Characterisation of methylphenidate-induced excitation in midbrain dopamine neurons, an electrophysiological study in the rat brain.

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Abstract: Methylphenidate (MPH) is a drug routinely used for patients with attention deficit and hyperactivity disorder (ADHD). Concerns arise about psychostimulant use, with dramatic increases in prescriptions. Besides, antipsychotic drugs are often administered in combination with MPH. In this study, we examine the consequences of MPH exposure in combination with dopamine D_2 receptor antagonism (eticlopride) on midbrain dopaminergic neurons in anaesthetised rodents, using in vivo extracellular single-cell electrophysiology. As expected, we show that methylphenidate (2 mg/kg, i.v.) decreases the firing and bursting activities of ventral tegmental area (VTA) dopamine neurons, an effect that is reversed with eticlopride (0.2 mg/kg, i.v.). However, using such a paradigm, we observed higher firing and bursting activities than under baseline conditions. Furthermore, we demonstrate that such an effect is dependent on dual alpha-1 and dopamine D_1 receptors, as well as glutamatergic transmission, through glutamate N-Methyl-D-aspartate (NMDA) receptor activation. Chronic MPH treatment during adolescence greatly dampens MPH-induced excitatory effects measured at adulthood. To conclude, we demonstrated here that a combination of methylphenidate and a dopamine D_2 receptor antagonist produced long-lasting consequences on midbrain dopamine neurons, via glutamatergic-dependent mechanisms.
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Abstract

Methylphenidate (MPH) is a drug routinely used for patients with attention deficit and hyperactivity disorder (ADHD). Concerns arise about psychostimulant use, with dramatic increases in prescriptions. Besides, antipsychotic drugs are often administered in combination with MPH. In this study, we examine the consequences of MPH exposure in combination with dopamine D₂ receptor antagonism (eticlopride) on midbrain dopaminergic neurons in anaesthetised rodents, using in vivo extracellular single-cell electrophysiology. As expected, we show that methylphenidate (2 mg/kg, i.v.) decreases the firing and bursting activities of ventral tegmental area (VTA) dopamine neurons, an effect that is reversed with eticlopride (0.2 mg/kg, i.v.). However, using such a paradigm, we observed higher firing and bursting activities than under baseline conditions. Furthermore, we demonstrate that such an effect is dependent on dual alpha-1 and dopamine D₁ receptors, as well as glutamatergic transmission, through glutamate N-Methyl-D-aspartate (NMDA) receptor activation. Chronic MPH treatment during adolescence greatly dampens MPH-induced excitatory effects measured at adulthood. To conclude, we demonstrated here that a combination of methylphenidate and a dopamine D₂ receptor antagonist produced long-lasting consequences on midbrain dopamine neurons, via glutamatergic-dependent mechanisms.
1 – Introduction

Methylphenidate (MPH) is the most prescribed psychostimulant for the treatment of attention deficit and hyperactivity disorder (ADHD) (Whitt et al., 2017). It is also occasionally prescribed in augmentation therapies in severely major depressed and bipolar patients (Adida and Azorin, 2014). The primary pharmacological effect of MPH is to increase central dopamine and norepinephrine neurotransmission, which both control executive and attentional function (Borodovitsyna et al., 2017; Del Campo et al., 2011; Engert and Pruessner, 2008). MPH interferes strongly with the synaptic reuptake of dopamine and norepinephrine (Ki=46–340 and 238–427 nM, for dopamine and norepinephrine transporters, respectively) and display weak affinity on other known receptors/transporters (Easton et al., 2007). This leads to robust increase in synaptic catecholamine level, which impact both presynaptic and postsynaptic receptors in dopamine/noradrenaline-rich regions. MPH is known to indirectly cause the activation of dopamine D2/D3 auto-receptors located in the cell body of dopamine neurons, inducing a large decrease in dopamine neuron firing activity of ventral tegmental area (VTA) dopamine neurons (De la Peña et al., 2018; Di Miceli et al., 2018; Shi et al., 2004; Shi et al., 2000). The consequence of this negative feedback is not really well understood. It has been proposed that low doses of MPH may reduce locomotor activity in ADHD patients through a similar mechanism of action (Fan and Hess, 2007). Recent data shows dopamine D2/D3 receptors-induced inhibition of firing activity is moderately but persistently lessened in rats that were chronically treated with MPH during adolescence (Di Miceli et al., 2018).

Another study from our laboratory performed in rodents also showed that acute MPH administration (3 mg/kg, iv) impacts the glutamatergic system by increasing N-methyl-D-aspartate (NMDA)-induced neurotransmission in the prefrontal cortex, partially through
dopamine D1 receptors (Di Miceli and Gronier, 2015). Therefore, strong interaction between MPH and glutamate neurotransmission may participate to the therapeutic effects and mechanism of action of MPH.

Growing evidence now shows that a significant number of patients are treated with psychostimulants concomitantly with antipsychotic medications (Olfson et al., 2006). It has been estimated that more than half of children on stimulants have been prescribed a concurrent antipsychotic at some point in their treatment (Scholle et al., 2018). Such combination, which may have disputable clinical consequence, is supported by the important co-morbidity between ADHD and other disorders commonly treated with antipsychotics such as bipolar disorders, Tourette’s syndrome and also off-labelled uses to tackle aggressiveness (Yanofski, 2010). Adverse effects are reported with the combined pharmacotherapy (Millichap and Yee, 2016; Ogutlu et al., 2018). Adverse effects were also reported when patients are switching from risperidone to MPH, producing ADHD-like symptoms (Hollis and Thompson, 2007; Sabuncuoglu, 2007).

