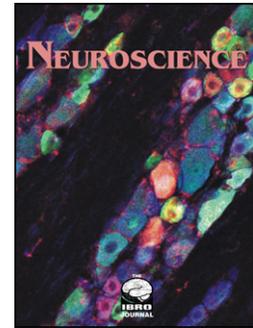


Accepted Manuscript

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PII: S0306-4522(09)01483-3
DOI: 10.1016/j.neuroscience.2009.09.007
Reference: NSC 11499

To appear in: *Neuroscience*

Received date: 29 June 2009
Revised date: 27 August 2009
Accepted date: 3 September 2009

Please cite this article as: Dommett, E.J., Overton, P.G., Greenfield, S.A., Drug therapies for attentional disorders alter the signal-to- noise ratio in the superior colliculus, *Neuroscience* (2009), doi: 10.1016/j.neuroscience.2009.09.007.

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Section Editor: Dr. Minoru Kimura

Drug therapies for attentional disorders alter the signal-to-noise ratio in the superior colliculus

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Abstract

Despite high levels of use, the mechanism of action of effective pharmacotherapies in Attention Deficit Hyperactivity Disorder (ADHD) is unknown. It has recently been hypothesized that one site of therapeutic action is the midbrain superior colliculus, a structure traditionally associated with visual processing, but also strongly implicated in distractibility, a core symptom of ADHD. We used male juvenile Wistar rats to examine the effects of therapeutically relevant doses of methylphenidate and d-amphetamine on collicular activity *in vitro*. Here we report a novel shared mechanism of the two drugs whereby they enhance the signal to noise ratio in the superior colliculus. The effects on the signal to noise ratio were mediated by serotonin via a pre-synaptic mechanism. This modulatory action would bias the system towards salient events and lead to an overall decrease in distractibility.

Keywords:

Attention Deficit Hyperactivity Disorder
Superior colliculus
Methylphenidate
D-amphetamine

Attention Deficit Hyperactivity Disorder (ADHD) is the most common neurodevelopmental disorder affecting an estimated 8-12% of children (Biederman & Faraone, 2005), with symptoms often persisting into adulthood (Spencer et al., 2002). Nonetheless, the pathophysiological changes which underlie ADHD are far from completely understood (Biederman, 2005), as are the mechanisms of action of the most commonly used pharmacotherapies – amphetamines and methylphenidate (Spencer et al., 2002). A central component of ADHD is an increase in distractibility (Douglas, 1983; Thorley, 1984) and amphetamine has been found to be effective in reducing distractibility in ADHD (Brown & Cooke, 1994; Spencer et al., 2001) and in healthy subjects (Halliday et al., 1990; Agmo et al., 1997). Behavioural evidence suggests that distractibility is intimately linked with the superior colliculus (SC), a major component of the subcortical visual system that is highly conserved across species (Overton et al., 1985). Work in a range of species has shown that collicular lesions lead to a decrease in distractibility (Goodale et al., 1978; Milner et al., 1978). Conversely, removal of prefrontal cortex control of the colliculus leads to an increase in distractibility in humans (Gaymard et al., 2003).

We have recently shown that d-amphetamine decreases multiunit and field potential responses to wholefield light flash stimuli in the superficial layers of the SC (Gowan et al., 2008). Hence, the SC may be a potential therapeutic target in ADHD, a proposal supported by an expanding body of evidence which suggests that the colliculus is dysfunctional in the disorder (Overton, 2008). However, our results are in contrast to earlier work in the cat (Grasse et al., 1993), where d-amphetamine enhanced single unit responses in the SC to stimuli presented within the excitatory region of the cells' receptive fields. Since the receptive fields of superficial layer SC

have suppressive surrounds (McIlwain & Buser, 1968; Binns & Salt, 1997), wholefield light stimuli (Gowan et al., 2008) covering both centre and surround are sub-optimal, whereas stimuli confined to the excitatory centre (Grasse et al., 1993) are considerably more effective. This raises the intriguing possibility that d-amphetamine depresses responses to stimuli which give relatively low levels of collicular activation and enhances responses to stimuli which give relatively high levels of activation, thus increasing the signal-to-noise ratio. This novel possibility was explored in the present study using an *in vitro* preparation in which visual afferents to the superficial layers were electrically stimulated at a range of intensities, providing low and high levels of SC activation. The evoked responses were exposed to therapeutically relevant doses of both d-amphetamine and methylphenidate.

Experimental Procedures

All procedures conducted were performed with both Institutional ethical approval and Home Office approval under the Animals (Scientific Procedures) Act (1986).

Slice Preparation: Juvenile male Wistar rats (Harlan Ltd., Bicester, UK), aged P20 to P35 (Andersen et al., 2008), were killed by decapitation under isoflurane anaesthesia (N=123). The brain was immediately placed in ice-cold 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) ringer containing (in mM): 120 NaCl, 5 KCl, 20 NaHCO₃, 6.7 HEPES acid, 3.3 HEPES salt, 2 CaCl₂, 2 MgSO₄, 1.2 KH₂PO₄ and 10 glucose, saturated with 95% O₂/5% CO₂. A coronal midbrain block was mounted onto a specimen plate with cyanoacrylate adhesive and placed in the slicing chamber submerged in HEPES ringer. 400µm coronal sections were prepared using a vibratome (VT1000S, Leica Microsystems Ltd., Milton Keynes, UK) and then

maintained at room temperature for one hour. Thirty minutes prior to recording, individual slices were transferred to a submersion recording chamber held at 34 °C, containing artificial cerebrospinal fluid (aCSF) circulating at 2 ml/min. The aCSF solution contained (in mM): 124 NaCl, 3.7 KCl, 26 NaHCO₃, 2.4 CaCl₂, 1.3 MgSO₄, 1.3 KH₂PO₄, and 10 glucose, saturated with 95% O₂/5% CO₂.

