.0ms, with an amplitude of 879 ± 89 spikes/sec. There was no significant association between chronic dose and whether a visual response was found at any intensity for all animals (4 mcd: χ^2 (3) = 2.46, p = 0.483, V = 0.138; 8 mcd χ^2 (3) = 1.50, p = 0.682, V = 0.108, 12 mcd χ^2 (3) = 7.76, p = 0.051, V = 0.245, 16 mcd χ^2 (3) = 2.81, p = 0.421,

V = 0.148 and 20 mcd χ^2 (3) = 3.94, p = 0.268, V = 0.175), indicating that chronic treatment with amphetamine did not impact on the likelihood of a visual response. A repeated measures ANOVA showed a significant main effect of STIMULUS INTENSITY on onset latency (Figure 3B, F (2.82, 214.40) = 445.33, p < 0.001, η_p^2 = 0.85). Within-subject contrasts showed a significant reduction in onset latency between each consecutive stimulus intensity (p<0.001). There was also a significant main effect of CHRONIC DOSE (F (3, 76) = 2.97, p = 0.037, η_p^2 = 0.11), although post hoc Tukey tests revealed only trend level differences between the 5 mg/Kg group and both the control group (p = 0.061), and 2 mg/Kg dose group (p = 0.081), with the onset latency being consistently faster in the control and low dose groups. There was no significant interaction effect between the STIMULUS INTENSITY and CHRONIC DOSE (F (8.46, 214.40) = 1.78, p = 0.079, η_p^2 = 0.07). There was however a significant main effect of STIMULUS INTENSITY on peak latency (Figure 3C, F (2.98, 226.72) = 3.87, p = 0.01, η_p^2 = 0.05). Within-subject contrasts revealed that only at the penultimate stimulus intensity was the peak latency significantly less than the first (p < 0.001). There was no significant main effect of CHRONIC DOSE (F (3, 76) = 0.626, p = 0.601, η_p^2 = 0.02), however there was a significant interaction effect between STIMULUS INTENSITY and CHRONIC DOSE (F (8.95, 226.72) = 2.35, p = 0.015, η_p^2 = 0.07). Tests of simple effects of STIMULUS INTENSITY within each CHRONIC DOSE level showed that peak latency changed significantly with stimulus intensity for the 5 mg/kg (F (4) = 2.71; p=0.006) and 10 mg/kg (F (4)= 3.37, p = 0.010) doses, but remained unaffected by stimulus intensity for the other dose groups. The simple effects test of CHRONIC DOSE within each level of STIMULUS INTENSITY revealed there were no significant differences between the different drug groups for all but the final (20 mcd) stimulus intensity (F (3) =3.77, p=0.014), where the 5 mg/kg dose had a significantly longer latency than the vehicle-treated group (p=0.045) and the 2 mg/kg group (p=0.038).

Finally, there was a significant main effect of STIMULUS INTENSITY on response amplitude (Figure 3D, F (1.89, 143.57) = 218.88, $p < 0.001$, $\eta_p^2 = 0.74$) with within-subject contrasts showing a significant increase in response amplitude with each consecutive rise in stimulus intensity ($p < 0.001$). There was no significant effect of CHRONIC DOSE (F (3, 76) = 0.69, p=0.563, $\eta_p^2 = 0.03$), and here was also no significant interaction effect (F (0.82, 143.57) = 0.79, p=0.576, $\eta_p^2 = 0.03$).

