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Methylphenidate Increases Cortical Excitability via Activation of Alpha-2 Noradrenergic Receptors

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Although methylphenidate (MPH), a catecholaminergic reuptake blocker, is prescribed for attention-deficit/hyperactivity disorder, there is a dearth of information regarding the cellular basis of its actions. To address this issue, we used whole-cell patch-clamp recordings to investigate the roles of various catecholamine receptors in MPH-induced changes in cortical neuron excitability. We bath-applied dopamine or noradrenaline receptor antagonists in combination with MPH to pyramidal cells located in deep layers of the infralimbic and prelimbic prefrontal cortices. Application of MPH (10 μM) by itself increased cortical cell excitability in slices obtained from juvenile rats. This MPH-mediated increase in excitability was lost when catecholamines were depleted with reserpine prior to recording, demonstrating the requirement for a presynaptic monoamine component. Antagonist studies further revealed that stimulation of alpha-2 noradrenergic receptors mediates the MPH-induced increase in intrinsic excitability. Dopamine D1 receptors played no observable role in the actions of MPH. We therefore propose that MPH is acting to increase catecholaminergic tone in the PFC, and thereby increases cortical excitability by mediating the disinhibition of pyramidal cells through mechanisms that may include activation of alpha-2 adrenoreceptors located in interneurons.
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INTRODUCTION

The prefrontal cortex (PFC) is critically involved in cognitive processes underlying working memory (WM), attention, and inhibition of responses to nonrelevant stimuli (Goldman-Rakic, 1996; Fuster, 2000). Studies in humans, non-human primates, and rats have shown that the PFC receives catecholaminergic inputs from the midbrain (Porrino and Goldman-Rakic, 1982; Levitt *et al*, 1984; Lewis *et al*, 1987; Lewis and Morrison, 1989; Miner *et al*, 2003) and a substantial amount of data supports the conclusion that dopamine (DA) and noradrenaline (NE) are involved in aspects of executive function (selective attention, response selection/control), as well as arousal (NE). These actions of DA and NE have been postulated to occur via their effects in the cortex. Although numerous studies have focused on the role of DA in the PFC (Sawaguchi *et al*, 1990; Arnsten *et al*, 1994, 1995; Yang and Seamans, 1996; Durstewitz *et al*, 1999; Lewis and O'Donnell, 2000; Lavin and Grace, 2001), there have been fewer studies analyzing the role of NE in modulating cortical activity relevant to cognitive performance.

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Methylphenidate (MPH) is one of the most widely prescribed drugs for the treatment of attention-deficit hyperactive disorder (ADHD) (Hunt *et al*, 1984; Hunt, 1987), a condition that includes deficits in executive functions (WM and attention). MPH blocks the reuptake of both DA and NE (Carlsson *et al*, 1966; Axelrod, 1970; Ferris *et al*, 1972; Hendley *et al*, 1972; Ross, 1978; Seeman and Madras, 1998). Through this action, both acute systemic administration and iontophoretic application of MPH evoke increases in cortical activity (Lacroix and Ferron, 1988). Moreover, MPH improves WM performance in healthy subjects (Elliot *et al*, 1997; Mehta *et al*, 2000) and several studies have shown that MPH increases overall speed and accuracy of attentional process in normal adults (Strauss *et al*, 1984; Solanto, 1998). However, there is a dearth of data regarding the receptor mechanisms mediating the effects of MPH on cortical neurons. The purpose of the present study was to determine the effects of MPH on prefrontal cortical activity in slices from juvenile rats and to examine the receptors involved in the actions produced by MPH. Or results show that MPH, via increases in catecholaminergic tone, increases cortical excitability acting through alpha-2 receptors.

METHODS

All procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals

published by the US Public Health Service, and was approved by the Medical University of South Carolina Animal Care and Use Committee.

Preparation of Brain Slices

Slices were prepared from juvenile (18–28 days) male Sprague–Dawley rats (Charles Rivers). The rats were deeply anesthetized with chloral hydrate (400 mg/kg), transcardially perfused with an oxygenated sucrose-substituted aCSF maintained at 1–5°C (in mM): 200 sucrose, 1.9 KCl, 1.2 Na_2HPO_4, 33 Na_2HCO_3, 6 MgCl, 0.5 $CaCl_2$, 10 glucose, and 0.4 ascorbic acid. Rats were decapitated and the brain was rapidly removed. Sections (350 μm) containing the PFC were obtained in the sucrose-substituted aCSF using a vibratome (Leica VT-1000S). The slices where then transferred to a holding chamber and incubated with oxygenated incubation buffer (125 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 1 CaCl, 4 MgCl, 15 sucrose, 10 dextrose, and 0.4 ascorbic acid) for at least 1 h at room temperature.