Recent evidence, including from our laboratory, suggests that psychostimulants can have an excitatory effect on dopamine neurons which is unmasked when the feedback inhibition is abolished by a D2-like receptor antagonist (Dela Peña et al., 2018; Shi et al., 2004; Shi et al., 2000; Shi et al., 2000). These studies identified adrenergic alpha-1 receptors as the driving mechanism (Dela Peña et al., 2018; Shi et al., 2000), together with the mesocortical circuitry (Dela Peña et al., 2018). However, these studies found a limited involvement of dopamine D1 receptors (Shi et al., 2000).
The present study was undertaken to better identify the mechanisms underlying this interesting circuitry in order to better evaluate its clinical relevance, knowing that MPH treatment is often combined with antipsychotics, all of which are, to some extent, dopamine D_{2}/D_{3} receptor antagonists. Using \textit{in vivo} extracellular single cell electrophysiology, we have examined the potential excitatory effect of MPH exposure combined with a selective dopamine D_{2} receptor antagonist (eticlopride) on the electrical activities of midbrain dopamine neurons by focusing on:

1) The possible receptors involved
2) The possible involvement of a glutamatergic tone
3) To what extent this effect can be mimicked by other non-stimulant drugs acting more selectively on dopamine or noradrenaline neurotransmission
4) The potential modulation by a chronic methylphenidate treatment during adolescence.

2 – Material and Methods

2.1 Animals
Male Sprague-Dawley rats were purchased from Charles River, UK. Naive animals were used at the beginning of the study. Animals were housed in groups of 2-4 per cage, maintained at 20-22°C with humidity rates above 40% under a 12:12 L/D cycle with lights ON at 07h00 AM. Food and water were provided \textit{ad libitum}. Animals were allowed a minimum of 3 days for acclimatisation. All experiments were performed during the light phase and with permission from both the UK Home Office and De Montfort University Ethics Committee.
2.2 Chronic MPH treatment

Chronic MPH treatment (5 mg/kg/day) was administered per os to early adolescent rats (PND 28). The treatment consisted in two daily doses of 2.5 mg/kg of MPH during 15 consecutive days. Each day, MPH was freshly dissolved into sucrose solutions (10% w/v in drinking water, 2 ml/kg) and given by hand to animals. Control animals therefore received 10% sucrose solutions (without MPH), twice a day at 2 ml/kg. Such a protocol was adapted from a previous study in rats (Atcha et al., 2010). Treatment was initiated following three days of training, where animals learnt to readily drink sucrose solutions (without MPH) from a syringe held by experimenters. Treatment was terminated after 15 consecutive days and was followed by 4-6 weeks of washout.

2.3 Single cell extracellular electrophysiology

Animals were initially deeply anaesthetized with chloral hydrate (400 mg/kg, intraperitoneal, with additional doses administered if necessary), secured to a stereotaxic frame and maintained at 36–37 °C with a heating pad. A catheter was inserted into the lateral tail vein to perform systemic drug administration. An incision was made across the top of the head and the edges of the skin drawn back to reveal the cranium. Bregma was identified and a hole was drilled through the bone at the coordinates of the ventral tegmental area (VTA), according to the atlas of Paxinos and Watson (Paxinos and Watson, 1997). Electrodes were manufactured in-house from borosilicate capillaries (1.5 mm, Harvard Apparatus Ltd., UK), pulled on a PP-830 vertical electrode puller (Narishige, Japan) and filled by hand with an electrolyte solution of NaCl 147 mM.

For microiontophoresis, multibarrel pipettes were filled with either: kynurenic acid (20 mM), dopamine (20 mM), MPH (20 mM), norepinephrine (20 mM), all dissolved into NaCl 147 mM, or a combination of the above and NaCl 2 M for current balancing. The central recording
channel contained NaCl at 147 mM. MPH, dopamine and norepinephrine were ejected as cations by applying positive currents at +10 nA while kynurenic acid was ejected as an anion with -10 nA currents. All drugs were retained by applying low intensity currents (2 to 4 nA) of opposite charge to the ejection currents. Electrode tips were broken down under an optical light microscope to an external diameter of 1-1.5 μm. Typical electrode resistances were in the range 4-8 MΩ. Outputs from the electrode were sent to a Neurolog AC pre-amplifier and amplifier (Digitimer, UK). If necessary, signal amplification was manually adjusted to record whole neuronal action potential amplitudes. Signals were filtered and sent to an audio amplifier, a Tektronix 2201 digital storage oscilloscope and a 1401 interface connected to a computer running Spike 2 (CED, UK) for data capture and analysis. Descent of the electrode was carried out using a hydraulic micromanipulator (Narishige, MO-103, Japan).

Putative midbrain dopamine neurons were identified according to the electrophysiological criteria summarised by Ungless and Grace in 2012 (Ungless and Grace, 2012). Only presumed dopaminergic neurons were selected in our study (Chenu et al., 2013; Grace and Bunney, 1984, 1983; Ungless et al., 2004; Valenti and Grace, 2009). These neurons displayed the following typical characteristics:

- a triphasic action potential lasting 2.5–3.5 ms often with a notch in the rising phase,
- a prominent negative compound and a time greater than 1 ms from the start of the depolarisation to the end of the repolarisation,
- a relatively low firing activity between 5–90 spikes/10 s with occasional bursting pattern.
A burst activity in these neurons was defined as at least two spikes occurring within 80 ms or less, followed by a silence period of at least 160 ms (Grace and Bunney, 1984; Overton and Clark, 1997; Paladini and Roeper, 2014).

2.4 Prefrontal cortex inhibition

Adapted from a previous study in rhesus monkeys (Tehovnik and Sommer, 1997), local prefrontal cortex inhibition was achieved by locally perfusing lidocaine (2% w/v in saline, pH 7) at a rate of 2 µl/min immediately prior to intravenous MPH challenge. Two and a half microliters of total volume was injected at each injection site. Four locations were chosen per hemisphere at the following coordinates (in mm from Bregma): anteroposterior/lateral/dorsoventral: 3.2/1/1.5, 3.2/1/3, 2/1/1.5 and 2/1/3. Following each lidocaine injections, 1 minute of diffusion was allowed before removing the Hamilton syringe. Electrophysiological recordings started typically 10-15 min following the injection procedure. Sham experiments (using vehicle) were not performed here, since such a procedure could have induced by itself some tissue damage.