Extracellular recording: Extracellular glass recording electrodes, broken back to give an impedance of 3-10 MΩ, filled with aCSF and 2% Pontamine Sky Blue, were placed in the superficial grey layer of the SC, and field potential (fEPSP) responses were evoked with electrical stimulation of the afferent optic fibres in the optic tract with concentric bipolar stimulating electrodes (FHC, Bowdoinham, ME; Figure 1A). Stimulation was triggered using Signal software (Cambridge Electronic Design [CED], Cambridge, UK) and produced by an isolated output unit built in house.

Data acquisition and analysis: Data were acquired and digitized at 20 KHz using a micro1401 acquisition system (CED) and stored directly on a computer for offline analysis. Field potentials were low pass filtered (dc-1 KHz) and amplified with a gain of 1000 (using a commercial amplifier [NeuroData Inc., Selden, NY] coupled to second amplifier and filter built in house). Stimulus-response curves were completed at the start of every experiment to calculate the intensity of the stimulation chosen relative to the intensity giving the maximum response. Baseline recordings (at a stimulation frequency of 0.033 Hz) were then made at the chosen intensity and assessed for stability (less than 10% variation of response amplitude over averages of 5 pulses). Only when recordings were stable for at least 15 min were drugs applied. Stimulation continued throughout and drugs were present for at least 30 min prior to measurements being made. All drugs, once added, were continuously perfused for the

duration of the experiment. Only a single drug concentration (see below) and a single drug application was used per slice. For assessing the effects of drugs on responses, stimulation intensity and frequency were held constant, and averages of 15 min of stable recording (30 pulses) in the presence of the drug were compared to the stable 15 min pre-drug period. Response sizes were determined by measuring the amplitude between the pre-stimulation baseline and trough.

For paired pulse depression (PPD) experiments, an inter pulse interval of 500 ms was chosen, due to the extended duration of the response to each pulse and previous use of this inter pulse interval in other collicular work (Platt & Withington, 1997). Paired pulse ratios were obtained by dividing the amplitude of the response to the second pulse by that of the first. For the analysis, stimulus intensities were grouped into high ($\geq 70\%$ of the intensity required to elicit the maximal response), low (those elicited with a stimulation intensity $\leq 30\%$ of the maximum) and medium (40-60% of the maximum).

To quantify AMPA and NMDA receptor-mediated contributions to the elicited responses, responses in the presence of the NMDA receptor antagonist AP5 (10 μM) were subtracted from responses in the absence of AP5 to give the size of the NMDA component. The remaining response was then exposed to the AMPA receptor antagonist CNQX (10 μM) to allow calculation of the AMPA component. Statistical analyses were conducted using linear regression analysis, t-tests or one-way ANOVA and probabilities of $p < 0.05$ (two tailed) were considered to be significant.

Drugs: All experiments were conducted in the presence of the GABA_A receptor antagonist bicuculline methiodide (10 μ M) in line with previous research (Isa et al., 1998). The bicuculline was applied prior to any recordings and after the slice had at least 30 minutes to equilibrate in the recording chamber. A further 30 minutes elapsed in the presence of bicuculline before recordings were made. All drugs were dissolved in distilled water (except for metergoline, which was dissolved in DMSO), to make stock aliquots at 1000x final concentrations and stored at -20 °C until required. Methylphenidate hydrochloride and d-amphetamine sulphate were obtained from Sigma-Aldrich (Gillingham, UK). All other drugs were from Tocris Biosciences (Bristol, UK). Although our study used rat brain tissue, which is likely to be both pharmacokinetically and pharmacodynamically (with regard to receptor efficacies) different to human brain tissue, the doses of methylphenidate and d-amphetamine used were guided by those used therapeutically in humans. At doses administered therapeutically, plasma levels of methylphenidate can reach approximately 40 ng/ml (Greenhill et al., 2001) and d-amphetamine around 120 ng/ml (McGough et al., 2003; Ricaurte et al., 2005). Brain levels of these substances reach higher concentrations than those found in plasma. Indeed, brain concentrations can be around 8 times the plasma concentration (methylphenidate, Gal et al., 1977; d-amphetamine, Riffée et al., 1978). Given these considerations, we chose concentrations of 100 ng/ml (0.37 μ M; low), 200 ng/ml (0.74 μ M; medium) and 300 ng/ml (1.11 μ M; high) for methylphenidate and 300 ng/ml (1.63 μ M; low), 600 ng/ml (3.26 μ M; medium) and 900 ng/ml (4.89 μ M; high) for d-amphetamine.