Whole-Cell Recordings

Individual slices were transferred to the recording chamber and neurons were visualized using an infrared upright microscope (Leica DMLFSA) equipped with a CCD video imaging camera (Hamamatsu C 2400). PFC pyramidal neurons were recognizable by their pyramidal-shaped cell body and the presence of a long apical dendrite projecting toward the pia. Current clamp recordings were performed using an Axopatch 200 B amplifier (Axon Instruments, Foster City, CA) with custom-made software. Electrodes were fabricated from borosilicate tubing (1.1 OD, Warner Instruments, Hamden, CT) using a horizontal Flaming/Brown puller (model P-97). The electrodes had resistances of 5–12 MΩ when filled with (in mM) 135 potassium gluconate, 3 KCl, 2 MgCl, 10 HEPES, 1 EGTA, 4 ATP, 0.3 GTP, 14 creatine phosphate, and 0.3% biocytin. The pH of the internal solution was 7.3. The signals were amplified and filtered at 10 kHz in bridge-balance mode and acquired on a computer at sampling intervals of 20–100 μs through a National Instrument Board (BNC-2090). Series resistances (2–6 MΩ after 'break-in') were 85% compensated. No additional corrections were made for junction potential between bath and pipette solutions. The access resistance was continuously monitored during recording. The recordings were preformed at room temperature.

The input resistance (IR) was assessed for each cell during all pharmacological treatments by injecting a series of hyperpolarizing pulses in increments of 40 pA. The slope of the resulting curve yields the IR value.

Excitability Measures

In order to evaluate changes in intrinsic excitability, a series of constant current pulses (10–300 pA) were injected into the cell to determine a current level that on average evoked three action potentials under baseline conditions. Once this current was determined, the same current was then used for the rest of the experiment. Spikes were evoked every 30 s for 10 min during baseline recordings, drugs were then bath-applied and after 5 min measures were taken again every 30 s for at least 10 more minutes. The membrane potential was constantly held at the value obtained during baseline recordings.

In order to control for the effects of repetitive injection of current pulses during the bath-application of drugs, as well as for the simple effects of time passage in any drug effects, the stimulation protocol was repeated in the absence of any drugs. This protocol is called control-recovery.

Drugs

The drugs used were MPH (1 and 10 μM; Sigma), clonidine (10 μM; Sigma), yohimbine (1 and 3 μM; Sigma), SCH 23390 (1–5 μM; Sigma), and propanolol (10 μM). These drugs were dissolved in aCSF and stored as concentrated stock solutions at −20°C. Prazosin (1 μM) was dissolved in 80% ethanol. All drugs were bath-applied. In a subset of experiments, CPP (10 μM), CNQX (10 μM), and bicuculline (10 μM) were added to the recording solution in order to prevent synaptic activity. The doses of MPH and clonidine used in the present experiments were selected following pilot experiments with dose–response curves (1–100 μM and 100 nM–100 mM, respectively). For other drugs, the doses were selected based on an analysis of the literature.

Catecholaminergic Depletion

In order to test the assumption that the effects observed after MPH administration were mediated by an increase in catecholaminergic tone, five animals were pretreated with reserpine (1.5 mg/kg, i.p.) 1.5 h before euthanasia. After the brain was removed, the slices were incubated in buffer containing reserpine (10–20 μM) until slices were transferred to the recording chamber. The significant degree of depletion of catecholamines produced by a similar treatment has been described previously (Heeringa and Abercrombie, 1995; Zimmer *et al*, 2002).

Statistical Analysis

In order to compare the effect of MPH on cortical excitability, paired Student's *t*-tests or repeated measures ANOVA (RM ANOVA) were used. Differences of alpha $\leqslant 0.05$ were considered significant. All the results are presented as mean $\pm$ SE.

RESULTS

A total of 125 cells were recorded in the infralimbic and prelimbic cortex. All neurons recorded were pyramidal cells, as identified by their electrophysiological and morphological characteristics under IRDC illumination and by subsequent biocytin labeling and immunohistochemistry. All the experiments were performed at the resting membrane potential values.

