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Potentiated gene regulation by methylphenidate plus fluoxetine treatment: Long-term gene blunting (*Zif268*, *Homer1a*) and behavioral correlates

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Abstract

Use of psychostimulants such as methylphenidate (Ritalin) in medical treatments and as cognitive enhancers in the healthy is increasing. Methylphenidate produces some addiction-related gene regulation in animal models. Recent findings show that combining selective serotonin reuptake inhibitor (SSRI) antidepressants such as fluoxetine with methylphenidate potentiates methylphenidate-induced gene regulation. We investigated the endurance of such abnormal gene regulation by assessing an established marker for altered gene regulation after drug treatments – blunting (repression) of immediate-early gene (IEG) inducibility – 14 days after repeated methylphenidate+fluoxetine treatment in adolescent rats. Thus, we measured the effects of a 6-day repeated treatment with methylphenidate (5 mg/kg), fluoxetine (5 mg/kg) or their combination on the inducibility (by cocaine) of neuroplasticity-related IEGs (*Zif268*, *Homer1a*) in the striatum, by in situ hybridization histochemistry. Repeated methylphenidate treatment alone produced modest gene blunting, while fluoxetine alone had no effect. In contrast, fluoxetine given in conjunction with methylphenidate produced pronounced potentiation of methylphenidate-induced blunting for both genes. This potentiation was seen in many functional domains of the striatum, but was most robust in the lateral, sensorimotor striatum. These enduring molecular changes were associated with potentiated induction of behavioral stereotypies in an open-field test. For illicit psychostimulants, blunting of gene expression is considered part of the molecular basis of addiction. Our results thus suggest that SSRIs such as fluoxetine may increase the addiction liability of methylphenidate. Key words: cognitive enhancer, dopamine, serotonin, gene expression, psychostimulant, SSRI antidepressant, striatum

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Conflict of interest

The authors declare no conflicts of interest.

Keywords

cognitive enhancer; dopamine; serotonin; gene expression; psychostimulant; SSRI antidepressant; striatum

Introduction

The use of psychotropic medications (e.g., psychostimulants, antidepressants) in children and adolescents is widespread. For example, the psychostimulant methylphenidate (Ritalin) is an effective agent to manage the symptoms of attention deficit/hyperactivity disorder (ADHD) [1,2]. ADHD is diagnosed in up to 7% or more of school-age children in the United States [2]. According to a recent study, in 2008 approximately 3 million children between 4 and 17 years of age in the US alone were treated with psychostimulant medications for ADHD [3].

There is also increasing abuse of such medical psychostimulants as “cognitive enhancers” by healthy children and adults [2,4,5]. While the exact magnitude of this medication abuse is difficult to determine [2,5], a recent estimate indicates that, in 2008 in the US, about 8.5% of the population age 12 and older reported past nonmedical use of prescription psychostimulants, and as many as 11 million prescriptions out of 38 million may have been diverted for nonmedical use [3]. This abuse can produce a particularly uncontrolled form of potentially high-level exposure, as these psychostimulants are often ground up and snorted or injected (e.g., [6–9]; see [10]).

Exposure to psychotropic drugs during development and maturation of the brain is of concern. Studies in animal models show that these drugs can induce abnormal neurobehavioral changes suggestive of an increased risk for drug addiction and other neuropsychiatric disorders later in life (for reviews, see [11–14]). Long-lasting neurobehavioral pathologies such as addiction are often mediated by changes in gene regulation [15]. Over the last decade, research in several labs has investigated the effects of exposure to medical psychostimulants on gene regulation in addiction-related neuronal systems, including the striatum [10]. These studies show that, for example, methylphenidate has the potential to alter the expression of many genes in a manner similar to illicit psychostimulants such as cocaine, but other genes appear less affected by methylphenidate than by cocaine [10,16].