Results

D-Amphetamine and methylphenidate alter SC responsiveness in a stimulation intensity-dependent fashion

The chosen stimulation intensities elicited a range of levels of SC activation: responses elicited by high intensities had significantly larger amplitudes than those elicited by low intensities (0.49 ± 0.03 vs 0.25 ± 0.04 mV respectively: ($t[101] = 5.12$, $p < 0.00001$). The effects of d-amphetamine and methylphenidate on these responses were correlated with the intensity of the eliciting stimulus – a substantial number of responses elicited by low intensities were reduced in amplitude whilst those elicited by higher intensities were largely unaffected (Figure 1B and C; Supplementary Figure 1). The relationship between stimulus intensity and drug effects was significant for all concentrations of d-amphetamine (900 ng/ml: $R^2 = 0.53$, $p < 0.001$; 600 ng/ml: $R^2 = 0.31$, $p < 0.05$; 300 ng/ml: $R^2 = 0.47$, $p < 0.001$) and methylphenidate (300 ng/ml: $R^2 = 0.35$, $p < 0.005$; 200 ng/ml: $R^2 = 0.28$, $p < 0.05$; 100 ng/ml: $R^2 = 0.41$, $p < 0.005$).

The effects of methylphenidate and d-amphetamine were mimicked by 5-HT and blocked by a 5-HT antagonist

The effects of d-amphetamine and methylphenidate on response amplitude were mimicked by the application of $1 \mu\text{M}$ 5-HT, which produced identical effects to the psychostimulants ($R^2 = 0.47$, $p < 0.005$; Figure 1D). In contrast, a higher concentration [$10 \mu\text{M}$] produced almost universal response suppression, but again this was more pronounced at low intensities ($R^2 = 0.73$, $p < 0.001$; Figure 1D). The role of 5-HT in the actions of d-amphetamine and methylphenidate was confirmed by the fact that their effects were completely abolished by prior application the 5-HT receptor antagonist metergoline ($10 \mu\text{M}$; Figure 2). In the presence of the antagonist, there was no

significant relationship between stimulation intensity and amplitude for either d-amphetamine ($R^2=0.09$, $p>0.05$) or methylphenidate ($R^2=0.12$, $p>0.05$).

The suppressive effects of d-amphetamine and methylphenidate are mediated pre-synaptically

We used paired pulse stimulation to determine whether application of the psychostimulants altered the probability of transmitter release and hence elicited effects through pre-synaptic actions. We found paired pulse depression (PPD) in the SC at all stimulus intensities under baseline conditions (high: $t[2]=18.92$, $p<0.01$; medium: $t[3]=3.03$, $p<0.05$; low: $t[2]=18.92$, $p<0.001$; Figure 3). In the presence of the two psychostimulants, PPD was significantly reduced at low intensities (d-amphetamine, $t[5]=3.10$, $p<0.05$; methylphenidate, $t[5]=3.61$, $p<0.05$), whilst unaffected at higher intensities (Figure 3), suggesting that response suppression at low intensities is caused, at least in part, by a reduction in release probability.

To assess whether there was an additional post-synaptic component to the effects of d-amphetamine and methylphenidate on collicular responses, we examined the effects of these drugs on receptor-mediated subcomponents of the synaptic response, the contribution of which may differentially change if post-synaptic changes occur at the receptor level. Stimulation-induced SC responses are mediated by excitatory amino acids, with α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor activation dominating at high intensities and N-methyl-D-aspartate (NMDA) receptor activation at low (Figure 4). D-amphetamine and methylphenidate had no significant effect on the AMPA:NMDA ratio at low intensities (Figure 4B; AMPA:NMDA ratio pre-drug 0.48 ± 0.13 ; d-amphetamine 0.65 ± 0.07 , $t[4]=1.66$, $p>0.05$; methylphenidate

0.84±0.22, $t[3]=1.21$, $p>0.05$), suggesting that post-synaptic changes at the receptor level had not taken place and that 5-HT mediated suppression of responses at low stimulus intensities was entirely due to pre-synaptic effects. However, at high intensities, in spite of a lack of change in release probability (see above), both drugs significantly reduced the AMPA:NMDA ratio (Figure 4B; AMPA:NMDA ratio pre-drug 13.2±1.7; d-amphetamine 0.9±0.09, $t[3]=7.06$, $p<0.05$; methylphenidate 1.1±0.4, $t[3]=6.82$, $p<0.05$). The implied post-synaptic changes appear largely without consequence in our paradigm.

Discussion

D-amphetamine and methylphenidate increased the signal-to-noise ratio in the SC by differentially affecting the impact of weak and strong activations: suppressing the former and retaining the latter. The effects of d-amphetamine and methylphenidate on collicular responses could be abolished by a 5-HT antagonist and mimicked by application of a low concentration of 5-HT itself. Mediation by 5-HT is perhaps not surprising given the known pharmacology of psychostimulants and monoaminergic innervation of the SC. That is to say, it is widely accepted that d-amphetamine and methylphenidate act to increase synaptic levels of the monoamines dopamine, noradrenaline and 5-HT (Azzaro et al., 1974; Holmes & Rutledge, 1976; Kuczenski & Segal, 1989; Easton et al., 2007). There is extensive serotonergic innervation of the rat SC (Parent et al., 1981), compared with lesser dopaminergic or noradrenergic projections (Weller et al., 1987). This serotonergic innervation also preferentially targets superficial layers (Parent et al., 1981), meaning that 5-HT is the preeminent monoamine in the superficial layers of the SC.

Whilst there are no previous reports of 5-HT changing the signal-to-noise ratio in the SC, there is evidence of monoamines fulfilling that role elsewhere in the brain, the classic example being dopamine-induced increases in signal-to-noise ratio in the striatum and frontal cortex (Rolls et al., 1984; Kiyatkin & Rebec, 1996). 5-HT itself has also been shown to affect the signal-to-noise ratio, causing a reduction in the ratio in the somatosensory cortex (Waterhouse et al., 1986) and thalamus (Funke & Eysel, 1993). However, previously reported examples of monoamine-mediated changes in the ratio, including those caused by 5-HT, have all arisen because of suppression of spontaneous background activity, producing a net increase in signal size. We however report a change in ratio due to an entirely novel mechanism: a change in the relationship between weak and strong signals (rather than signal and background), suppressing weak signals and retaining strong signals.