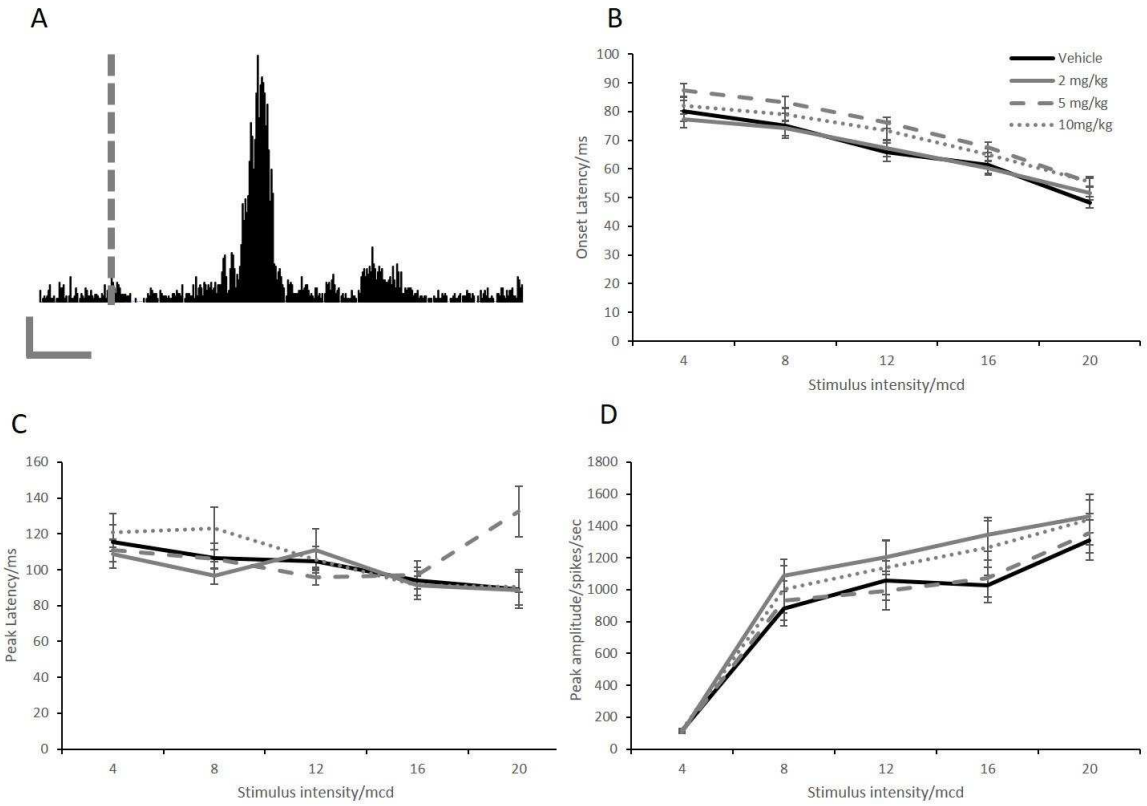

Figure 3: A typical multiunit response to a visual stimulus in the superficial layers of the colliculus in a vehicle-treated animal. Example at mid intensity (12 mcd) delivered at time shown by dashed line. Vertical scale bar = 100 spikes/sec; horizontal scale bar = 50 ms (A). Line graphs to show the relationship between stimulus intensity and onset latency (B), peak latency (C) and peak-to-peak amplitude (D) for the different chronic treatment groups. Analysis and data is shown for recordings only where responses were found at all intensities (Total sample N=80: Vehicle N=23, 2 mg/kg N=19, 5 mg/kg N=21 and 10 mg/Kg N=17). A representative key is shown in (B). In all cases numerical data presented is as the mean ± SEM.

3.2 Chronic amphetamine does not affect spine types or densities

Five different spine types were identified within the colliculus: thin, stubby, mushroom, filopodia and branched (Figure 4A). A repeated measures ANOVA was used to analyse the proportion of these different spine types. This revealed no significant main effect of CHRONIC DOSE ($F(3, 15) = 0.63$, $p = 0.610$, $\eta_p^2 = 0.11$), and no interaction effect ($F(5.87, 29.35) = 0.90$, $p = 0.506$, $\eta_p^2 = 0.153$). However, there was a significant main effect of SPINE TYPE (Figure 4B, $F(1.96, 29.35) = 73.24$, $p < 0.001$, $\eta_p^2 = 0.83$). Contrasts revealed that there were no significant differences between the percentage of thin and stubby spines overall ($p=0.995$). However, there were significantly more thin and stubby spines than any other type ($p \leq 0.001$) of spine. Additionally, there were more mushroom spines than both filopodia and branched ($p < 0.001$) and more filopodia than branched ($p < 0.001$). As well as there being no differences between groups in terms of the types of spines present, a One-Way ANOVA found no significant differences in spine density between different chronic treatment groups (Figure 4C, $F(3, 15) = 1.05$, $p = 0.401$, $\eta_p^2 = 0.17$).