Effects of MPH on Intrinsic Excitability

The overall effect of MPH administration in all the cells tested in the PFC ($n = 30$) was a significant increase in intrinsic excitability ($n = 30$ cells; control: 3.9 ± 0.3 spikes/

pulse; MPH: 5.1 ± 0.57 spikes/pulse; $p < 0.0007$, paired *t*-test; Figure 1 and Table 1). MPH administration decreased intrinsic excitability in only 2/30 cells (6.6%) and did not affect excitability in 33.3% of the cases (10/30 cells). The amplitude of the AHP and the IR were measured in a subgroup of 10 cells, and it was found that MPH administration did not produce a significant effect in any of these parameters.

Administration of 1 μM MPH did not change cortical activity ($n = 10$; control: 2.8 ± 0.3 spikes/pulse; MPH: 2.7 ± 0.61 spikes/pulse; Table 1).

When the same protocol was repeated in the absence of MPH, repetitive injections of current pulses did not increase cell excitability ($n = 5$; control 1: 4.1 ± 0.9 spikes/pulse; control 2: 4.4 ± 0.9 spikes/pulse; Figure 1). Furthermore, these experiments demonstrate that prolonged

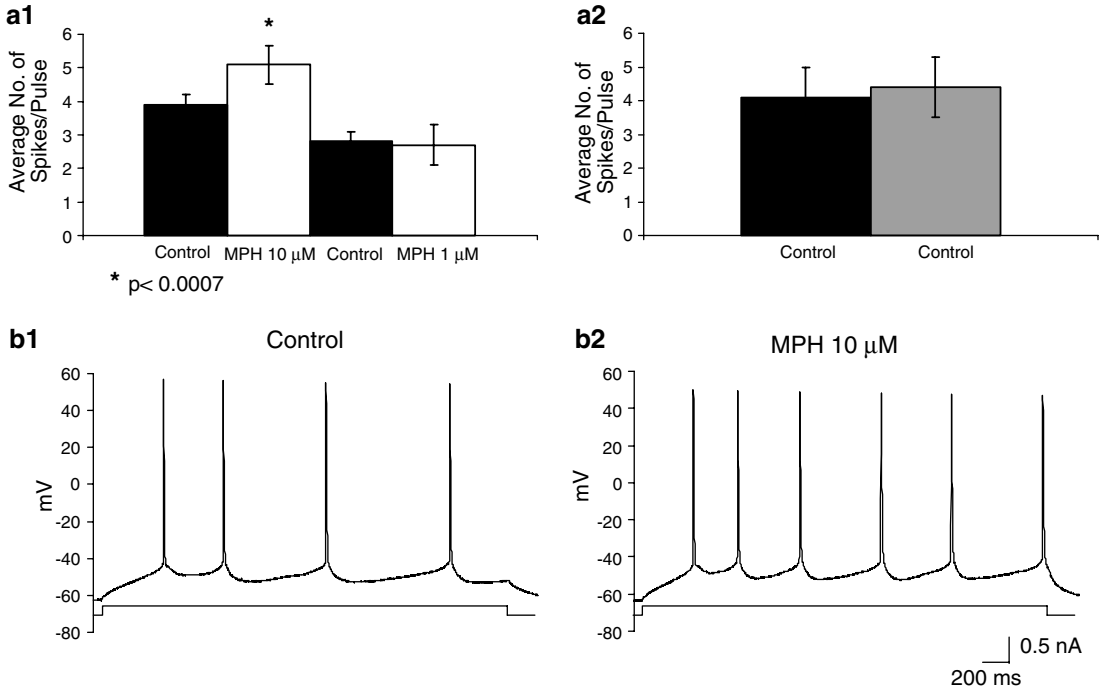

Figure 1 Bath-administration of MPH (10 μM) results in significant increases in cortical excitability. (a1) MPH mediates a significant increase in cortical excitability ($n = 30$, $p < 0.0007$). In contrast, administration of lower concentrations of MPH (1 μM) did not evoke significant changes in cortical excitability. The bar graphs depict the average number of spikes/pulse. (a2) Control-recovery experiments show that repetitive injection of current pulses does not induce an increase in the evoked activity of cortical cells. In addition, this experiment provides control for the temporal aspects of the experiments, demonstrating that the length of the recording does not affect intrinsic excitability. (b1) Representative traces of the average number of spikes evoked during control recordings and (b2) after 5 min of MPH (10 μM) administration.