The means by which a single neurotransmitter (5-HT) could produce differential effects on visual signals in the SC was uncertain; hence, we explored the mechanism further with a particular focus on whether the effects of 5-HT were pre- or post-synaptically mediated. To achieve this, we examined the impact of d-amphetamine and methylphenidate on the AMPA and NMDA receptor-mediated components of the SC responses and collicular responses to paired pulses. In line with previous research (Platt & Withington, 1997), we found that paired pulse stimulation of the visual input to the SC resulted in depression of the response to the second pulse (PPD), indicating a decrease in transmitter release to this pulse. Under baseline conditions, this PPD was far greater in response to paired pulses of low intensity stimulation in contrast to those of medium and high intensity. Given the depressed response to the second pulse is normally associated with a lack of easily releasable transmitter following the

response to the initial pulse, it is likely that stimulating at medium or high intensities has the effect of mobilising some of the harder to release stores of transmitter (Zucker, 1989), thus overcoming the depressed response to the second pulse.

If the psychostimulant-induced suppression of responses found at low intensities was due to pre-synaptic changes, it would be expected that PPD would be reduced in the presence of the drugs, indicating a decrease in transmitter release to the primary pulse (Rice & Cragg, 2004). This was indeed found to be the case, suggesting that the suppressive effects of d-amphetamine and methylphenidate to low stimulation intensities are at least partly mediated pre-synaptically. This conclusion is supported by the presence of pre-synaptic 5-HT receptors on optic afferents in the SC (Segu et al., 1986; Mooney et al., 1996), activation of which results in a decrease in release (Mooney et al., 1996).

Further results suggest that the suppressive effects of d-amphetamine and methylphenidate to low stimulation intensities are exclusively mediated pre-synaptically. Examining the effects of these drugs on receptor-mediated subcomponents of the synaptic response, we found that at low stimulation intensities, d-amphetamine and methylphenidate did not change the AMPD:NMDA ratio, suggesting that post-synaptic changes at the receptor level had not taken place and that 5-HT mediated suppression of responses at low stimulus intensities was entirely due to pre-synaptic effects. However, at high intensities, in spite of a lack of change in release probability (as revealed by the PPD work), both drugs significantly reduced the AMPA:NMDA ratio. This augmentation of the NMDA receptor-mediated component of the response appears to be a novel action for 5-HT, which suppresses

NMDA receptor-mediated responses elsewhere in the central nervous system (Marcoli et al., 1997; Liang et al., 1998). Although without consequence in our paradigm, it is possible that these post-synaptic excitatory amino acid receptor changes may produce response enhancements in other paradigms where relatively high levels of collicular activation are achieved, and may potentially underlie the amphetamine-related enhancements in collicular visual processing seen by Grasse et al. (1993; see Introduction).

At a behavioural level, the effect of d-amphetamine and methylphenidate on the signal-to-noise ratio in the SC could explain how these drugs improve sustained attention (Mackworth, 1965; Elliott et al., 1997; Bizarro et al., 2004) and improve distractibility (Halliday et al., 1990; Brown & Cooke, 1994; Agmo et al., 1997; Spencer et al., 2001; Faraone et al., 2007). The SC is widely acknowledged to be important for the generation of saccades (Sparks, 1999), with superficial layer neurons responding more vigorously to a stimulus if it is the target for a saccade (Goldberg & Wurtz, 1972). In terms of recent conceptualisations of the manner in which bids for motor expression, by systems (like the colliculus) capable of specifying actions, are processed by the brain (Redgrave et al., 1999; Gurney et al., 2001), enhanced activity can be seen as putting a stronger ‘bid’ into the central selection device, hypothesised to be the basal ganglia. In the case of the superficial layers of the SC, this can occur either via direct ascending projections to the thalamus 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 also project to the thalamus (McHaffie et al., 2005). A stronger bid is more likely to win against competitors and generate a motor output (enabled through a reduction in tonic inhibition of the SC deep layers

arising from basal ganglia output structures, see Chevalier & Deniau, 1990) and hence an eye movement. By enhancing SC responses, the likelihood of eye movements could be increased. Conversely, by depressing responses in the SC, the likelihood of eye movements would be reduced. By effectively reducing responsiveness - bids - by weak stimuli, psychostimulants have the effect of biasing the system so that distractions only occur to particularly salient stimuli, thus leading to a reduction in overall distractibility and a correlative improvement in sustained attention.

The results of the present study not only provide crucial insights into the mechanism by which d-amphetamine and methylphenidate decrease distractibility and improve sustained attention in normal subjects but also suggest that these drugs may at least in part act in the SC to the same effect in ADHD: a suggestion which is in line with evidence reviewed elsewhere (Overton, 2008) that the SC is dysfunctional in ADHD. Consistent with the possibility that the colliculus may be dysfunctional in the disorder, and that 5-HT induced modulation of the SC is therapeutically important, 5-HT selective drugs, such as fluoxetine, appear to have therapeutic efficacy in ADHD (Barrickman et al., 1991; Gibson et al., 2006). Furthermore, ADHD patients have been reported to show alterations in 5-HT metabolism (Hoshino et al., 1985). If psychostimulants do achieve their therapeutic effects on distractibility by an action at the level of the SC, then the development of drugs which dampen stimulus-related activity in this region could represent an important novel path for the development non-addictive pharmacotherapies for ADHD.