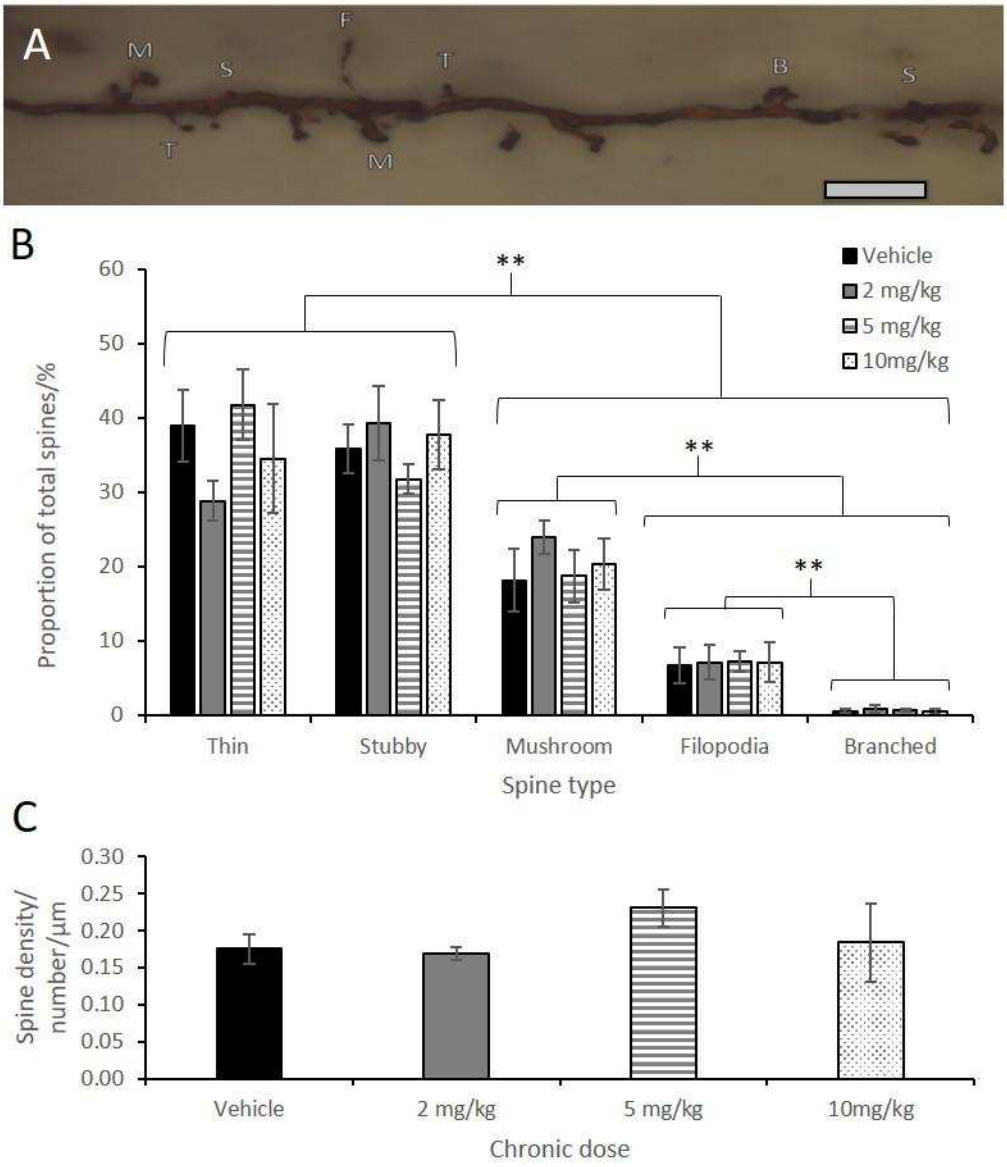

Figure 4: Examples of different dendritic spines in the superior colliculus (Scale bar = 5 µm) T=thin, S=stubby, M=mushroom, F=filopodia and B=branched (A). The proportion of different spine types in the colliculus showed a predominance of thin and stubby spines but no differences between chronic drug groups (B). There were also no significant differences in spine density (C). In all cases numerical data presented is as the mean ± SEM. ** p<0.001.

3.3 Chronic amphetamine increases the size of synaptophysin-positive puncta

Analyses of synaptophysin measures (Figure 5A and B) using One-Way ANOVA showed no significant differences in the number of synaptic puncta ($F(3, 17) = 0.75$, $p = 0.536$, $\eta_p^2 = 0.12$), the percentage area covered by puncta ($F(3, 17) = 1.07$, $p = 0.389$, $\eta_p^2 = 0.16$) or the density

of the puncta (F (3, 17) = 0.86, p = 0.482, η_p^2 = 0.13) between the chronic treatment groups. However, there was a significant difference in the average size of the puncta (Figure 5C, F (3, 17) = 10.45, p < 0.001, η_p^2 = 0.65). Post hoc Tukey tests revealed that the vehicle-treated 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).