Table 1 Effects of Specific DAergic and NErgic Drugs on Pyramidal Cell Properties

	MP (mV)			IR (MΩ)			Number of spikes/pulse		
Treatment	**Control**		**Treatment**	**Control**		**Treatment**	**Control**		**Treatment**
Control–MPH (10 μM, $n = 30$)	72.3 ± 0.2		72.2 ± 0.1	463.3 ± 47.8		435.0 ± 48	**3.9 ± 0.3**		**5.1 ± 0.5***
Control–MPH (1 μM, $n = 10$)	71.3 ± 0.8		71.2 ± 0.4	359.2 ± 27.2		402.1 ± 37.8	2.8 ± 0.3		2.7 ± 0.6
Control–MPH (reserpine)	70.1 ± 0.6		69.4 ± 0.5				4.0 ± 0.7		4.3 ± 0.9
Control–prazosine–MPH	73.0 ± 0.1	73.5 ± 0.4	72.7 ± 0.2	445 ± 41.2	429.3 ± 40.3	405.4 ± 53.1	**2.8 ± 0.2**	**3.9 ± 0.5**	**4.9 ± 0.9****
Control–yohimbine–MPH	72.9 ± 1.1	72.2 ± 1.9	72.3 ± 1.2	488.2 ± 82	439.8 ± 59.5	527.4 ± 60	3.4 ± 0.4	3.7 ± 0.5	3.2 ± 0.8
Control–propanolol–MPH	71.8 ± 2.5	71.8 ± 2.1	71.9 ± 2.0	423.4 ± 41.1	267 ± 92.9	420 ± 50.8	4.2 ± 0.8	5.2 ± 1.5	5.1 ± 1.4
Control–SKF 38390–MPH	72.3 ± 0.1	72.6 ± 0.2	71.9 ± 0.2	345.1 ± 64	232 ± 85	327 ± 81.1	**3.1 ± 0.3**	**3.7 ± 0.7**	**5.4 ± 0.9*****
Control–clonidine	71.8 ± 0.4		72.1 ± 0.3	575.3 ± 56.9		418.9 ± 62.7	**3.4 ± 0.6**		**6.1 ± 0.7******

Bold values indicate significant changes.
*$p < 0.0007$.
**$p < 0.01$.
***$p < 0.03$.
****$p < 0.000005$.

recordings by themselves did not induce a change in cell activity.

Effects of MPH in Catecholamine-Depleted Slices

It is known that MPH increases the levels of catecholamines by blocking the NE reuptake transporter in the PFC (Axelrod, 1970; Carlsson *et al*, 1966; Ferris *et al*, 1972; Hendley *et al*, 1972; Ross, 1978; Sonders *et al*, 1997). Thus, the observed increases in cortical excitability elicited by MPH may result from an increased concentration on extracellular catecholamines. To test this hypothesis, animals were pretreated with reserpine, an inhibitor of the vesicular monoamine transporter. In slices harvested from animals pretreated with reserpine, MPH (10 μM) failed to increase cortical excitability ($n = 10$ cells; control: 4.0 ± 0.7 spikes/pulse; MPH: 4.3 ± 0.9 spikes/pulse; Figure 2). This result strongly suggests that the MPH-mediated increase in cortical excitability derives from an increase in the extracellular concentration of catecholamines. Reserpine treatment did not, however, affect the basic electrophysiological properties of pyramidal cells.

Receptors Involved in the MPH-Mediated Increase in Excitability

To identify the receptors underlying the MPH effect on cortical excitability, we tested the effects of specific NErgic or DAergic receptor antagonists. We tested the effects of bath-application of the specific alpha-1 antagonist prazosin (1 μM), the specific alpha-2 antagonist yohimbine (2 μM), the specific beta antagonist propanolol (10 μM), and the specific DA D1 antagonist SCH 23390 (1–5 μM). We tested only a specific DA D1 antagonist, but not a DA D2 antagonist, since only D1 receptor activation has been shown to increase cortical excitability in rat PFC slices (Yang and Seamans, 1996; Lavin and Grace, 2001).

NErgic Antagonists

Pretreatment with yohimbine (2 μM) prevented the increase in excitability mediated by MPH (10 μM) ($n = 10$ cells; control: 3.4 ± 0.4 spikes/pulse; yohimbine: 3.7 ± 0.5 spikes/pulse; MPH: 3.2 ± 0.8 spikes/pulse; RM ANOVA $F(8, 2) = 0.17$, $p < 0.83$; Figure 3).