2.5 Drugs

All drugs were purchased from Sigma (Sigma-Aldrich, UK) except for atomoxetine (Sequoia Research, UK) and SCH23390 (Tocris, UK). All drugs were dissolved into saline (NaCl 147 mM).

2.6 Data analysis

All data are expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed using paired or unpaired Student’s t-tests or one/two-way analysis of variance (ANOVA), followed by Neuman–Keuls (one-way ANOVA) or Bonferroni tests (two-way
ANOVA), as appropriate. Probabilities smaller than 0.05 were considered significant (p<0.05). n values refer to the number of neurons tested. Only one neuron per animal was investigated in cases of intravenous drug administrations.

The mean basal firing activity was evaluated after neurons achieved stable firing rates, generally after 2-5 min of recording. Pre-drug values of firing rate were obtained by averaging the firing rate over a period of at least 4 min immediately prior to the intravenous administration while post-drug values were obtained by averaging the firing over a period of 5 min following drug administration. Burst are reported as the % of all spikes in burst events.

**Supplementary Table 1** summarises all the statistical tests performed in the present study, together with post-hoc results.

### 3 – Results

The animals used in the present study were either completely naïve animals and control animals (treated with vehicle) that were used in the chronic treatment studies (see last part of the results section). The two groups of animals presented similar characteristics for their electrophysiological responses and were therefore pooled together.

#### 3.1 Hidden excitatory effects on dopamine neurons by methylphenidate

Methylphenidate (MPH), injected intravenously at 2 mg/kg, significantly (p<0.001, Tukey’s post hoc test after significant repeated measures one-way ANOVA, F(2,26)=31.97, p<0.001) reduced the firing of VTA dopamine neurons (**Fig. 1A**). All bursting activities observed in basal conditions were completely suppressed following MPH administration (**Fig. 1B**). The dopamine D₂ receptor antagonist eticlopride (0.2 mg/kg) not only rescued both firing and bursting activities, but significantly induced a further activation of firing (64%, p<0.001, **Fig.**
1A) and burst activity (167%, p<0.05, Fig. 1B) over baseline levels. Twelve out of 14 neurons tested with the combination of 2 mg/kg of MPH and 0.2 mg/kg of eticlopride (MPH/eti) increased their firing by more than 40% (40-236%) from baseline. Burst activity was increased by an average of 122% in the neurons that initially displayed some burst firing (n=5), while 6 out of 9 neurons that were initially in a single-spike firing mode transitioned into burst firing neurons once administered with the combination of MPH/eti. The remaining three neurons presented no burst activities at baseline and after the combination MPH/eti.

Besides, in neurons that were not completely silenced by MPH (n=9, firing rates > 0 following MPH exposure), we observed a strong correlation (r²=0.958) between initial response to MPH (% of inhibition from baseline firing) and later response to eticlopride (% of change following eticlopride) (Supplemental Figure S1).

In experiments where administration of eticlopride was performed first, followed by MPH, overshoot of firing rates and burst activities were observed (Fig. 2A-C, one-way ANOVA: [firing] F(1.139,7.972)=35.96, p<0.001, [burst] F(1.089,7.62)=15.52, p<0.01). Alone, neither eticlopride nor saline administration did affect the electrical activities of midbrain dopamine neurons (Fig. 2D-F, one-way ANOVA: [firing] F(1.313,13.13)=0.369, p=0.61, [burst] F(1.672,16.72)=0.05, p=0.93).

Such an excitatory effect was not observed when the selective dopamine D₂ receptor agonists quinpirole (20 µg/kg) or apomorphine (40 µg/kg) were administered instead of methylphenidate (Fig. 3), where firing and burst activity return to initial baseline level after eticlopride treatment (non-significant post-hoc tests after repeated measures one-way ANOVA: [firing] F(1.313,6.567)=17.79, p<0.01, [burst] F(1.621,8.106)=10.71, p<0.01), as observed previously (Eddine et al., 2015; Zhang et al., 1992). Interestingly, some neurons (2/6) presented firing activity reaching values within 8-12 Hz range and burst activity reaching 95% of all spikes in
burst, while they were previously silenced by dopamine agonists (Fig. 3D). In some cases, delayed responses are observed, as shown on Figure 3D.

These results confirm previous observations that the combination of MPH and D2 receptor antagonist (such as eticlopride) produce overshoot of firing rate and burst activity (Dela Peña et al., 2018; Shi et al., 2000).

3.2 Effects of other selective catecholamine uptake inhibitors

In this series of experiments, we have tested, in eticlopride pre-treated animals, the effects of the very selective dopamine reuptake inhibitor GBR12909 (vanorexine) and the noradrenaline reuptake inhibitor atomoxetine, alone and in combination. In contrast to what was previously observed regarding MPH/eti, cumulative administrations of the non-stimulant ADHD drug atomoxetine (4-5 mg/kg) did not change the firing (t(5)=0.64, p=0.55) and burst activities (t(5)=0.41, p=0.69) of dopaminergic neurons (Fig. 4A-B) in eticlopride-treated animals, but also in naive animals (Supplemental Figure S2). Similarly, when GBR12909 (2 mg/kg) was administered in eticlopride-treated animal (Fig. 4C-D), only non-significant effects were observed. A subsequent administration of atomoxetine following GBR-12909 did not induce any significant effect either (Fig. 4C-D). [firing] F(1.296,6.479)=0.573, p=0.52, [burst] F(1.378,6.888)=0.239, p=0.714). These results demonstrate the specificity of MPH to induce excitatory effects after dopamine D2 receptor antagonism.