Differences between methylphenidate and cocaine are likely related to their differential neurochemical effects; methylphenidate inhibits dopamine and norepinephrine transporters (among other effects), while cocaine also inhibits reuptake of serotonin, in addition to dopamine and norepinephrine (see [16]). A facilitatory role for serotonin in addiction-related gene regulation is well established (e.g., [17–19]). Would therefore increasing serotonin action by treatment with selective serotonin reuptake inhibitor (SSRI) antidepressants in combination with methylphenidate produce more pronounced (“cocaine-like”) gene regulation? This notion is supported by our findings showing that the SSRIs fluoxetine or citalopram potentiate gene regulation by methylphenidate in the striatum [20]. Such potentiation of methylphenidate-induced gene regulation was first demonstrated for acute

induction of immediate-early genes (IEGs; *c-Fos*, *Zif268*) [21,22] and neuropeptides (substance P, dynorphin) [23]. Further studies showed that fluoxetine also potentiates increases in dynorphin, enkephalin and 5-HT1B receptor expression [24], as well as blunting of IEG induction [25], produced by repeated methylphenidate treatment, in striatal neurons.

Blunting (repression) of IEG inducibility is one of the best-established neuroadaptations produced by repeated cocaine/amphetamine treatment (see [10]). Blunting refers to the finding that a psychostimulant challenge given after repeated psychostimulant pretreatment produces attenuated (blunted) IEG induction compared with induction by the first psychostimulant administration (see [10]). We previously found that repeated treatment with methylphenidate alone produced some blunting of *c-Fos* and *Zif268* inducibility, whereas another IEG, *Homer1a*, was minimally or not affected [26,27]. This latter finding is in contrast to effects of repeated cocaine treatment, which produces robust blunting for *c-Fos* and *Zif268*, but also *Homer1a* [28]. Our further study demonstrated that adding fluoxetine to the repeated methylphenidate treatment robustly potentiated gene blunting for both *Zif268* and *Homer1a* [25], thus mimicking cocaine effects.

Gene blunting by psychostimulants is thought to be mediated by epigenetic modifications [29] or other neuroadaptations [10] and is typically long-lasting. For example, gene blunting produced by the 5-day repeated cocaine treatment endured for more than 3 weeks [28]. In our recent study [25], we examined IEG blunting one day after the repeated methylphenidate +fluoxetine treatment in adolescent rats, for comparison with our earlier findings [26]. In the present study, we investigated whether such IEG blunting endures. Using the same experimental design, again in adolescent rats, we assessed IEG (*Zif268*, *Homer1a*) induction by a cocaine challenge 14 days after the repeated methylphenidate+fluoxetine treatment. As in our previous studies [25,26], this design mimicked a situation in which an individual exposed to methylphenidate and/or SSRIs during adolescence subsequently abuses cocaine. Our results show that blunting of both IEGs was still very robust 14 days after the methylphenidate+fluoxetine pretreatment, especially in the lateral striatum.

Methods

Subjects

Male Sprague–Dawley rats (five weeks old at the beginning of the drug treatment; Harlan, Madison, WI, USA) were housed 2–3 per cage under standard laboratory conditions (12:12h light/dark cycle; lights on at 07:00h) with food and water available ad libitum. Experiments were performed between 13:00 and 17:00h. Prior to the drug treatment, the rats were allowed one week of acclimation during which they were repeatedly handled. All procedures met the NIH guidelines for the care and use of laboratory animals and were approved by the Rosalind Franklin University Animal Care and Use Committee.

Drug treatments

Rats received 6 daily injections of vehicle (i.p., V), methylphenidate HCl (5 mg/kg, MP; in 0.02% ascorbic acid, 1 ml/kg; Sigma, St. Louis, MO, USA), fluoxetine HCl (5 mg/kg, FLX; Sigma), or methylphenidate plus fluoxetine (MP+FLX). Fourteen days after the last

treatment, they received a cocaine challenge (25 mg/kg, C; cocaine HCl, Sigma) or vehicle (groups V/C, MP/C, FLX/C, MP+FLX/C, V/V, n=6–9). After the injection, the rat was placed in an open-field apparatus (43 × 43 cm), and locomotion (ambulatory distance) and stereotypy ('stereotypy 1') counts were measured for 40 min with an activity monitoring system (Truscan, Coulbourn Instruments, Allentown, PA, USA). These 'stereotypy' counts reflect local, repetitive movements (e.g., head bobbing and focused sniffing).