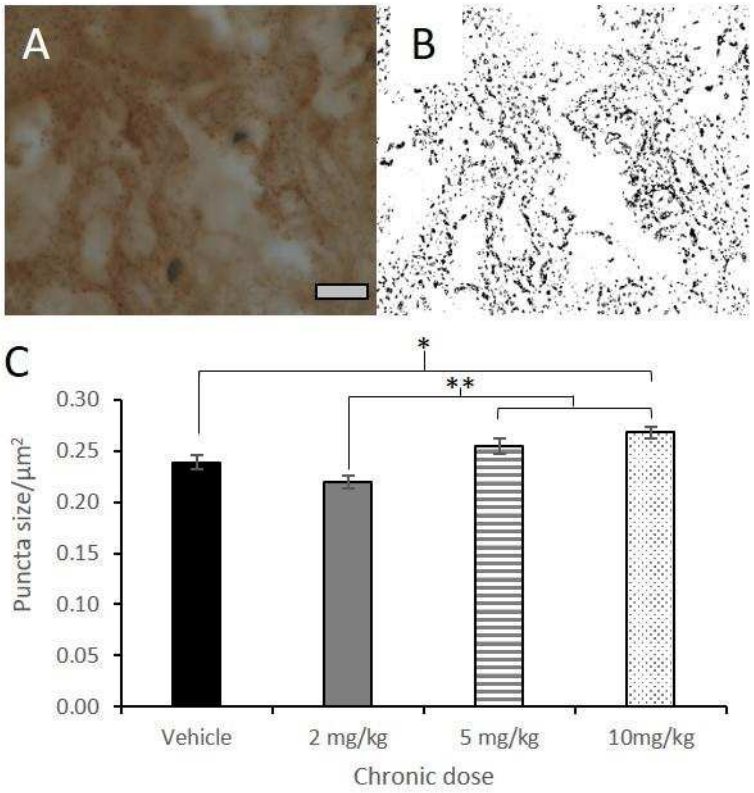

Figure 5: An example of synaptophysin stained tissue, merged through the z-axis (A). An image following thresholding to display just the stained synaptophysin puncta (B). Scale bar = 10 µm. Variation in puncta size between the different drug groups (C). In all cases numerical data presented is as the mean ± SEM. * p<0.05, ** p<0.001.

4. Discussion

In line with previous studies, we recorded complex LFP responses in the superficial layers of the SC in response to whole-field light flashes (Clements et al., 2014; Dyer and Annau, 1977;

Gowan et al., 2008). The response parameters in the vehicle-treated control group were consistent with previous work in drug-naïve dark-adapted rats for both latencies (Dyer and Annau, 1977; Hetherington et al., 2017) and peak-to-peak amplitude (Clements et al., 2014). We found a relationship between stimulus intensity and onset latency, whereby the response was faster at higher stimulus intensities, which has been previously demonstrated in the absence of chronic amphetamine treatment (Clements et al., 2014; Dyer and Annau, 1977). This relationship was found for all drug groups, and therefore appears unaffected by dose, even though all doses of amphetamine significantly increased onset latency. The finding that chronic amphetamine treatment significantly increased onset latency during withdrawal has parallels with acute administration work where it has been found to increase the latencies of LFPs at higher doses (Gowan et al., 2008). By contrast, although peak latency was affected by chronic dose, amphetamine did not produce any systematic variation from the vehicle-treated control group for this parameter, in line with acute amphetamine effects (Clements et al., 2014).

As would be expected from the literature (Gowan et al., 2008) we reported that peak-to-peak amplitude of the LFP response was increased with increasing stimulus intensity. However, we found a significant interaction between drug dose and stimulus intensity, which suggested that the increase in peak-to-peak amplitude of the LFP response with increasing stimulus intensity was driven largely by increases in the 5 mg/kg and 10 mg/kg groups at the higher stimulus intensities. Given that the LFP response is best described as representing 'peri-synaptic activity', which includes post-synaptic potentials (Ekstrom, 2010; Logothetis, 2008), these data suggest that chronic amphetamine treatment with a high dose of amphetamine enhances incoming signals to the colliculus, albeit only for those high intensity signals.