Acknowledgements

This work was funded as part of the James Martin 21st Century School at Oxford University. EJD is a fellow of the James Martin Institute for the Future of the Mind and a Junior Research Fellow at Somerville College. The authors would like to thank Dr Ian Devonshire for assistance in data analysis.

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Figure Legends

Figure One: (A) Experimental paradigm showing stimulating (Stim) and recording (Rec) electrode positions (OT: optic tract, SGS, stratum griseum superficiale, SO stratum opticum, SGI, stratum griseum intermediale). (B) The effects of d-amphetamine (900 ng/ml) and (C) methylphenidate (300 ng/ml) on collicular responses varied with stimulus intensity. Points below the dotted line indicate a decrease in response and points above indicate an increase. Insert images show typical traces for low and high stimulation intensities in the presence (blue=amphetamine, red=methylphenidate) and absence (black) of the psychostimulants. Similar effects

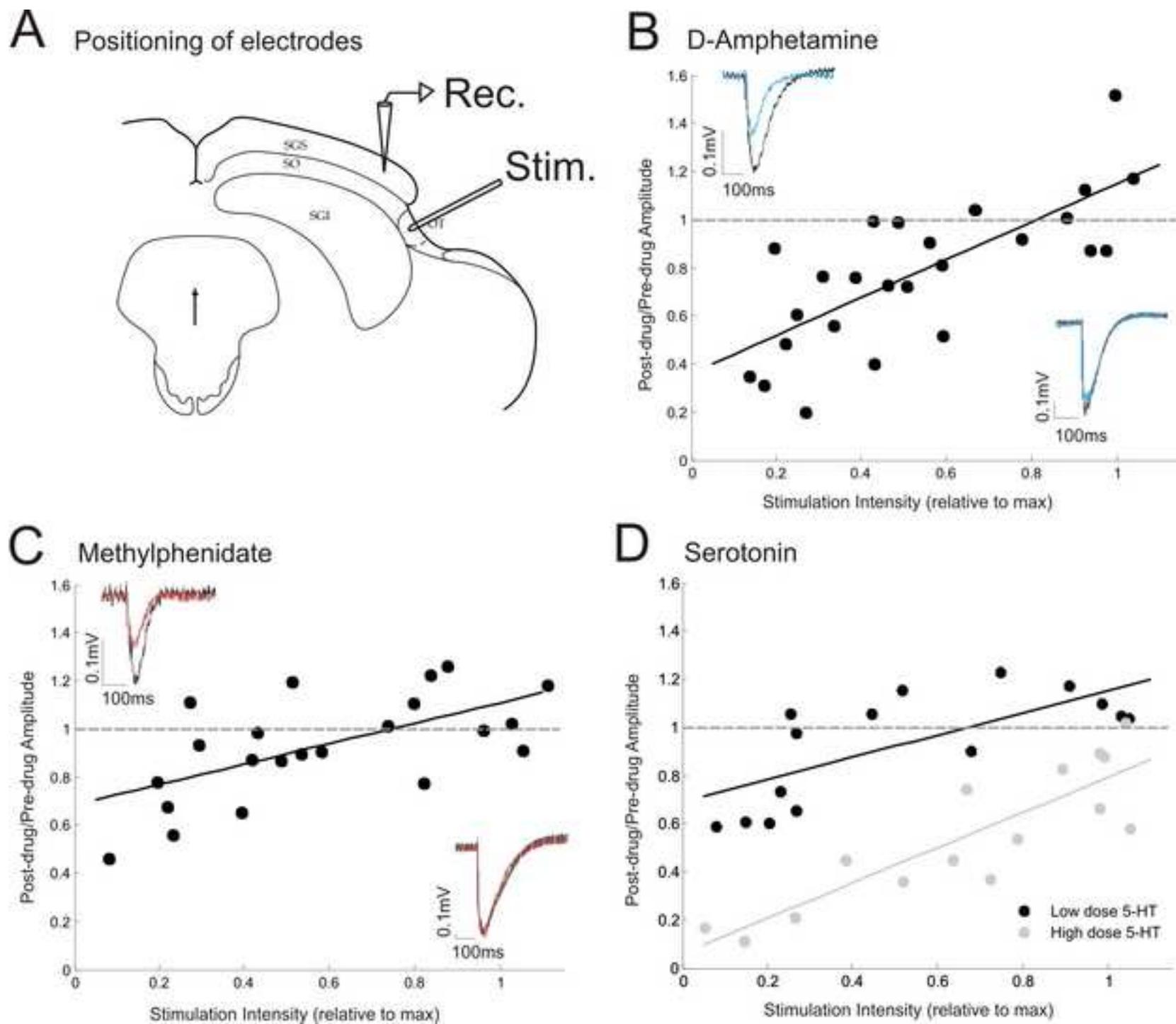
were found with a low concentration (dose) of 5-HT (1 μ M), but a high concentration of 5-HT (10 μ M) was universally depressant (D).