Tissue preparation and in situ hybridization histochemistry

The rats were killed with CO₂ 40 min after the vehicle or cocaine challenge injection. The brain was rapidly removed, frozen in isopentane cooled on dry ice and then stored at –30 °C until cryostat sectioning. Coronal sections (12 µm) were thaw-mounted onto glass slides (Superfrost/Plus, Daigger, Wheeling, IL, USA), dried on a slide warmer and stored at –30 °C. In preparation for the in situ hybridization histochemistry, the sections were fixed in 4% paraformaldehyde/0.9% saline for 10 min at room temperature, incubated in a fresh solution of 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% saline (pH 8.0) for 10 min, dehydrated, defatted for 2 × 5 min in chloroform, rehydrated, and air-dried. The slides were then stored at –30 °C until hybridization.

Oligonucleotide probes (48-mers; Invitrogen, Rockville, MD, USA) were labeled with [33P]-dATP as described earlier [30]. The probes had the following sequence: *Zif268* (*Egr1*), complementary to bases 352–399, GenBank accession number M18416; *Homer1a*, bases 1493–1540, AB003726. One hundred µl of hybridization buffer containing labeled probe (~3 × 10⁶ cpm) was added to each slide. The sections were coverslipped and incubated at 37 °C overnight. After incubation, the slides were first rinsed in four washes of 1X saline citrate (150 mM sodium chloride, 15 mM sodium citrate), and then washed 3 times 20 min each in 2X saline citrate/50% formamide at 40 °C, followed by 2 washes of 30 min each in 1X saline citrate at room temperature. After a brief water rinse, the sections were air-dried and then apposed to X-ray film (BioMax MR-2, Kodak) for 5–9 days.

Analysis of autoradiograms

Gene expression in the striatum was assessed in sections from 3 rostrocaudal levels [rostral, approximately at +1.6 mm relative to bregma [31]; middle, +0.4 (Fig. 1); caudal, –0.8] in a total of 23 sectors mostly defined by their predominant cortical inputs (see [30,32]). Eighteen of these sectors represent the caudate-putamen, and 5 the nucleus accumbens (Fig. 2).

Hybridization signals on film autoradiograms were measured by densitometry (NIH Image; Wayne Rasband, NIMH, Bethesda, MD, USA). The films were captured using a light table (Northern Light, Imaging Research, St. Catharines, Ontario, Canada) and a Sony CCD camera (Imaging Research). The "mean density" value of a region of interest was measured by placing a template over the captured image. Mean densities were corrected for background by subtracting mean density values measured over white matter (corpus callosum). Values from corresponding regions in the two hemispheres were then averaged. The illustrations of film autoradiograms displayed in Figure 1 are computer-generated images, and are contrast-enhanced where necessary.

Statistics

Treatment effects were determined by one-factor ANOVA. Newman-Keuls post hoc tests were used to describe differences between individual groups (Statistica, StatSoft, Tulsa, OK, USA). The distribution of changes in gene induction throughout the striatum was illustrated by maps (Fig. 2). For these maps, the difference in gene induction (blunting) (vs. acute cocaine, V/C) in a given sector was expressed as the percentage of the maximal difference observed for either probe (% max.). The regional distributions of the fluoxetine potentiation of *Zif268* versus *Homer1a* blunting, and of *Zif268* or *Homer1a* blunting (present study) versus *Zif268* or *Homer1a* blunting seen one day after the repeated treatment [25] were compared by Pearson correlations.