Multiunit responses were similar in onset and peak latency as well as peak amplitude in our control group to those found in previous studies (Clements et al., 2014; Gowan et al., 2008). A similar relationship to that found for LFP responses was demonstrated for stimulus intensity and onset latency, with the latency decreasing as intensity increased. Also similar to the LFP data we found an increase in response amplitude with increasing stimulus intensity. However, unlike the LFP data this was found for all groups and not a result of preferential increases in the groups treated with the higher amphetamine doses. Also, unlike LFP responses, we found that peak latency decreased, albeit only at the highest stimulus intensity and largely due to a decrease in the animals treated with the higher doses of amphetamine. It is possible to speculate that chronic treatment with higher doses of amphetamine, whilst increasing the strength of incoming excitatory signals, suppresses the impact of those signals on collicular activity via some form of neuroadaptation, resulting in a mismatch of effect on LFP and multiunit activity. One possible mechanism for this is that amphetamine decreases the number of transient voltage-gated sodium channels, increasing the threshold for action potential generation in the colliculus. This has been found previously for other brain regions following chronic treatment with amphetamine, albeit given intraperitoneally (Peterson et al., 2006). This suppression would be consistent with our behavioural effects previously reported which suggest that chronic amphetamine treatment reduces activity in the colliculus and suppresses collicular dependent behaviour (Turner et al., 2018a).

There were no significant differences between groups in terms of dendritic spine density or expression of different spine types. This is perhaps surprising given previous research revealing significant increases in spine density in rodents following chronic amphetamine treatment (Robinson and Kolb, 1997, 1999). However, in this previous work, data indicated that the increase in density may have come from an increase in branched spines which were

not prevalent in the colliculus. Additionally, the increases seen previously in the prefrontal cortex and nucleus accumbens have not been replicated in all other areas (Selemon et al., 2007) indicating this may not be a common action of amphetamine but specific to these limbic and cortical structures. In the present context, the lack of impact of chronic amphetamine on spine parameters suggests that whatever neuroadaptations underlie chronic amphetamine's suppressive effect on collicular activity in response to excitatory inputs, this is not mediated by post-synaptic structural changes.

Despite not revealing any effects of chronic drug administration on dendritic spines in the SC, our study is, as far as we are aware, the first investigation of the SC using the adapted rapid Golgi method and therefore, the general pattern of spine types is of interest. The most prevalent spine types within the SC were stubby and thin spines, often suggested to be the earliest in development from filopodia (Harris, 1999). 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). However, there is also evidence to suggest that spines do not develop along a single directional pathway with the exact role of filopodia unclear and therefore this interpretation should be considered with caution (Hering and Sheng, 2001). Future studies may consider further analysis of spine types at multiple time points to better understand these characteristics of collicular morphology. Additionally, such studies should differentiate between different neuron types within the colliculus to best understand any impact of changes on the function of local circuitry.

The present study did reveal changes in synaptophysin staining indicating some alterations to synaptic functioning. Whilst there were no significant differences in the number of synaptic puncta, area they covered or density, 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. The impact of amphetamine on synaptophysin could indicate that the colliculus is sensitive to amphetamine-induced remodelling (Bisagno et al., 2004). Previous work reporting an increase in puncta size in another brain region associated it with synaptic enhancement, suggesting that neurons contacted by presynaptic terminals with a larger synaptophysin content are likely to show increased mEPSC frequency (Misra et al., 2010). This is consistent with our electrophysiology results which indicated that at the higher doses there was a greater amplitude of LFP response to high stimulus intensities. However, as suggested above, this increased incoming signal may be compensated for by a decreased response within the colliculus, removing any corresponding increase in multiunit activity. It is not possible from the current data to establish which, if either, of these changes occurred first i.e. whether amphetamine increased the synaptophysin content which was then compensated for by a reduction in the number of transient voltage-gated sodium channels or vice versa. However, given that acute administration of amphetamine has been previously found to reduce multiunit responses (Gowan et al., 2008), it is possible to speculate that this was the initial effect of amphetamine. This is also in line with previous work showing that a reduction in dopamine causes an increase in visual responses (Rolland et al., 2013), such that amphetamine, by increasing dopamine levels would suppress activity. Indeed, we have previously hypothesized that one mechanism of action of amphetamine in conditions such as ADHD, where it reduces

