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Fluoxetine potentiation of methylphenidate-induced neuropeptide expression in the striatum occurs selectively in direct pathway (striatonigral) neurons

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Abstract

Concomitant therapies combining psychostimulants such as methylphenidate and selective serotonin reuptake inhibitors (SSRIs) are used to treat several mental disorders, including attention-deficit hyperactivity disorder/depression comorbidity. The neurobiological consequences of these drug combinations are poorly understood. Methylphenidate alone induces gene regulation that mimics partly effects of cocaine, consistent with some addiction liability. We previously showed that the SSRI fluoxetine potentiates methylphenidate-induced gene regulation in the striatum. The present study investigated which striatal output pathways are affected by the methylphenidate + fluoxetine combination, by assessing effects on pathway-specific neuropeptide markers. Results demonstrate that fluoxetine (5 mg/kg) potentiates methylphenidate (5 mg/kg)-induced expression of substance P and dynorphin, markers for direct pathway neurons. In contrast, no drug effects on the indirect pathway marker enkephalin were found. Because methylphenidate alone has minimal effects on dynorphin, the potentiation of dynorphin induction represents a more cocaine-like effect for the drug combination. On the other hand, the lack of an effect on enkephalin suggests a greater selectivity for the direct pathway compared with psychostimulants such as cocaine. Overall, the fluoxetine potentiation of gene regulation by methylphenidate occurs preferentially in sensorimotor striatal circuits, similar to other addictive psychostimulants. These results suggest that SSRIs may enhance the addiction liability of methylphenidate.

Keywords

methylphenidate; psychostimulant; SSRI; striatum; dynorphin; substance P

INTRODUCTION

Methylphenidate (Ritalin) is a Schedule II psychostimulant that is widely used to treat the symptoms of attention-deficit hyperactivity disorder (ADHD), a behavioral disorder diagnosed in up to 7% of school-age children in the US (DSMMD 2000; Kollins 2008). During the past decade, there has been a rise in the use of selective serotonin reuptake inhibitors (SSRIs) in combination with methylphenidate for the treatment of various mental disorders, including ADHD/depression (Safer *et al.* 2003; Bhatara *et al.* 2004) and ADHD/bipolar comorbidities (Kollins 2008), and refractory major depression (e.g., Nelson 2007;

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Ishii *et al.* 2008; Ravindran *et al.* 2008). Methylphenidate is also given in conjunction with SSRIs as an acceleration treatment (e.g., Lavretsky *et al.* 2003), or to treat sexual dysfunction related to SSRIs (e.g., Csoka *et al.* 2008). Moreover, the increasing use of methylphenidate as a “cognitive enhancer” (Greely *et al.* 2008) to study or for recreational purposes (Kollins *et al.* 2001; Swanson and Volkow 2008; Steiner and Van Waes 2012) constitutes an additional source for potential uncontrolled co-exposure in patients treated with SSRI antidepressants. This is of concern because the molecular effects of such drug combinations are largely unknown.

Methylphenidate changes the function of cortico-basal ganglia circuits. Some of these changes are mediated by altered gene regulation in projection neurons of the striatum (Yano and Steiner 2007). These molecular effects are mainly a result of excessive stimulation of dopamine receptors (Yano *et al.* 2006; Alburges *et al.* 2011), but glutamate and serotonin also play a role in psychostimulant-induced gene regulation (see Steiner 2010). Methylphenidate binds to and blocks dopamine (and norepinephrine) transporters, but, contrary to cocaine, does not affect serotonin function (see Yano and Steiner 2007). This may explain why methylphenidate mimics many but not all gene regulation effects of cocaine (a dopamine/norepinephrine/serotonin reuptake inhibitor) (Yano and Steiner 2007). In agreement with this notion, we recently demonstrated that fluoxetine and citalopram, two SSRI antidepressants that increase serotonin function, potentiate methylphenidate-induced expression of immediate-early genes (transcription factors; *zif* 268, *c-fos*) in the striatum and render these molecular changes more cocaine-like (Steiner *et al.* 2010; Van Waes *et al.* 2010). Present throughout most of the striatum, this potentiation was most robust in its sensorimotor parts.

These immediate-early genes serve as molecular markers to identify sites and magnitudes of drug actions. Consistent with the observed regional distribution of the potentiation, the methylphenidate + SSRI combination also enhanced behavioral stereotypies, which are associated with dysfunction in sensorimotor striatal circuits (Van Waes *et al.* 2010). Moreover, given their role as transcription factors, these gene products may mediate drug-induced neuroplastic changes (Knapska and Kaczmarek 2004). Indeed, such acute gene induction in striatal circuits is directly correlated with molecular changes in these circuits after repeated treatments, as shown for methylphenidate (e.g., Brandon and Steiner 2003; Cotterly *et al.* 2007) and cocaine (e.g., Steiner and Gerfen 1993; Willuhn *et al.* 2003; Unal *et al.* 2009). These acute effects thus also identify circuits prone for neuroplastic changes induced by chronic treatments.