3.3 The excitatory component of MPH depends on both D1 and α1 receptors

Pre-administration of the α1 receptor antagonist prazosin (1.5 mg/kg) did not prevent MPH/eti excitatory effects (Fig. 5A-C). [firing] t(6)=3.198, p<0.05, [burst] t(6)=3.092, p<0.05). Similarly, pre-administration of the dopamine D1 receptor antagonist SCH23390 (0.6 mg/kg)
did not prevent MPH/eti excitatory effects (Fig. 5D-F, [firing] t(5)=2.743, p<0.05, [burst] t(5)=1.738, p=0.14). However, if neurons were pre-treated with SCH23390, the excitatory response induced by the combination MPH/eti faded progressively following the administration of prazosin (Fig 5F), showing that D₁ and α₁ receptors could be both required for the excitatory effect of MPH/eti. To further investigate such an observation, we found that the excitatory effect of MPH/eti was completely prevented in all neurons tested (7/7, p<0.01 Fisher’s exact test) when combined administrations of both prazosin (1.5 mg/kg) and SCH23390 (0.6 mg/kg) were performed prior to MPH/eti challenge (Fig 5G-I, [firing] t(6)=0.679, p=0.52, [burst] t(6)=1.631, p=0.15). On their own, prazosin (Fig. 5J-K) or SCH23390 (Fig. 5L-M) had only minimal effects on the electrophysiological properties of VTA dopaminergic neurons ([prazosin, firing] F(1.829,9.143)=0.438, p=0.64, [prazosin, burst] F(2.387,11.94)=1.036, p=0.39, [SCH23390, firing] F(1.435,8.61)=9.264, p=0.01, [SCH23390, burst] F(1.824,10.94=0.084, p=0.90).

3.4 Role of glutamatergic neurotransmission in MPH-induced excitatory effects

We then examined whether MPH/eti-induced excitatory effects could be related to glutamate receptor activation, since we previously demonstrated a role of glutamatergic NMDA receptors in the neuronal responses to psychostimulants in the prefrontal cortex (Di Miceli and Gronier, 2015). Intravenous administration of the selective NMDA receptor antagonist MK801 (0.5 mg/kg) completely prevented any activation of firing or burst activity (Fig. 6A-B-C, non-significant post-hoc tests after repeated measures one-way ANOVA: [firing] F(1.593,6.373)=21.99, p<0.01, [burst] F(1.056,4.222)=3.082, p=0.15) following MPH and D₂ receptor antagonism. At this dose of 0.5 mg/kg, MK801 administration did not alter baseline firing (F(1.038,3.114)=1.06, p=0.38) or burst activities (F(1.17,3.509)=0.151, p=0.76) of midbrain dopamine neurons (Fig. 6D-E).
3.5 Involvement of local glutamate and catecholamine receptors

These results prompted us to examine the possible involvement of local glutamatergic and catecholamine receptors on the MPH-mediated activation of midbrain dopamine neurons. Local application of MPH at 10 nA, via microiontophoresis, significantly (post-hoc test after two-way ANOVA, F(1,20) = 37.8, p < 0.001) induced firing rate inhibitions (Supplemental Figures S3A).

Following intravenous exposure to MPH/eti, iontophoretically-applied MPH induced significant (post-hoc test after two-way ANOVA, F(1,20) = 98.03, p < 0.001) firing rate increases (Supplemental Figures S3A).

Under baseline conditions, kinurenic acid (KA), dopamine (DA) and norepinephrine (NE) all induced firing rate inhibitions of midbrain dopaminergic neurons (Supplemental Figures S3B-D, post-hoc tests after one-way ANOVA, F(3,69) = 10.57, p < 0.001).

Furthermore, iontophoretic application of kynurenic acid (KA), an NMDA receptor antagonist, at low currents (5-10 nA) was found to block the activation of dopamine neurons induced by the local application of NMDA (not shown). When applied solely, and in the absence of NMDA in the adjacent iontophoretic channels, it also reduced neuronal firing rates in 10 out of 13 neurons tested (Supplemental Figure S3B-C), indicative of some tonic activation of NMDA receptors in dopamine neurons.

3.6 Role of the PFC in MPH-induced excitatory effects

We examined the possibility that descending glutamatergic pathways, originating from the prefrontal cortex (Carr and Sesack, 2000; Geisler et al., 2007; Miller, 2014; Wanchoo et al., 2009), may have a role in MPH/eti-induced excitatory effects on midbrain dopamine neurons. Under prefrontal cortex inactivation (Fig 7A), using lidocaine microperfusions adapted from previous studies (Barnes et al., 2000; Wenzel et al., 2011), the combination MPH/eti did not
significantly increase the firing and burst activity of the dopamine neurons tested (Fig 7B-C), although some heterogeneity was observed. These results suggest a possible involvement of a functional link between the prefrontal cortex and the VTA in the excitatory effects of methylphenidate following D₂ receptor blockade.

A summarising diagram of these results can be found on Figure 9.

3.7 Consequence of a chronic MPH treatment during adolescence

We tested the excitatory response to the combination MPH/eti in a group of young adult rats that were previously exposed to MPH during adolescence (administered in sucrose solution in the drinking water). All treated rats were investigated 4–6 weeks after the last MPH administration that lasted 15 days (5 mg/kg/day) and was initiated during early adolescence (Di Miceli et al., 2018). In chronically treated rats during adolescence, the combination of MPH/eti was significantly dampened for both firing rates (Fig. 8A, post-hoc test after two-way ANOVA, F(1,42)=42.45, p<0.001) and burst activities (Fig. 8B, post-hoc test after two-way ANOVA, F(1,42)=29.01, p<0.001) at adulthood. Control animals (treated with vehicle) presented similar responses to naïve animals and were thus pooled together into the same group.

4 – Discussion

Here, we have demonstrated that MPH, following dopamine D₂ receptor blockade, exerts strong excitatory effects on ventral tegmental area dopamine neurons. This excitatory input involves a combination of activation of noradrenergic α₁ and dopaminergic D₁ receptors, through a complex circuitry that may partially involve prefronto-cortical structures and activation of a glutamate excitatory drive onto dopamine neurons, engaging NMDA receptors. Interestingly,
this effect is significantly dampened in subjects that were previously treated with MPH during their adolescence.