By contrast, pretreatment with the alpha-1 antagonist prazosin (1 μM) did not prevent the MPH-mediated increase in excitability ($n = 10$ cells; control: 2.8 ± 0.2 spikes/pulse; prazosin: 3.8 ± 0.5 spikes/pulse; MPH: 4.9 ± 0.9 spikes/pulse; RM ANOVA, $F(2, 20) = 3.78$, $p < 0.04$; Figure 3). Indeed, Fisher's *post hoc* analysis indicates that MPH produces significant increases in cortical excitability ($p < 0.01$) relatively to control under conditions of alpha-1 receptor blockade.

Pretreatment with the beta antagonist propanolol (10 μM) also failed to prevent the MPH-mediated increase in cortical excitability ($n = 10$; control: 4.2 ± 0.8 spikes/pulse; propanolol: 5.2 ± 1.5 spikes/pulse; MPH: 5.1 ± 1.4 spikes/pulse; RM ANOVA $F(2, 16) = 1.09$, $p < 0.35$; Figure 3).

DAergic Antagonists

Similarly, pretreatment of the cortical slices with SCH 23390 (5 μM) did not prevent the MPH-mediated increase in excitability ($n = 10$; control: 2.8 ± 0.3 spikes/pulse; SCH 23390: 4.0 ± 06 spikes/pulse; MPH: 6.1 ± 0.7 spikes/pulse; RM ANOVA $F(2,14) = 9.7$, $p < 0.02$). Fisher's *post hoc* analysis shows that MPH produces a significant increase in excitability ($p < 0.0006$) when compared with control values under conditions of D1 receptor blockade (Figure 3).

NE Agonists

The experiments described above indicate that alpha-2 adrenoreceptors mediate the excitatory effects of MPH. In order to further test the effects of the alpha-2 receptors on cortical activity, the effects of a specific agonist of alpha-2 NErgic receptors (clonidine, 10 μM) were tested. Clonidine administration produced a significant increase in cortical excitability in 75% of the cells tested (15/20) (control: 3.4 ± 0.6 spikes/pulse; clonidine: 6.1 ± 0.7 spikes/pulse; paired *t*-test, $p < 0.0006$; Figure 4).

Role of Local Cortical Networks in the MPH-Mediated Increases in Excitability

In order to investigate the role of the local network on the effects mediated by MPH, recordings were performed in the presence of the glutamate and $GABA_A$ blockers, CNQX (10 μM), CPP (10 μM), and bicuculline (10 μM). In 10 pyramidal neurons tested under these conditions, MPH (10 μM) administration did not increase intrinsic excitability (control: 2.3 ± 0.3 spikes/pulse; MPH: 2.0 ± 0.4 spikes/pulse; Figure 5). This result suggests that the excitatory effects mediated by the increase in catecholamines resulting from MPH administration are indeed mediated through changes in cortical networks.

In summary, MPH administration significantly increased cortical excitability. When specific DA or NE receptor antagonists were administered prior to MPH, only alpha-2 blockade prevented the MPH-mediated increases in excitability.

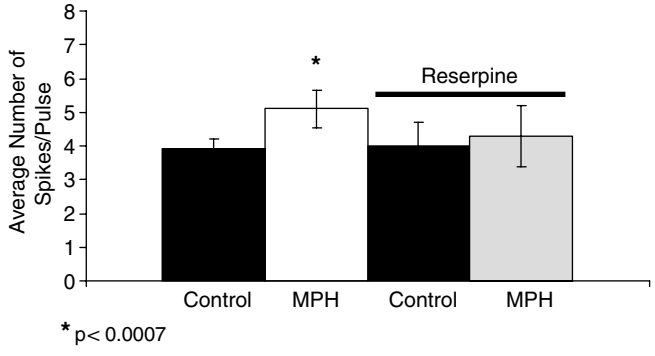

Figure 2 MPH did not increase cortical excitability in reserpine-treated tissue. Bath-administration of MPH (10 μM) mediates significant increases in cortical excitability by increasing catecholaminergic tone. In contrast, if the tissue is pretreated with reserpine, MPH is unable to increase cortical excitability. The histogram represents the increases in average number of spikes/pulse.