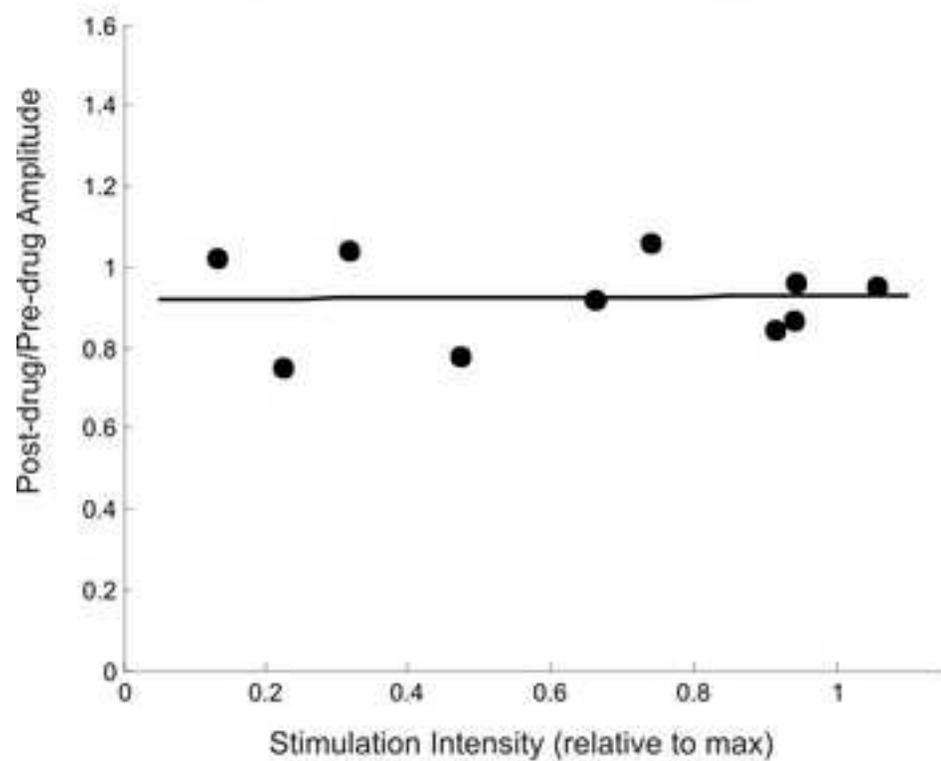
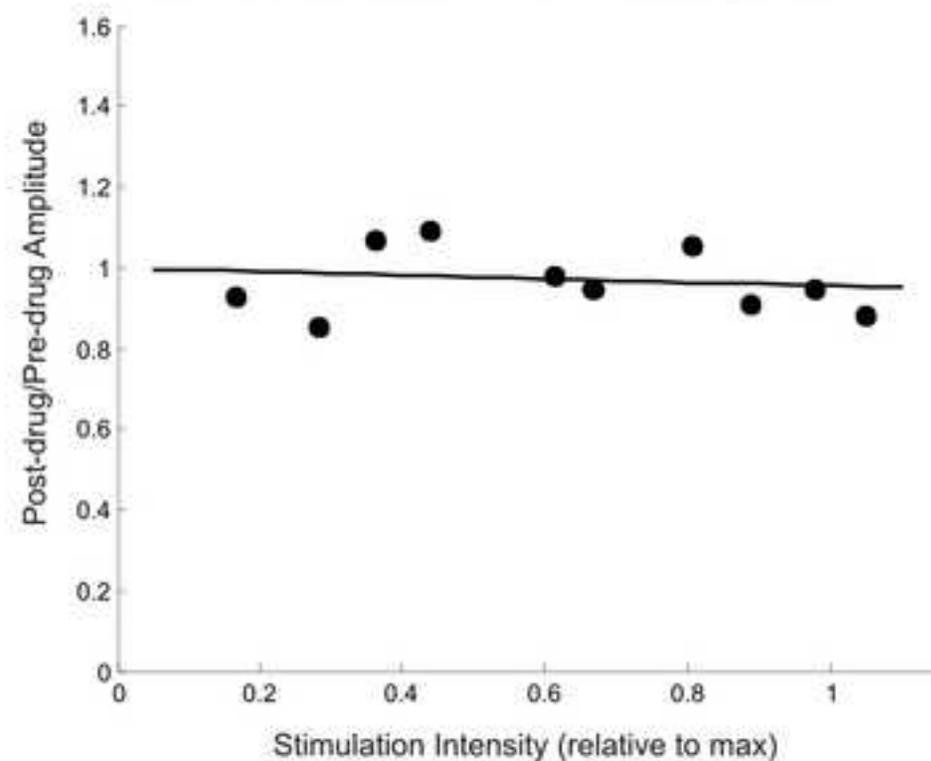
Figure Two: In the presence of the 5-HT receptor antagonist metergoline (10 μ M), neither d-amphetamine (900ng/ml; A) nor methylphenidate (300ng/ml; B) had any effect on collicular responses elicited by optic tract stimulation.