As expected, an acute administration of MPH induces inhibitions of both the firing and burst activities of midbrain dopamine neurons as observed before with other psychostimulants (Einhorn et al., 1988; Panin et al., 2012). Pharmacologically, MPH is known to block reuptake of dopamine (Markowitz and Patrick, 2008; Volz et al., 2008), leading to increased extracellular dopamine concentrations (Wilens, 2008) followed by activation of inhibitory dopamine D2 autoreceptor on midbrain dopamine neurons (Centonze et al., 2002; Shi et al., 2000; Viggiano et al., 2004). A previous study has determined an IC50 value of 1.9 mg/kg using MPH on midbrain dopamine neurons (Choong and Shen, 2004), in excellent agreement with the present study, where 2 mg/kg of MPH induced a 45% inhibition of firing.

The firing inhibition produced by either MPH or D2 receptor agonists can be reversed by selective D2 antagonists (Ackerman et al., 1993; Dela Peña et al., 2018; Eddine et al., 2015; El Mansari et al., 2010; Federici et al., 2005; Shi et al., 2000; Shi et al., 2000; Tepper et al., 1997).

However, in the current study, firing and burst activity rescuing revealed excitatory effects of MPH on these neurons (overshoot). A few studies have brought out the fact that psychostimulant administrations (cocaine, amphetamine and MPH), followed by D2 receptor antagonism, lead to such effects (Dela Peña et al., 2018; Shi et al., 2004; Shi et al., 2000). We found in the present study that such an effect requires simultaneous α1 and D1 receptor activation as only the combination of an α1 and D1 receptor antagonists can totally prevent such effects. Our results differ from the aforementioned studies, as the authors found only a partial effect of α1 receptor blockade (Shi et al., 2004; Shi et al., 2000). According to some studies, adrenergic α1 receptor inhibition reduced bursting activity as well as regularised the firing rates.
of VTA dopamine neurons (Grenhoff and Svensson, 1993), an effect that is not reported in the present study.

On the other hand, acute exposure of the very selective norepinephrine uptake inhibitor atomoxetine did not induce any change in firing or burst activity of midbrain dopamine neurons, alone or in combination with D₂ receptor blockade. This is in partial disagreement with the study of Linnér et al showing that reboxetine, another selective norepinephrine uptake inhibitor, causes increases in burst activity, but not firing activity, of dopamine neurons (Linnér et al., 2001), while both Wong et al (Wong et al., 2000) and Milan et al (Millan et al., 2001) reported no effects. There is no obvious explanation for this discrepancy with our study, as both reboxetine and atomoxetine have very similar pharmacological profiles. Interestingly, according to Linnér’s study, reboxetine exposure, at high doses (20-25 mg/kg, i.v.), increased dopamine levels in the PFC (Linnér et al., 2001), which may be related to its potential ability to increase bursting discharges of VTA dopamine neurons. However, this effect was not believed to be mediated through activation of excitatory α₁ receptors located on dopamine neurons, as intra-VTA administration of reboxetine failed to increase dopamine release in terminal areas (Linnér et al., 2001). Nevertheless, our data indicates that the sole blockade of the norepinephrine transporter (NET) is not likely to explain the large rise in electrical excitability of dopamine neurons observed following the MPH/etic combination. Interestingly, combining (after D₂ receptor blockade) the action of selective dopamine transporter (DAT) blocker (GBR12909) with a selective NET blocker (atomoxetine) did not cause, either, the same level of activation of dopamine neurons as MPH. Though there is no obvious explanation for this inconsistency, it shows that this excitatory effect maybe only be a characteristic of psychostimulants, which may differentially affect the dopaminergic and noradrenergic system and cause different neuronal adaptations compared to the other selective monoamine reuptake blockers.
Our study demonstrates that activation of both dopamine and norepinephrine pathways are required to promote psychostimulant-induced dopamine cell activation. Noradrenergic neurotransmission may play a crucial role in the regulation of VTA dopamine neurons, mainly through direct connexions from the locus coeruleus to the VTA, as shown by previous anatomical and electrophysiological studies involving electrical stimulations (Grenhoff et al., 1993; Jones and Moore, 1977; Simon et al., 1979). VTA projections onto the locus coeruleus have also been detected (Beckstead et al., 1979; Deutch et al., 1986; Samuels and Szabadi, 2008; Simon et al., 1979; Swanson, 1982), although they remain very sparse (Swanson, 1982).

Interactions between the locus coeruleus and the VTA may be crucial in mediating the effects of psychostimulants such as amphetamines in terminal areas (Karim et al., 2017). Noradrenergic modulation of midbrain dopamine neurons is not clearly characterised. Contradictory data exist in the literature regarding the direct $\alpha_1$ adrenergic receptor modulation on dopamine neurons. While some $\alpha_1$ noradrenergic receptors are present post-synaptically on dopamine neurons (Mitrano et al., 2012; Rommelfanger et al., 2009), immunohistochemical study showed that $\alpha_1$ adrenergic receptors are also co-localized with GABA-positive terminals in the VTA (Mitrano et al., 2012). *In vivo* electrophysiological studies using iontophoresis techniques found either robust excitatory effects (Goertz et al. 2015) or mild inhibitory effects (Pradel et al., 2018) following local application of $\alpha_1$ receptor agonists. Recent evidence from *in vitro* electrophysiological studies suggests that $\alpha_1$ noradrenergic agonist can increase glutamatergic and decreases GABAergic synaptic transmission onto VTA dopamine neurons by interacting with $\alpha_1$ receptors located on glutamate and GABA terminals adjacent to dopamine cells (Velasquez-Martinez et al., 2020, 2015). Such a combined effect may be able to potentiate the glutamatergic tone on dopamine neurons, partially by removing tonic GABAergic inhibition.
In the ventral tegmental area, burst activity is regulated by local neurotransmission and each bursting activity releases greater quantities of dopamine at terminals versus single spike activity (Bean and Roth, 1991; Gonon, 1988; Oster et al., 2015). Alternate firing modes between bursting and single discharges are vital to maintain balanced D₁ and D₂ receptor occupancies (Dreyer et al., 2010). Local glutamatergic tone is, in part, responsible for the generation of bursting patterns (Chergui et al., 1993; Kuznetsov et al., 2006; Tong et al., 1996). Our results clearly demonstrate the role for NMDA receptors in mediating the hidden MPH-induced excitatory effects. Many studies have found that burst activity is strongly regulated by afferent PFC connexions (Lodge, 2011; Murase et al., 1993; Omelchenko and Sesack, 2007; Overton and Clark, 1997; Patton et al., 2013; Svensson and Tung, 1989), but other structures may be equally involved (Grace, 2016). Pre-treatment with MK801 at 0.5 mg/kg (a dose that does not affect dopamine firing activity) completely abolished MPH-induced excitatory effects. In the present study, we confirmed the presence of a direct tonic activation of NMDA receptors on the majority of dopamine neurons tested with the iontophoretic applications of kynurenic acid.