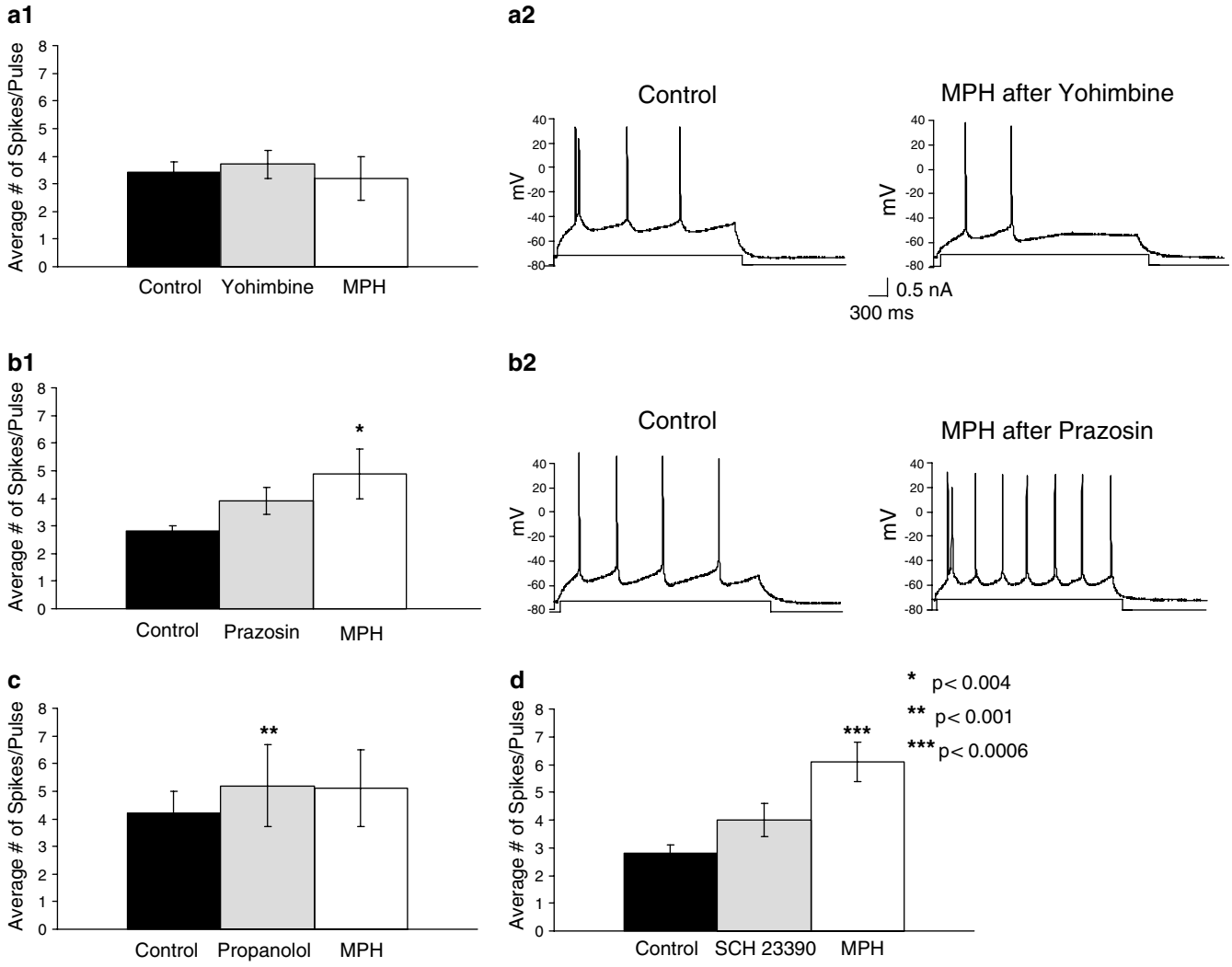

Figure 3 Alpha-2 antagonists block the MPH-mediated increases in cortical excitability. (a1) The alpha-2 antagonist yohimbine (2 μM) blocks the MPH-mediated effects in cortical excitability. (a2) Representative traces showing the preventive effects of yohimbine administration. (b1) The alpha-1 antagonist prazosin (1 μM) did not prevent the increase in excitability mediated by MPH (10 μM, $p<0.004$). (b2) Representative traces showing the MPH-mediated increases in cortical excitability following prazosin administration. (c) The beta antagonist propanolol (10 μM) induce a significant increase in cortical excitability ($p<0.001$) that is sustained following MPH administration (10 μM). (d) The DA D1 antagonist SCH 23390 (5 μM) did not prevent the MPH-mediated increases in cortical excitability.