distractibility, may be to suppress collicular activity (Brace et al., 2015a; Clements et al., 2014; Hetherington et al., 2017; Overton, 2008). If this were to be the case, the upregulation of synaptophysin could be a neuroadaptation to moderate the impact of amphetamine on collicular output. The existence of this compensatory balance may explain the evidence in the literature that amphetamine has sustained efficacy as a treatment for ADHD without evidence of improved efficacy over time (Fredriksen et al., 2014). However, since synaptophysin changes have been demonstrated in response to withdrawal from amphetamine treatment (admittedly from a shorter period of i.p. administration) (Subramaniam et al., 2001), we have to acknowledge that the changes in synaptophysin in our study may have been an acute response to withdrawal. That aside, the preservation of collicular visual output in the face of enhanced collicular input suggests that some of the changes that attend chronic amphetamine administration that prevent a worsening of symptoms may take place in the SC.

There are several limitations to the current study must be acknowledged. Firstly, whilst every effort has been made to ensure doses are therapeutically relevant and administered using an appropriate method to best mirror human use, we did not measure blood plasma levels, something that should be considered in future research. Secondly, the age of the animals in the present study (adult) would limit the applicability of the results to children with ADHD. However, given that the condition is now accepted to be present in adults as well children and the two cohorts are deemed distinct (Moffitt et al., 2015) these results do still have relevance to the clinical population. Thirdly, we did not examine the effects on the colliculus during chronic treatment and only in withdrawal and therefore to further dissect the effects of chronic treatment from the effects of withdrawal, future studies should consider collecting measurements during both phases. Finally, whilst research in animals has contributed much

to our understanding of the superior colliculus and the structure is highly conserved across mammalian species, there are variations in sensory processing between rodents, as used here, and humans, and the impact of such processing on attentional processes including distractibility. For example, rodents do not rely on vision as a primary sense and instead use their whiskers to explore the environment (Castro-Alamancos and Favero, 2016; Lee et al., 2016), although with training they can use their visual system as effectively on attentional tasks (Lee et al., 2016). Although the intermediate layers of the colliculus contribute to whisker input processing, it cannot be assumed that effects of amphetamine equivalent to that found here in the visual layers would be found in the rodent's dominant sensory system. To date, this has not been investigated, although other psychostimulant treatment has been found to alter barrel cortex responses to whisker stimulation (Bekavac and Waterhouse, 1995), indicating this could be worth investigating.

Based on the current findings and the limitations described there are several future directions that could be considered. Prior to any further work investigating potential mechanisms of action of orally administered amphetamine, studies should be undertaken to directly compare the pharmacological effects and pharmacokinetics of the drug in humans and rats to ensure comparability. Additionally, to unpack the cellular mechanisms of action it would be helpful to consider conducting in vitro studies to investigate the effects of chronic amphetamine on the transient voltage-gated sodium channel currents, as has been completed for acute treatment in the prefrontal cortex (Peterson et al., 2006). Similarly, whilst we opted to randomly sample neurites for Golgi staining within the superficial layers for this study, the first of its kind; further work should consider systematic analysis of neurites from specific types of neurons within the colliculus in response to both vehicle treatment and amphetamine treatment of different durations. Finally, the work conducted here was

completed during a discrete withdrawal period and, therefore, it is not possible to definitively distinguish between the effects of different phases of withdrawal. As such future work should considered staggered experimentation with animals experiencing different durations of treatment and withdrawal.

5. Conclusion

In conclusion, we have shown, for the first time, the effects of chronic amphetamine treatment on visual responses in the colliculus in withdrawal. Results indicate that chronic amphetamine enhances the visual input to the SC, but that this does not translate into an enhanced visual output from the SC. Additionally, we have described the five different types of dendritic spine present in the superficial layers of the colliculus and shown these to be unaffected by amphetamine treatment, indicating the previous effects of amphetamine on dendritic measures may be region specific. Finally, we have found changes in synaptic integrity, as measured by synaptophysin puncta size. Collectively these results suggest that chronic amphetamine's enhancement of visual input to the SC is in part mediated by presynaptic remodelling involving synaptophysin. Furthermore, drug-induced suppression of collicular output activity (needed to balance out the effect of the enhanced input) does not involve gross postsynaptic structural alterations. We suggest that because chronic amphetamine produces changes at the collicular level that seem to balance out, this potentially stabilises the structure and may prevent the worsening of symptoms in disorders like ADHD.

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