Lidocaine is a blocker of fast voltage-gated sodium channels (Catterall, 2002; Courtney, 1975; Sheets and Hanck, 2003; Vedantham and Cannon, 1999; Yeh and Tanguy, 1985). It has been frequently used to induce cortical inactivation in animal models (Ahn and Phillips, 2002; Martin, 1991; Sara and Hervé-Minvielle, 1995; Tehovnik and Sommer, 1997; Uehara et al., 2007; Valenti and Grace, 2009). When lidocaine was slowly perfused within the PFC, we observed dampening of the excitatory effects of MPH following D₂ receptor antagonism, though not observed in all of the neurons tested. This highlights the possible involvement of descending PFC glutamatergic connexions onto the VTA in response to MPH/eti administration, in line with other studies which clearly demonstrated that PFC inactivation, using non-specific (electric) or specific (ibotenic acid, indirect GABAₐ agonist) lesions,
prevented MPH- (Lee et al., 2008), cocaine- (Li et al., 1999; Pierce et al., 1997) or methamphetamine- (Ramos et al., 2005) induced behavioural sensitisations. In good concordance with these data, our previous electrophysiological studies demonstrated a strong excitatory effect of MPH and D-amphetamine on prefrontal cortex pyramidal neurons (Di Miceli and Gronier, 2015; Gronier, 2011; Gronier et al., 2010), an effect that can propagate activation on VTA dopamine neurons. Interestingly, these effects were partially prevented by selective D_{1} receptor antagonist (SCH23390), while D_{2}, β_{1} or α_{1} receptor antagonists had generally only marginal effects, though significant blocking effects could still be observed in a minority of neurons tested (Gronier, 2011). In addition, in another electrophysiological study, we have established that systemic or local administration of a dopamine D_{1} receptor agonist (A77636) significantly increases firing and burst activity of PFC neurons (Gronier et al., 2013). Therefore, an excitatory glutamate drive from the PFC to VTA triggered by dopamine D_{1} receptor stimulation on PFC neurons may contribute to the activation of VTA dopamine neurons following the MPH/eti combination. However, this could not be the only mechanism involved as significant (but possibly lower) activation are still occurring following D_{1} receptors blockade. Involvement of α_{1} receptor is also required, as only the combination of α_{1}/D_{1} antagonism can completely block MPH/etic-induced excitatory effect in the present study. It is likely that the involvement of α_{1} adreno receptors in MPH/etic-induced excitatory effect of VTA dopamine cells takes place in another structure. One attractive hypothesis would involve α_{1} receptors located on GABA terminals adjacent to the VTA dopamine neurons, which negatively regulate GABA release (Velasquez-Martinez et al., 2015). Administration of methylphenidate may indirectly cause their activation, enhancing excitability of dopamine neurons. Reducing the GABAergic tone by α_{1} receptor activation may disinhibit dopamine neurons and prompt some glutamate-dependent activation in cells that receive convergent
glutamate and GABA inputs. When one of the key receptors (either the α₁ of D₁ receptors) has been shut down, dopamine receptor activation may still occur, but at a lower level (interestingly, prazosin tends to have a more robust effect on blocking activation on firing activity, while SCH23390 may be more efficient at reducing burst activation).

However, more complex mechanisms may also be involved. Though the PFC is believed to carry an excitatory drive to the VTA (Dela Peña et al., 2018), the functional interaction between the two structures at the cellular level is complex. In the VTA, not all dopamine neurons are in direct synaptic contact with PFC neurons. According to histological studies, it is preferentially the mesocortical dopamine neurons and some GABA projecting neurons, but not the mesolimbic dopamine neurons, that receive a direct glutamate innervation from the PFC (Carr and Sesack, 2000; Gao et al., 2007). It is likely that a significant proportion of the neurons we have tested in our study were not mesocortical dopamine neurons (Breton et al., 2019). It is not clear how mesolimbic dopamine neurons can be modulated by cortico-tegmental projections (Jackson et al., 2001). Inhibition or activation of these neurons have been observed following electrical or chemical stimulation of the PFC (Gao et al., 2007; Jackson et al., 2001; Murase et al., 1993).

Strong dampening of MPH/eti-induced excitatory effects were observed at adulthood in rats previously treated with MPH (5 mg/kg/day) during 15 days at adolescence. These effects could be due to altered mesocortical circuitry, as previously observed (Urban et al., 2012), although striking differences between in vivo and ex vivo studies are to be noted (Di Miceli and Gronier, 2015; Urban et al., 2012). Long-term consequences of chronic MPH treatment was observed following chronic MPH exposure during adolescence, with adult rats presenting altered cognitive behaviour (Pardey et al., 2012), behavioural sensitisation or tolerance (Jones and
Dafny, 2014) and altered responses to dopamine D₂ receptor challenge (Di Miceli et al., 2018).