DISCUSSION

We have shown that MPH mediates increases in excitability in cortical pyramidal neurons obtained from infant rats. The MPH-mediated increases were not observed when experiments were performed in reserpine-treated tissue, suggesting that the increase in cortical excitability is mediated by an increase in the concentration of catecholamines. Surprisingly, our results indicate that the mechanisms underlying the MPH modulation of cortical excitability involve activation of alpha-2 NE receptors.

Effects of MPH on Cortical Excitability

Our study reveals that MPH significantly increased excitability on cortical pyramidal neurons in normal animals. Moreover, our results suggest that the increase in cortical excitability is the result of MPH enhancing the concentration of catecholamines in the synaptic cleft. Furthermore, the fact that specific NE antagonists induce increases in cortical excitability further suggests the presence of a catecholaminergic tone in the slice.

The specific alpha-2 antagonist yohimbine was the only catecholaminergic antagonist that was able to suppress the excitatory effects of MPH. Alpha-2 receptors are located pre- and postsynaptically, and the alpha-2A subtype is most dense in the PFC and in the locus coeruleus (LC) (Aoki *et al*, 1994, 1998). Activation of alpha-2 receptors has been shown to hyperpolarize neurons through Gi proteins, as shown in the LC (Egan *et al*, 1983; Andrade and Aghajanian, 1985; Williams *et al*, 1985). This hyperpolarization is associated with increases in K^+ conductances and decreases in Ca^{2+} currents. Nonetheless, our results have shown that (1) the excitatory actions of MPH are mediated through alpha-2 receptors and (2) the alpha-2 agonist clonidine increased cortical excitability.