Gene regulation in the striatum induced by psychostimulants such as cocaine occurs preferentially in the subtype of neurons that express D1 dopamine receptors and project to the substantia nigra (direct pathway neurons) (Steiner 2010; Lobo and Nestler 2011). This selectivity was first demonstrated by studies that assessed drug actions on neuropeptides that are differentially localized in the different striatal output pathways (Steiner and Gerfen 1998). Direct pathway neurons predominantly express the neuropeptides substance P and dynorphin. In contrast, the other subtype of striatal projection neurons, those that mostly express D2 receptors and project to the globus pallidus (indirect pathway), contain enkephalin. These neuropeptides have often served as markers to differentiate effects of experimental manipulations between these striatal output pathways (Steiner and Gerfen 1998). Thus, drugs such as cocaine and amphetamine produce robust changes in expression of substance P and dynorphin (i.e., the direct pathway), while expression of enkephalin (i.e., the indirect pathway), is less affected (Yano and Steiner 2007; Steiner 2010).

In the present study, we assessed in adolescent rats the effects of the methylphenidate + fluoxetine combination on these neuropeptide markers in order to determine which striatal

output pathway is affected by this drug treatment. Moreover, to determine which functional domains are involved, gene expression was mapped, by *in situ* hybridization histochemistry, in 23 striatal sectors mostly defined by their predominant cortical inputs (see Willuhn *et al.* 2003). These sectors designate specific corticostriatal circuits. Our results show that fluoxetine robustly potentiates methylphenidate-induced expression of substance P and dynorphin, but not enkephalin, suggesting selective effects on the direct pathway. Fluoxetine potentiation was most robust in sensorimotor striatal circuits.

MATERIALS AND METHODS

Subjects

Male Sprague–Dawley rats (35 days old at the time of the drug treatment; Harlan, Madison, WI, USA) were housed 2 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 a single intraperitoneal injection of vehicle (V), methylphenidate HCl (2 mg/kg, MP2, or 5 mg/kg, MP5; in 0.02% ascorbic acid, 1 ml/kg; Sigma, St. Louis, MO, USA), fluoxetine HCl (5 mg/kg, FLX; Sigma), or methylphenidate plus fluoxetine (n=5–8 per group).

Tissue preparation and *in situ* hybridization histochemistry

The rats were killed with CO₂ 90 min after the 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 [35S]-dATP as described earlier (Steiner and Kitai 2000). The probes had the following sequence: substance P, complementary to bases 128–175, GenBank accession number X56306; dynorphin, bases 862–909, M10088, and enkephalin, bases 436–483, M28263. 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

Striatal gene expression was assessed in sections from three rostrocaudal levels, rostral (approximately +1.6 mm relative to bregma, Paxinos and Watson 1998), middle (+0.4) and caudal (–0.8) (Fig. 1), in a total of 23 sectors mostly defined by their predominant cortical

inputs (see Willuhn *et al.* 2003). Eighteen of these sectors (Fig. 2) represent the caudate-putamen and 5 represent the nucleus accumbens (medial and lateral core, medial, ventral and lateral shell) (see Yano and Steiner 2005a).

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. Maximal hybridization signal is black.

Statistics

Treatment effects were determined by two-factor ANOVA with methylphenidate (0, 2, and 5 mg/kg) and fluoxetine (0, 5 mg/kg) as between-subject variables. Newman-Keuls post hoc tests were used to describe differences between individual groups (Statistica, StatSoft, Tulsa, OK, USA). For illustrations of topographies (maps, Fig. 2), significant ($P < 0.05$) changes in gene expression were expressed as the percentage of the maximal change observed for that particular probe (% max.). The changes in neuropeptide expression (substance P, dynorphin, enkephalin) and those in immediate-early gene expression (zif 268, c-fos; as reported in Van Waes *et al.* 2010) in these 23 striatal sectors were compared by Pearson correlations.

RESULTS

Methylphenidate, but not fluoxetine, increases substance P expression in the striatum

Fluoxetine (5 mg/kg) alone had no statistically significant effect on the expression of any of the assessed neuropeptides. However, for dynorphin, several sectors showed a tendency for decreased expression after fluoxetine treatment. To facilitate a comparison of the effects of methylphenidate + fluoxetine versus methylphenidate alone across the different neuropeptides, data were thus expressed relative to their respective controls (vehicle for methylphenidate, fluoxetine alone for methylphenidate + fluoxetine).