Results

Cocaine induces *Zif268* and *Homer1a* expression in the striatum

As shown before (e.g., [25,28]), acute administration of cocaine (i.e., the cocaine challenge in vehicle-pretreated animals, V/C) induced *Zif268* and *Homer1a* expression on all three rostrocaudal levels of the striatum (middle: Figs. 1 and 3; rostral, caudal: data not shown). Thus, for *Zif268*, a statistically significant increase in expression was observed in 17 of the 23 striatal sectors (caudate-putamen: 16/18; nucleus accumbens: lateral shell), and for *Homer1a*, in 16 sectors (caudate-putamen: 15/18; nucleus accumbens: lateral shell) ($P < 0.05$, V/C vs. V/V). The most robust cocaine-induced IEG expression occurred in dorsal and lateral (sensorimotor) sectors of the middle (Figs. 1 and 3) and caudal striatum (not shown), as reported previously (e.g., [28,30]; see [10]). In contrast, on those levels, no significant gene induction was seen in the ventral sectors (middle, Figs. 1 and 3), as seen before (e.g., [25,28]). Also confirming previous findings (e.g., [25,28]), in the nucleus accumbens, maximal acute cocaine-induced IEG expression was seen in the lateral shell (data not shown).

Gene blunting after repeated treatment with methylphenidate and/or fluoxetine for cocaine-induced *Zif268* and *Homer1a* expression in the striatum

Repeated pretreatment with methylphenidate (5 mg/kg) alone (MP/C) produced borderline attenuation (blunting) of cocaine-induced *Zif268* expression. This effect was statistically significant ($P < 0.05$, vs. V/C) in only two of the 23 striatal sectors (rostral: dorsomedial; middle: medial sector; Figs. 2A and 3A), although a similar tendency was present in several other sectors with a robust acute *Zif268* response (V/C) (e.g., lateral striatum) (Fig. 3A). The effect of methylphenidate pretreatment on *Homer1a* induction was similar. Significant blunting in MP/C animals was observed in two sectors (rostral: dorsomedial, medial; Fig. 2B), with a similar tendency again present in more sectors (Fig. 3B).

In contrast to methylphenidate, repeated pretreatment with fluoxetine (5 mg/kg) alone had no significant impact on subsequent IEG induction by cocaine, neither in the caudate-putamen nor in the nucleus accumbens (Figs. 1, 2 and 3). None of the 23 sectors showed significant changes in *Zif268* or *Homer1a* induction by cocaine in the fluoxetine-pretreated group ($P > 0.05$, FLX/C vs. V/C; Figs. 2 and 3), although a tendency for increased gene induction was observed in some sectors (Fig. 3).

However, when given in conjunction with methylphenidate, fluoxetine potentiated gene blunting by repeated methylphenidate treatment in the striatum (Figs. 1, 2 and 3). This fluoxetine potentiation of blunting was reflected by a greater proportion of the 23 striatal sectors displaying significantly attenuated *Zif268* or *Homer1a* induction by cocaine after the methylphenidate+fluoxetine pretreatment ($P<0.05$, MP+FLX/C vs. V/C), compared with methylphenidate alone (MP/C vs. V/C) (*Zif268*: 7 sectors vs. 2 sectors; *Homer1a*: 11 vs. 2; Figs. 2, 3). Direct statistical comparisons showed that *Zif268* and *Homer1a* blunting were significantly more robust in the MP+FLX/C group than in the MP/C group in 3 and 4, respectively, of the 23 sectors ($P<0.05$, Fig. 2, POT).

The tendency for increased gene induction after pretreatment with fluoxetine alone (FLX/C) also resulted in more sectors showing significantly attenuated gene induction in the MP+FLX/C group vs. FLX/C, compared to MP+FLX/C vs. MP/C (POT) (*Zif268*: 8 sectors vs. 3 sectors; *Homer1a*: 12 vs. 4; Figs. 2, 3).

It was of interest to compare gene blunting after the 14-day withdrawal (present study) with blunting after the 1-day withdrawal period [25]. This comparison showed that, after the 1-day withdrawal, MP+FLX/C animals displayed blunted *Zif268* and *Homer1a* induction (vs. V/C) in 15 and 12 sectors, respectively, or significant potentiation (POT) of blunting (MP+FLX/C vs. MP/C) in 9 and 8 of the 23 sectors (see Fig. 3 in [25]), whereas after the 14-day withdrawal (present study), these numbers were 7 and 11 or 3 and 4 (see above). Overall, IEG blunting thus receded with extended withdrawal, but we note that, especially in the lateral striatum, blunting and its potentiation remained very robust even 14 days after the 6-day pretreatment. For example, for MP+FLX/C in the ventrolateral sector on the middle level (maximal blunting, Fig. 4), induction for both *Zif268* and *Homer1a* was still less than 50% of that in the V/C group (Fig. 3), up from 25–30% after the 1-day withdrawal [25], and 50–60% of MP/C (potentiation), up from 40–50%.