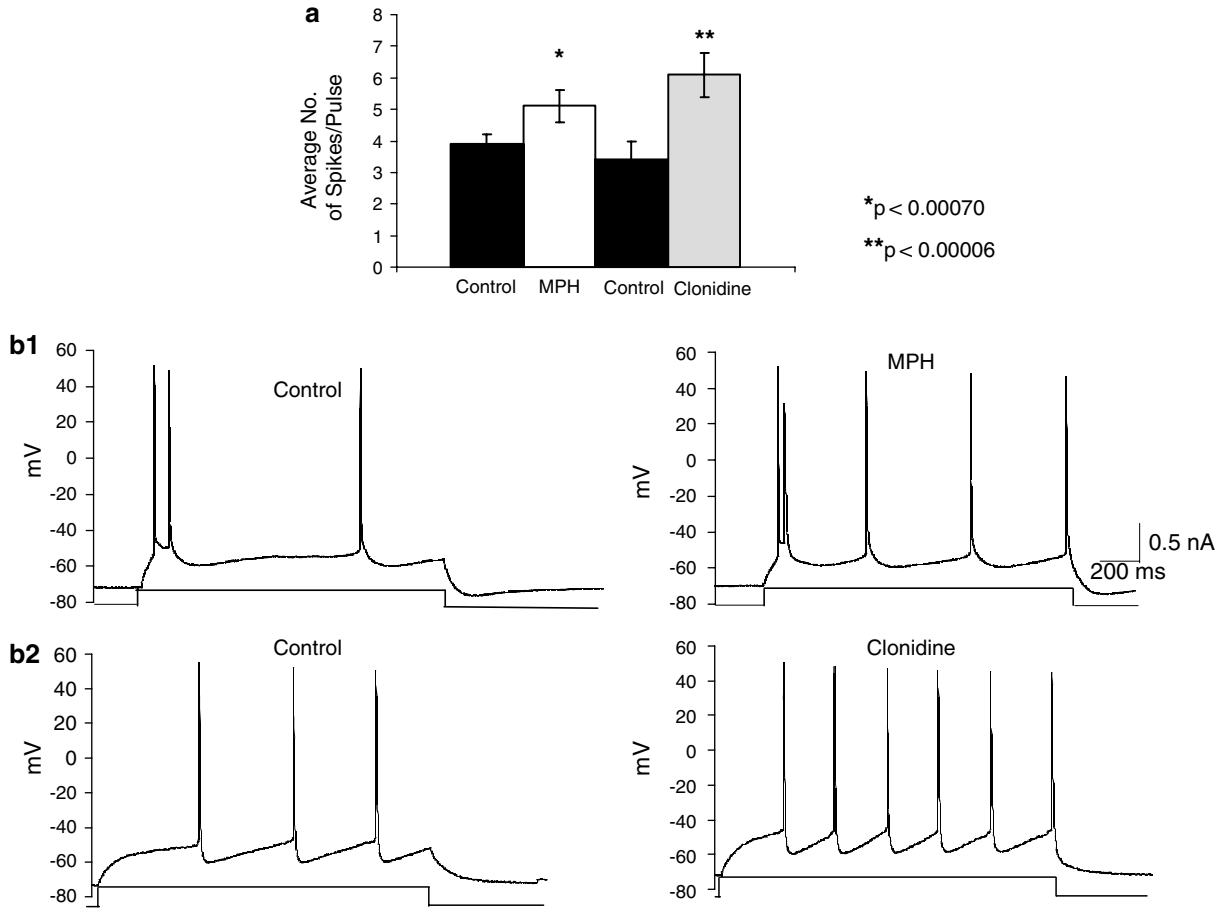

Figure 4 The specific alpha-2 agonist clonidine mimicked the increase in cortical excitability mediated by MPH. (a) The histogram indicates that both the reuptake blocker MPH and the specific alpha-2 agonist clonidine increased cortical excitability. (b1) Representative traces of the average number of spikes evoked during control recordings and following MPH (10 μM) administration. (b2) Representative traces of the average number of spikes evoked during control recordings and following clonidine (10 μM) administration.

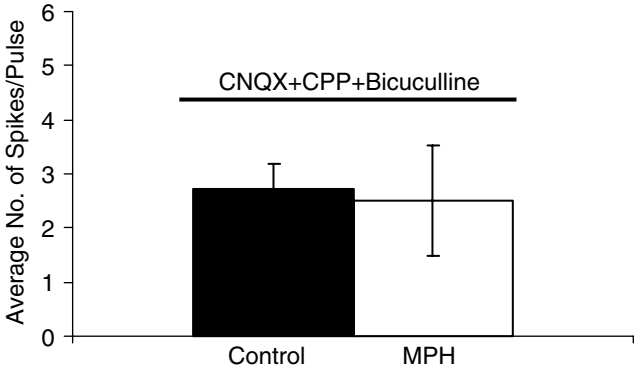

Figure 5 When the $GABA_A$ and glutamate blockers, bicuculline (10 μM), CNQX (10 μM), and CPP (10 μM), were added to the bath, MPH administration did not increase cortical excitability.

How to explain this discrepancy? We hypothesize that MPH is increasing cortical activity by disinhibiting pyramidal neurons. That is, MPH is increasing the catecholaminergic tone in the slice, and as a consequence, higher concentrations of NE in the synaptic cleft now are able to activate pre- and postsynaptic receptors. Activation of presynaptic alpha-2 receptors in the slice will have minimal consequences; however, activation of postsynaptic alpha-2 NER located in interneurons could hyperpolarize GABA neurons and therefore disinhibit pyramidal cells. Indeed, our experiments with glutamate and GABA blockers suggest that the increases in excitability elicited by MPH administration are mediated through changes in local cortical networks. Moreover, preliminary whole-cell recordings of pyramidal cells in the presence of bicuculline indicate that administration of the alpha-2 agonist clonidine fails to elicit increases in cell excitability (Lavin, 2004). Moreover, clonidine administration decreases activity of fast spiking interneurons (Lavin, 2004), indicating that at least part of the increase in excitability produced by clonidine was mediated by interneurons.

In summary, the present results support the hypothesis that MPH, by increasing the level of catecholamines in the cortex, produces a disinhibition of pyramidal cells and thereby an increase in cortical excitability. The presence of a catecholaminergic tone in the slice can be further suggested by our results showing that in tissue pretreated with reserpine, MPH failed to produce an increase in cortical excitability. Moreover, the MPH-mediated increases in excitability are mediated by activation of alpha-2 NER.

Elliot *et al* (1997) have suggested that MPH enhances cognitive performance, WM, and sustained attention in normal volunteers. Moreover, Mehta and colleagues (2000) showed that the beneficial effects of MPH on WM in normal volunteers correlate with increased activity in the dorsolateral PFC and the parietal cortex. Thus, even in a normal system, MPH seems to improve attention and WM function. Therefore, it is tempting to speculate that by increasing cortical excitability, MPH optimizes cortical function including activity related with WM and attention. Moreover, by increasing cortical excitability, MPH could facilitate the response of neurons to strongly correlated synaptic inputs, therefore 'selecting' the activity of relevant stimuli over nonrelevant stimuli.

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