Interestingly, intracellular studies have shown that the α₁-adrenoreceptor-mediated activation on glutamatergic transmission is no longer present after a chronic cocaine treatment, and this desensitization persists after the withdrawal period (while the inhibitory effect of α₁-adrenoreceptor on GABA release remains) (Velasquez-Martinez et al., 2020). Therefore, psychostimulant-induced dopamine cell activation may be a relevant mechanism involved in sensitization to drugs of addiction. Whether this persistent desensitisation, observed in our study, can be associated with a change in drug abuse behaviour during later life in patients previously treated with MPH during adolescence is a very interesting question that deserve further investigation. Interestingly, chronic MPH treatment also disrupted PFC activity in freely behaving animals (Salek et al., 2012; Yang et al., 2007).

To conclude, we propose that MPH firstly activates both dopamine D₁ and adrenergic α₁ receptors, which, in turn, trigger glutamate release, likely in the midbrain, inducing in fine NMDA receptor activation and therefore enhancement of the firing rates and burst of VTA dopaminergic neurons. Such an excitatory input of MPH may involve, in part, PFC-VTA connections. Finally, chronic MPH treatment during adolescence dampened the responses to MPH/eti at adulthood, in parallel to previous observations from our laboratory (Di Miceli et al., 2018), highlighting possible long-term consequences of chronic MPH exposure. In addition, our observations highlight the fact that glutamate activation of dopamine neurons may be a predominant process, which can develop more particularly in patients concomitantly treated with psychostimulant and antipsychotic (possibly 40% of patients treated with MPH). It will remain to examine how it could impact on the therapeutic effects and long-term consequences of psychostimulant use in these patients (Olfson et al., 2006; Scholle et al., 2018). Therefore, careful clinical monitoring of these patients should be performed.
Competing interests:

None.

Acknowledgements:

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Figure legends:

Figure 1: MPH-induced autoreceptor-mediated negative feedback on midbrain dopamine neurons.

(A-B): Two mg/kg of MPH significantly decreased the firing and burst activities of midbrain dopamine neurons. Dopamine D2 receptor antagonism with eticlopride (0.2 mg/kg) successfully rescued MPH-induced firing rate decreases and even further increased the firing and burst activities to levels higher than under baseline condition. Following eticlopride, firing activity and percentage of spikes firing in burst were increased by an average of 65 and 167% from baseline levels, respectively. (C): Representative recording in one neuron with the corresponding sequence of intravenous challenges. An action potential waveform is represented for reference. The horizontal bar represents 1 ms. Here and in the following figures n values represent the number of neurons recorded. **p<0.01 vs. baseline, $$$p<0.001 vs. MPH, Neuman-Keuls tests after significant repeated measures ANOVA.

Figure 2: Alleviation of negative feedback by initial eticlopride exposure.

(A-B): Initial eticlopride administration reveals MPH-induced excitatory effects on firing and burst activity of dopamine neurons. (C): Representative recording in one neuron with the corresponding sequence of intravenous challenges. (D-E): Alone, vehicle or eticlopride did not alter the electrical activities of midbrain dopamine neurons. (F): Representative recording in one neuron with the corresponding sequence of intravenous challenges. ***p<0.001 vs. baseline and $$$p<0.001 vs. MPH, Neuman-Keuls tests after significant repeated measures ANOVA.
Figure 3: Comparison with other dopaminergic agonists; specificity of MPH to induce excitatory effects.

(A-B): Intravenous challenges with 20 µg/kg of quinpirole, a dopamine D₂ receptor agonist, induced significant firing rate decreases (p<0.05, Neuman Keuls test after significant repeated measures ANOVA). Such decreases could be reversed with 0.2 mg/kg of eticlopride (dopamine D₂ receptor antagonist), which rescued both firing rates and burst activities to baseline levels. Subsequent MPH exposure (2 mg/kg) increased both firing and burst activities above baseline levels. Representative recording in one neuron with the corresponding sequence of intravenous challenges (C) and in another neuron with the dopamine D₂ receptor agonist apomorphine (D), showing particularly high level of activation following MPH administration. *p<0.05, **p<0.01, vs. baseline, $$$p<0.01$ vs. quinpirole, &p<0.05, &&p<0.01 vs. eticlopride, Neuman-Keuls tests after significant repeated measures ANOVA.

Figure 4: Absence of excitatory effects by the selective norepinephrine uptake inhibitor atomoxetine and dopamine uptake inhibitor GBR-12909.

Intravenous exposure to the selective norepinephrine reuptake inhibitor atomoxetine (ATX) (A, B) at 5 mg/kg or to the selective dopamine uptake GBR-12909 at 2 mg/kg (C, D), or to the combination of the two drugs (C, D) did not alter the firing or the burst activities of midbrain dopamine neurons in eticlopride-treated animals.

Figure 5: Methylphenidate requires both adrenergic alpha-1 and dopamine D₁ receptors to exert its excitatory effects.

When the α₁ receptor antagonist prazosin was pre-administered (1.5 mg/kg), MPH (2 mg/kg) still exerted excitatory effects on the firing rates (A) and burst activities (B) of VTA dopamine neurons and following dopamine D₂ receptor antagonism (eticlopride 0.2 mg/kg). (C): Representative recording in one neuron with the corresponding sequence of intravenous challenges. (D-E): Similar results were observed using the dopamine D₁ receptor antagonist SCH23390, pre-administered at 0.6 mg/kg. (F): Representative recording of a neuron pre-treated with SCH23390, which excitatory response induced by the combination MPH/eti faded progressively following the administration of prazosin (G-I): Both prazosin and SCH23390, administered as a combined pre-treatment, totally prevented MPH/eticlopride-induced activation of firing rate and burst activities in of the recorded neurons (8/8). Alone, prazosin (J-
K) or SCH23390 (L-M) did not alter firing or burst activities of VTA dopamine neurons. *p<0.05 vs. baseline, Neuman-Keuls tests after significant repeated measures ANOVA.

Figure 6: Role of glutamatergic neurotransmission in MPH-induced excitatory effects.
(A-B): Pre-treatment with the selective glutamatergic antagonist MK801 (0.5 mg/kg) completely prevented MPH/eti-induced excitatory effects. (C): Representative recording in one neuron with the corresponding sequence of intravenous challenges. Alone, MK801 (0.5 mg/kg) did not affect the firing (D) or burst (E) activities of midbrain dopamine neurons. **p<0.01 vs. baseline and $$p<0.01$ vs. MPH, Neuman-Keuls tests after significant repeated measures ANOVA.