Regional distribution of fluoxetine potentiation of methylphenidate-induced gene regulation

Our correlation analysis confirmed that the regional distribution in the striatum of the potentiation of blunting by fluoxetine was very similar for *Zif268* and *Homer1a* ($r=0.892$, $P<0.001$; Fig. 4). This analysis further shows that the potentiation of blunting after the 14-day withdrawal period (present study) occurred in the same striatal sectors as that after the 1-day withdrawal [25] (*Zif268*: $r=0.791$, $P<0.001$; *Homer1a*: $r=0.818$, $P<0.001$; Fig. 4). Overall, in the present as in our previous study, the potentiation was most robust in, but not limited to, sectors of the sensorimotor striatum (Fig. 4, open diamonds), with maximal potentiation present in the ventrolateral sector of the middle striatum (Figs. 2, 3, and 4).

Potentiation of methylphenidate-induced gene regulation is associated with a potentiated increase in stereotypies

We assessed behavioral consequences of the methylphenidate+fluoxetine pretreatment in an open-field test. Our results show that stereotypy counts during the 40-min observation period after the cocaine injection were affected (Fig. 5). All groups displayed increasing stereotypy scores over these 40 min. Overall, there was a strong tendency for a main effect

of drug treatment ($P=0.056$). This effect was significant for the first 15 min ($P<0.01$) and was produced by higher scores in the MP+FLX/C group than in the MP/C (potentiation), FLX/C and V/C groups ($P<0.05$ each; Fig. 5). The MP+FLX/C animals thus displayed an accelerated increase in stereotypy levels in the first part of the test period. Ambulation counts were not significantly affected, although the MP+FLX/C group tended to have lower counts early in the observation period (data not shown). These behavioral results mirrors our earlier finding of potentiated stereotypy counts induced by acute MP+FLX vs. MP treatment [22].

Discussion

Our work demonstrates that administration of fluoxetine in conjunction with repeated methylphenidate treatment potentiates methylphenidate-induced gene regulation in the striatum [20]. Previously, we showed such potentiation of gene regulation for increases in opioid peptide expression (dynorphin, enkephalin [24]), as well as for blunting of IEG induction by a cocaine challenge [25]; these were measured within 1 day after the repeated treatment. The present study is the first to demonstrate that such potentiated gene blunting after repeated methylphenidate+fluoxetine treatment endures for at least 2 weeks past the treatment, similar to effects of repeated cocaine treatment [28]. Overall, the magnitude of *Zif268* and *Homer1a* blunting was comparable to that found after the 5-day repeated cocaine (25 mg/kg) treatment [28]. The present rats were treated during adolescence (postnatal days 35–40); these molecular changes thus endured towards young adulthood of the animals. Our findings complement other recent findings showing that methylphenidate+fluoxetine pretreatment in juvenile rats produced molecular changes in midbrain dopamine neurons and behavioral alterations that in part were still present (or even more robust) in adult animals [33] (see below).

IEG blunting

The functional consequences of blunted IEG inducibility in striatal neurons remain unclear. However, the integrity of neurons depends on balanced regulation of gene expression to ensure that cellular components with limited half-lives are replenished and normal cellular plasticity is maintained. It can be assumed that drug-induced gene blunting results in disrupted cell homeostasis/function (see [10,34]). Such gene blunting serves as a marker for neuroadaptations after these drug treatments [10].

The two IEG markers that we investigated, *Zif268* and *Homer1a*, are both directly involved in neuronal plasticity. *Zif268* encodes a zinc finger transcription factor that regulates the activity of other genes [35]. *Zif268* is implicated in several long-term neurobehavioral changes induced by psychostimulants. For example, studies showed that *Zif268* is critical for place preference conditioning by cocaine [36] and for reconsolidation of cocaine memories [37,38]. *Zif268* also contributes to processes underlying cocaine-induced behavioral sensitization [36], as well as other aspects of neuroplasticity [35].