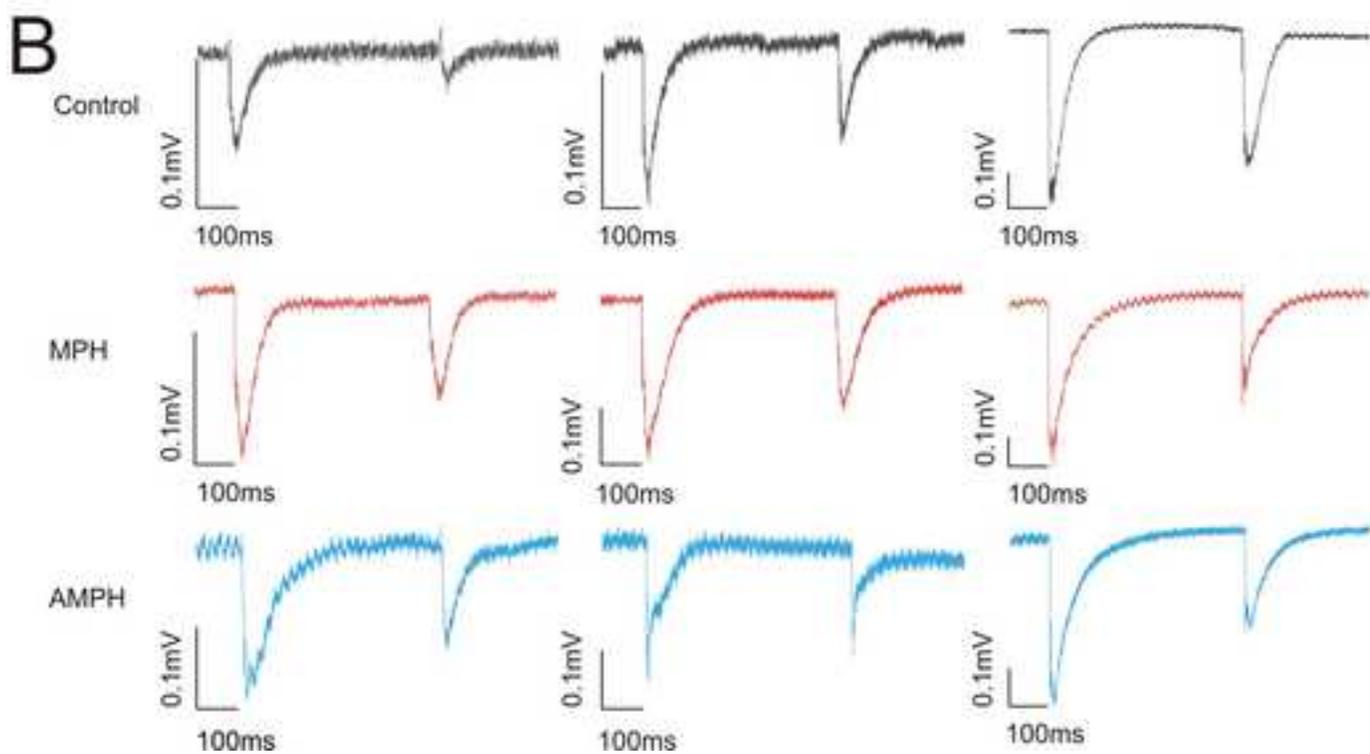
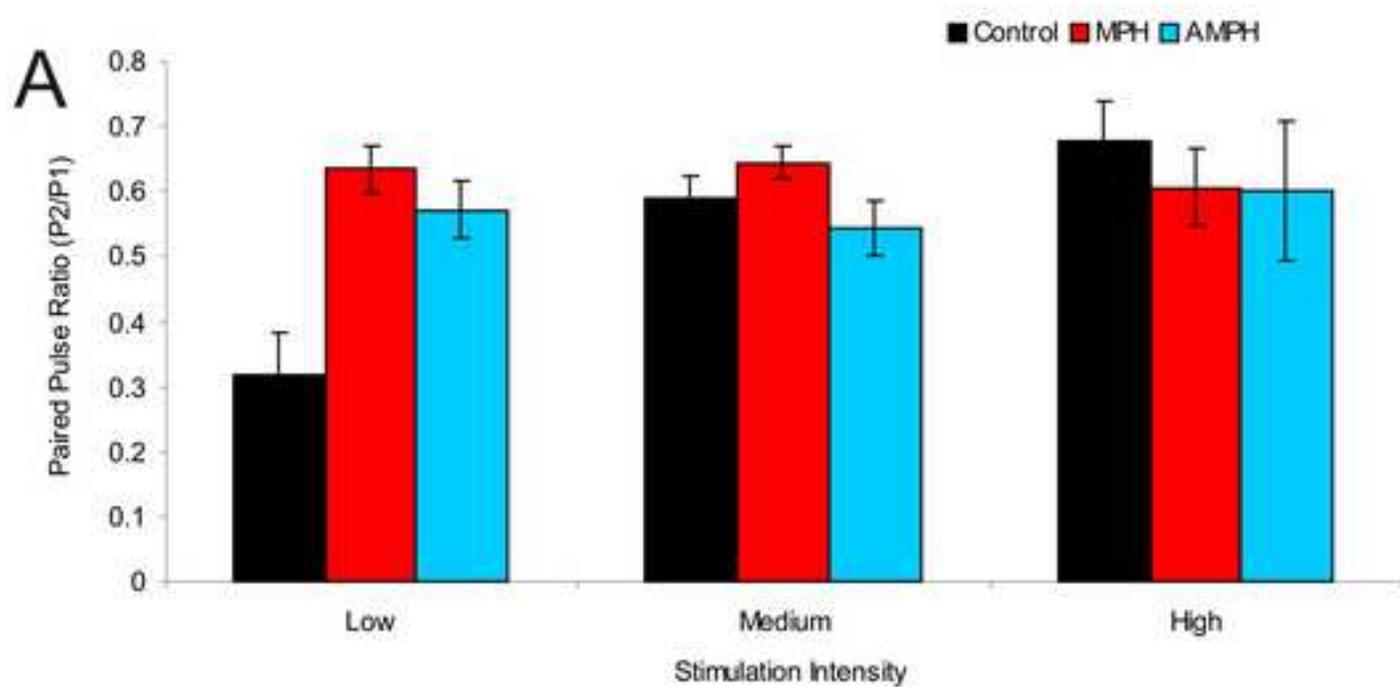
Figure Three (A) The paired pulse depression (PPD) evident in the SC at baseline was suppressed by methylphenidate (MPH; 300 ng/ml) and d-amphetamine (AMPH; 900 ng/ml) at low stimulation intensities only: * $p < 0.05$ vs MPH and AMPH. (B) Typical traces showing PPD for each of the three stimulation intensities and drug conditions.