Figure 7: Functional mesocortical connexions are required for MPH-induced excitatory effects.
(A): Locations of intra-PFC lidocaine perfusions (2% w/v in saline), performed before intravenous MPH challenge. A total of 2.5 µl of lidocaine were injected into 8 locations. For each hemisphere, two coordinates respective to Bregma (anteroposterior/lateral) were chosen (+2.2/1; +3.2/1), each injected at two depths (1.5 and 3.0 mm below cortical surface). Bleu dots indicate microperfusion locations. Dotted lines represent anatomical delimitations. Adapted from Paxinos and Watson, 1997. Such a protocol reduced (but not abolished) MPH/eti-induced excitatory effects (non-significant compared to baseline levels) on the firing (B) and burst activities (C) of midbrain dopamine neurons. **p<0.01 vs. baseline, Neuman-Keuls tests after significant repeated measures ANOVA.

Figure 8: Blunted responses to MPH/eti at adulthood following chronic MPH treatment during adolescence.
At adulthood, the responses of both firing rates (A) and burst activities (B) to MPH/eti were strongly dampened following chronic treatment with MPH (5 mg/kg/day for 15 days) during adolescence. ***p<0.001 vs. baseline, two-way ANOVA. ns: non-significant.
Figure 9: Summary of the results from the current study. In line with our recent electrophysiological studies in the PFC (Di Miceli and Gronier, 2015; Gronier, 2011; Gronier et al., 2013), we suggest that MPH will elicit an excitatory effect on PFC pyramidal neurons, which is dependent on the synergic activation of alpha1 and D1 receptors on prefrontal pyramidal neurons, thus stimulating a glutamatergic drive to the PFC, likely mediated by NMDA receptors, explaining the very large range increases in firing and burst activity.

Supplementary Table 1. Statistical tests and results performed in the present study.

Supplemental Figure S1: Direct relationship between responses to MPH and eticlopride. Strong correlation ($r^2=0.958$) between initial response to MPH (abscissa) and subsequent response to eticlopride (ordinate).

Supplemental Figure S2: ATX does not alter the firing or burst activities of midbrain dopaminergic neurons. Cumulative doses of 4 mg/kg of ATX do not alter the firing rates (A) or bursting activities (B) of midbrain dopaminergic neurons in naïve animals. (C): Representative electrophysiological recording.

Supplemental Figure S3: Influence of locally-applied MPH, catecholamines and kinurenic acid on midbrain dopaminergic neurons. (A): Under baseline conditions, microiontophoretic applications of MPH (10 nA) induced firing rate inhibition, an effect that reverses (firing rate activation) following MPH/eticlopride intravenous exposure. $***p<0.001$ vs. 0 nA, $p<0.05$ vs. baseline at 10 nA, after two-way ANOVA. (B): Microiontophoresis of kynurenic acid (KA), dopamine (DA) and norepinephrine (NE) induced robust firing rate inhibitions. $**p<0.01$, $***p<0.001$ vs. 0 nA, Neuman-Keuls tests after significant one-way ANOVA. (C): Representative firing rate recording depicting inhibition by kynurenic acid. Representative firing rate recording depicting inhibitions with MPH, DA and NE under baseline condition (D).
References


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https://doi.org/10.1016/j.brainres.2007.01.108


https://doi.org/10.1016/S0006-3495(85)83965-5


https://doi.org/10.1016/0006-8993(92)91091-r
Figure 1

Panel A: Baseline comparison of firing rate (spikes/10sec) and bursts (% of spikes in burst) across different conditions:
- Baseline
- MPH 2 mg/kg
- Eticlopride 0.2 mg/kg

Panel B: Statistical analysis showing significant differences with asterisks indicating levels of significance.

Panel C: Time course of firing rate (spikes/10sec) and bursts (Hz) over time, with arrows indicating drug administration.

Legend:
- **: Significant difference
- $$$: Highly significant

Figure 1
Figure 2
Figure 3

(A) Firing (spikes/10sec) and Bursts (% of spikes in burst) under different conditions:
- Baseline
- Quinpirole (20 µg/kg)
- Eticlopride (0.2 mg/kg)
- MPH (2 mg/kg)

(B) Histogram showing firing rate and number of spikes in bursts.

(C) Time series of firing rate and bursts.

(D) Graph showing the effect of Apomorphine (20 µg/kg) and Eticlopride (0.2 mg/kg) on firing rate and bursts.

Figure 3
Figure 4
Figure 5
Figure 6
Figure 7

A

B

C

Firing (spikes/10sec)

Baseline
MPH
2 mg/kg
Eticlopride
0.2 mg/kg

Bursts (% of spikes in burst)

n=7

Figure 7
Figure 8

A

B

*** ns

 baseline MPH/eti

*** ns

 baseline MPH/eti

 Naive and controls n=28

 Chronic MPH during adolescence n=16

 Naive and controls n=28

 Chronic MPH during adolescence n=16
Baseline methylphenidate eticlopride

VTA neuron

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Baseline methylphenidate eticlopride

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**Hypothesis**

D2R antagonist

MPH → VTA

α1R-mediated release of glutamate?

MPH → α1R/D1R-mediated activation pyramidal neurons

Gronier, 2011
Gronier et al., 2013
Di Miceli & Gronier, 2015

D1R: dopamine D1 receptor
D2R: dopamine D2 receptor
α1R: adrenergic alpha 1 receptor
NMDA-R: N-methyl-D-aspartate receptor

PFC: prefrontal cortex

Figure 9
Conflict of interest:
None.
Ethical Statement

Experiments were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the UK Home Office. All protocols were carried out under project and personal licenses issued by the UK Home Office under the UK Animals (Scientific Procedures) Act 1986 and were also approved by the Committee on the Ethics of Animal Experiments of De Montfort University (Protocol 60/4333).
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Supplementary Material

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