Homer1 is a member of a family of scaffolding proteins (Homer/Vesl proteins) that are present in the postsynaptic density and help cluster glutamate receptors and link these to internal calcium stores. *Homer1* also plays a role in receptor trafficking and other

mechanisms of synapse and dendrite structuring and plasticity [39–41]. The truncated IEG isoform, *Homer1a*, seems to act as a dominant negative regulator of glutamate signaling [42], by promoting disassembly and turnover of the signaling complex [39,40]. This is seen as a necessary stabilizing process [43] during activity-dependent synaptic plasticity [42]. Reduced (blunted) inducibility of *Homer1a* would thus be expected to impair activity-dependent synaptic plasticity. Various previous studies have implicated altered *Homer1* expression in drug-induced abnormal neuroplasticity related to addiction (for review, see [44]), although the exact consequences at the cell physiological level remain to be determined. In summary, our findings indicate lasting neuroadaptations after this methylphenidate+fluoxetine combination treatment.

Striatal circuits affected and behavioral correlates

As in our previous studies, we mapped changes in gene regulation throughout the striatum in order to identify the functional domains (corticostriatal circuits) affected by these treatments. The regional distribution of these effects was similar to that seen before (e.g., [22,25]). While the fluoxetine potentiation of methylphenidate-induced gene regulation was widespread and occurred on rostral to caudal levels of the striatum, these effects were most robust on the middle level and were maximal in the lateral (sensorimotor) striatum. Given that the maximal effects of methylphenidate alone are centered more medially [10], involving striatal circuits that receive inputs from prefrontal cortical areas [30], the addition of fluoxetine produces a shift in the most impacted corticostriatal circuits towards the lateral, sensorimotor striatum. These effects are thus also more “cocaine-like” than those of methylphenidate alone (see [10]).

The lateral striatum is critical for stimulus-response (habit) learning [45,46], and drug-induced molecular changes in this part of the striatum are implicated in aberrant habit formation and compulsive behavior in drug addiction (e.g., [47–49]). There is also evidence for a role of the lateral striatum in relapse to drug seeking after previous drug exposure (e.g., [50,51]) (see [10], for discussion).

The behavioral consequences of methylphenidate+fluoxetine treatment remain to be fully determined. A previous study found that fluoxetine given in conjunction with acute methylphenidate enhanced methylphenidate-induced locomotor activity in an open-field test in rats [52]. In our earlier study, using different methylphenidate and fluoxetine doses than Borycz et al. [52], we found that fluoxetine potentiated behavioral stereotypies induced by acute methylphenidate [22]. Our present findings show a similar effect, that is, facilitated induction of stereotypies by the cocaine challenge after repeated methylphenidate +fluoxetine treatment. Behavioral stereotypies are thought to reflect dysfunction in sensorimotor striatal circuits [53]. Of note, in our study, potentiated gene regulation was most pronounced in the ventral portion of the lateral sensorimotor striatum, which processes cortical information critical for oral control and forelimb and head movements ([54]; see also [55]). Abnormal forelimb/neck/head movements contributed to the stereotypies measured here.

Potential clinical relevance

The observed methylphenidate+fluoxetine-induced changes in gene regulation in the striatum mimic to some degree molecular effects of psychostimulants such as cocaine [10], which are considered part of the molecular basis of psychostimulant addiction (e.g., [47,56,57]). Some of these molecular effects are induced by methylphenidate alone [10], but our results show that fluoxetine can potentiate these effects and induce others [20]. In this context, our findings suggest that fluoxetine may enhanced the addiction liability of methylphenidate.

This is consistent with findings in preclinical studies. For example, repeated pretreatment with cocaine or amphetamine in animal models facilitates subsequent psychostimulant seeking and self-administration, suggesting an increased abuse/addiction liability (c.f., [10]). Similarly, several previous studies found that repeated pretreatment with methylphenidate facilitated the subsequent acquisition of cocaine self-administration in rats [58–60].