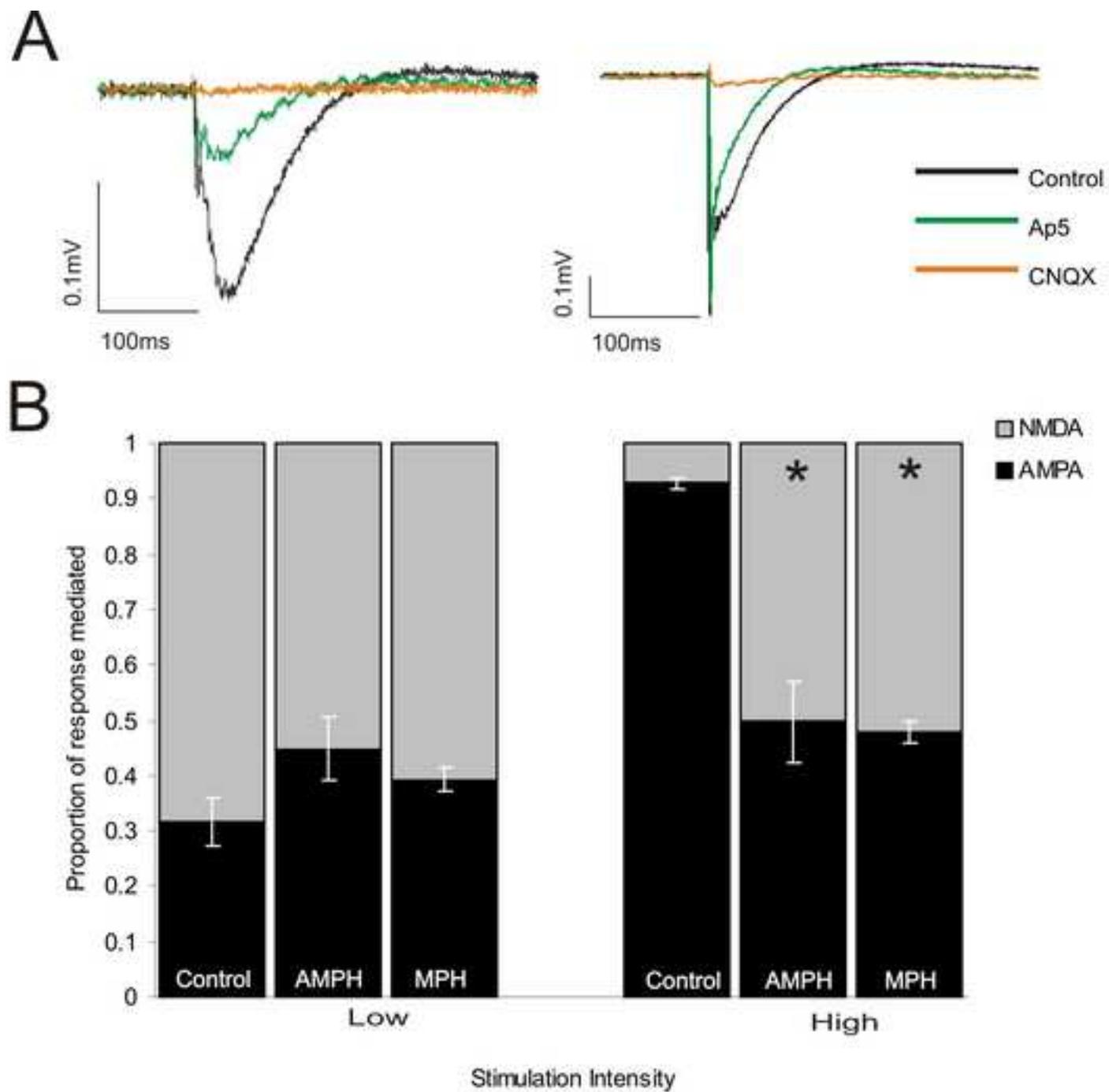
Figure Four (A) shows typical traces in response to high and low intensity stimulation during control (baseline) conditions and in the presence of an NMDA receptor antagonist (AP5, 10 μ M) or an AMPA receptor antagonist (CNQX, 10 μ M). (B) In the presence of methylphenidate (MPH; 300 ng/ml) and d-amphetamine (AMPH; 900 ng/ml), the NMDA receptor-mediated component of the high intensity responses was significantly increased: * $p < 0.05$ vs Control.

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A D-Amphetamine + 5-HT antagonist**B** Methylphenidate + 5-HT antagonist



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Supplementary Figure One: As with higher concentrations, lower concentrations of both d-amphetamine (300ng/ml, A; 600ng/ml; B) and methylphenidate (100ng/ml, C; 200ng/ml, D) also altered collicular responses in a manner which was dependent on the stimulus intensity.