It is presently unknown whether pretreatment with SSRIs in conjunction with methylphenidate will modify subsequent cocaine self-administration in rats. However, a recent study showed that methylphenidate+fluoxetine pretreatment in juvenile rats modifies place preference conditioning by cocaine in adulthood [33]. Thus, adding fluoxetine to repeated methylphenidate pretreatment produced enhanced preference for the cocaine-paired environment, in contrast to the cocaine avoidance expressed by the methylphenidate only-pretreated controls [33]. This finding of an enhanced cocaine preference (sensitivity) later in life does suggest an increased abuse/addiction risk for the drug combination (for review, see [10]). Moreover, these methylphenidate+fluoxetine-pretreated rats also displayed enhanced sensitivity to natural reward (sucrose) and other effects suggestive of deficits in mood-related behaviors and reactivity to stress as adults ([33]; see [61]).

The above preclinical studies may thus indicate potential health risks for methylphenidate +SSRI co-exposure in humans. How widespread is methylphenidate+SSRI co-exposure resulting from pharmacological treatments or cognitive enhancer use? SSRIs such as fluoxetine are often the first-line treatment for several depressive and anxiety disorders [62] and are given to millions of patients in the United States alone every year. As discussed, methylphenidate is used both in the treatment of conditions such as ADHD and as a recreational drug and cognitive enhancer [2,4,5]. Combination therapies of methylphenidate plus an SSRI are indicated for several conditions, including ADHD/depression comorbidity [2,63,64], which occurs with up to 40% prevalence in pediatric ADHD [65,66]. These combination treatments can be effective in patients who do not adequately respond to individual drugs (e.g., [67]). In addition, methylphenidate is also combined with SSRIs as augmentation therapy and as acceleration treatment for SSRIs in major depressive disorder (e.g., [68–71]) and other indications. It is currently unknown how much accidental methylphenidate+SSRI co-exposure occurs as a result of cognitive enhancer use by patients on SSRIs.

The addiction liability of methylphenidate alone is more limited compared with illicit psychostimulants [72], and it remains unclear whether proper medical use of drugs such as methylphenidate (i.e., in low oral doses, which produce slower bioavailability and lower

serum levels) increases the risk for subsequent substance use disorder in humans [2,73,74], at least within a few years of the treatment when these studies were conducted. To our knowledge, there are no studies yet that assessed the risks of cognitive enhancer use.

There are apparently also no clinical studies that addressed the potential of enhanced risks after methylphenidate+fluoxetine co-exposure. If there is such a risk, it will likely be greater for methylphenidate use as a cognitive enhancer or recreational drug, which often involves higher doses and routes of administration (snorting, intravenous injection [6,8,9]) that result in faster uptake and higher drug serum levels. Both of these are risk factors for addiction-related gene regulation ([75]; see [10], for discussion). Future studies will have to determine whether co-exposure of methylphenidate with SSRIs indeed increases the addiction liability of methylphenidate, as our gene regulation effects may suggest [10].

Conclusions

Repeated methylphenidate treatment in adolescent rats produces changes in gene regulation in the striatum that mimic to some degree molecular effects of cocaine [10]. These neuronal changes are associated with a facilitation of subsequent cocaine seeking and taking in the cocaine self-administration model in rats [58,60]. Our present study shows that adding an SSRI antidepressant (fluoxetine) to repeated methylphenidate treatment potentiates methylphenidate-induced gene regulation (gene blunting) and that this effect can endure into adulthood, similar to other neuronal effects [33]. Future studies will have to determine whether such co-exposure with fluoxetine will further facilitate cocaine taking in rats, and whether these preclinical effects signify an increased risk for substance use disorder after such drug co-exposure in humans.

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Figure 1.

Fluoxetine potentiates repeated methylphenidate-induced gene blunting in the striatum. Illustrations of film autoradiograms depict *Zif268* (A) and *Homer1a* expression (B) in coronal sections from the middle striatum in rats that received 6 daily injections of vehicle (V), fluoxetine (5 mg / kg; FLX), methylphenidate (5 mg / kg; MP), or methylphenidate +fluoxetine (MP+FLX), followed 14 days later by a vehicle injection (V) or a cocaine challenge (25 mg/kg; /C). The maximal hybridization signal is in black.

Figure 2.

Topography of fluoxetine-potentiated gene blunting in the striatum. Maps depict the distribution of blunting [i.e., the difference (decrease) vs. acute cocaine, V/C] for *Zif268* (**A**) and *Homer1a* induction (**B**) in the rostral, middle and caudal striatum in rats treated with 6 daily injections of fluoxetine (5 mg / kg; FLX/C), methylphenidate (5 mg / kg; MP/C), or methylphenidate+fluoxetine (MP+FLX/C), followed 14 days later by the cocaine challenge. The differences between MP+FLX/C and MP/C groups (POT) and between MP+FLX/C and FLX/C groups (right) are also shown. The data are normalized relative to the maximal decrease observed (% of max.) for either gene. Sectors with a significant decrease ($P<0.05$) vs. acute cocaine controls (V/C) are shaded as indicated. Sectors without significant difference are in white. Caudateputamen: c, central; d, dorsal*; dc, dorsal central; dl, dorsolateral*; dm, dorsomedial; m, medial; v, ventral; vc, ventral central; vl, ventrolateral*. Nucleus accumbens: mC, medial core; lC, lateral core; mS, medial shell; vS, ventral shell; lS, lateral shell. *sensorimotor sectors; see [32].

Figure 3.

Fluoxetine potentiation of repeated methylphenidate-induced *Zif268* and *Homer1a* blunting in the middle striatum. Mean density values (mean $\pm$ SEM) for *Zif268* (A) and *Homer1a* expression (B) in rats that received 6 injections of vehicle (V), fluoxetine (5 mg / kg; FLX), methylphenidate (5 mg / kg; MP), or methylphenidate+fluoxetine (MP+FLX), followed 14 days later by a cocaine challenge (25 mg/kg; /C), are given for the medial (m), ventral (v), dorsal (d), central (c), dorsolateral (dl), and ventrolateral (vl) sectors. Values in vehicle controls (V/V) are indicated by the broken lines. The potentiation of blunting displayed a medial-lateral gradient, with the most robust blunting ventrolaterally. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. V/C or FLX/C; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, MP+FLX/C vs. MP/C (potentiation).

Figure 4.

Relationship between the potentiation of blunting of *Zif268* induction vs. that of *Homer1a* induction 14 days after repeated methylphenidate+fluoxetine pretreatment (left), and for *Homer1a* blunting at 14 days vs. 1 day after repeated pretreatment (right). The scatterplots show that the potentiation (i.e., the differences in induction between MP+FLX/C and MP/C animals) is correlated between *Zif268* and *Homer1a* on withdrawal day (WD) 14 ($r=0.892$; left) (present study), and, for *Homer1a*, between WD14 and WD1 [25] ($r=0.818$; right), across the 23 striatal sectors. The values are expressed as the percentages of the maximal potentiation for each gene. Note that, for both comparisons, the maximal values (at 100/100) represent gene blunting in the ventrolateral sector on the middle level. The generally lower values for WD14 compared with WD1 (right) reflect diminished blunting after the 14-day withdrawal period. The open diamonds indicate sensorimotor sectors. *** $P<0.001$.

Figure 5.

Drug effects on open-field behavior. Stereotypy counts (mean $\pm$ SEM) over 40 min (left) and for min 1–15 (right) after the cocaine injection are shown for animals that received 6 injections of vehicle (V), fluoxetine (5 mg / kg; FLX), methylphenidate (5 mg / kg; MP), or methylphenidate+fluoxetine (MP+FLX), followed 14 days later by the cocaine challenge (25 mg/kg; /C) and the open-field test. Fluoxetine potentiated methylphenidate-induced stereotypies at the beginning of the test. * $P < 0.05$ vs. V/C or FLX/C; # $P < 0.05$, MP+FLX/C vs. MP/C (potentiation).