Consistent with our previous findings (Yano and Steiner 2005b), administration of methylphenidate alone induced a dose-dependent increase in substance P expression most prominently in the middle and caudal striatum (Figs. 1, 2, 3). Whereas the lower dose (2 mg/kg) produced tendencies that did not reach statistical significance (Fig. 3), the higher dose (5 mg/kg) caused a significant increase in six of the 23 striatal sectors (middle level: medial, dorsal, dorsolateral, and central sectors; caudal: medial, dorsal; Figs. 2 and 3).

In contrast, and also in accordance with our previous study (Yano and Steiner 2005b), neither dynorphin nor enkephalin expression was significantly affected by 2–5 mg/kg of methylphenidate alone, although several sectors showed a tendency for increased dynorphin expression with the higher dose ($P > 0.05$ for all sectors) (Figs. 1, 2, 3).

Fluoxetine potentiates methylphenidate-induced substance P and dynorphin, but not enkephalin, expression

Although fluoxetine (5 mg/kg) alone had no significant effect on neuropeptide expression (see above), when given in conjunction with methylphenidate, the SSRI robustly potentiated methylphenidate-induced substance P and dynorphin expression (Figs. 1, 2, 3). Statistically significant potentiation was observed for the high dose of methylphenidate (5 mg/kg) (Figs.

2, 3). For substance P (Fig. 2A), this fluoxetine potentiation was reflected by significantly increased expression in 12 of the 23 striatal sectors after the methylphenidate + fluoxetine (MP5+FLX) treatment, as opposed to only 6 sectors after methylphenidate alone (MP5). Moreover, substance P induction was significantly more robust in 9 sectors in the MP5+FLX group, compared with the MP5 group (POT5; Fig. 2A).

Similarly, for dynorphin (Fig. 2B), the methylphenidate + fluoxetine (MP5+FLX) combination significantly increased expression in 7 striatal sectors, compared with 0 sectors for methylphenidate alone (MP5) (Figs. 2B and 3). The direct comparison of these two groups showed significantly greater dynorphin induction in the MP5+FLX group than in the MP5 group in 9 of the 23 striatal sectors (POT5; Fig. 2B).

In contrast to substance P and dynorphin, enkephalin expression in the striatum was not affected by the methylphenidate + fluoxetine combination ($P>0.05$ for all striatal sectors; Figs. 2C and 3).

Regarding the regional selectivity, our results show that the fluoxetine potentiation of methylphenidate-induced neuropeptide expression was present on all three rostrocaudal levels and occurred in many sectors of the caudate-putamen (Fig. 2). Indeed, our correlation analysis indicated that the regional distribution of the potentiation was similar for substance P and dynorphin ($r=0.560$, $P<0.01$, Fig. 4C). In contrast, in the various nucleus accumbens sectors, no effects of methylphenidate, either alone or in combination with fluoxetine, were found for neuropeptide expression (Table 1).

Regional distribution of fluoxetine potentiation: neuropeptides versus immediate early genes

We recently reported that fluoxetine also potentiates methylphenidate-induced immediate-early gene expression (zif 268, c-fos) in corticostriatal circuits (Van Waes *et al.* 2010). The expression of these immediate-early genes has a greater dynamic range than that of neuropeptides and thus allows a more accurate determination of the effects in individual striatal sectors (corticostriatal circuits). Our results with these immediate-early gene markers showed that the most robust effects of the methylphenidate + fluoxetine combination occurred in sensorimotor circuits of the caudate-putamen (Van Waes *et al.* 2010) (Fig. 4A).

It was thus of interest to compare the regional distribution of the potentiation for neuropeptides with that of these immediate-early genes. This analysis also allowed a comparison of gene regulation by methylphenidate + fluoxetine at two different time points, 40 min (zif 268, c-fos; Van Waes *et al.* 2010) versus 90 min (neuropeptides, present results) after drug administration. Our results show that, across the 23 striatal sectors, the regional distribution of the potentiation for both substance P and dynorphin induction was positively correlated with that of the immediate-early genes c-fos and zif 268 (substance P x c-fos, $r=0.511$, $P<0.05$; substance P x zif 268, $r=0.542$, $P<0.01$, Fig. 4B; dynorphin x c-fos, $r=0.433$, $P<0.05$; dynorphin x zif 268, $r=0.520$, $P<0.05$). Indeed, as was the case for the immediate-early genes (Fig. 4A), the potentiation of neuropeptide induction was greatest in sensorimotor sectors (Fig. 4B, C). The regional distribution of the fluoxetine potentiation was thus invariant across survival times (40 vs. 90 min) and gene classes (neuropeptides vs. immediate-early genes) and was maximal in sensorimotor striatal circuits.

DISCUSSION

The goal of this study was to evaluate the effects of concomitant administration of the psychostimulant methylphenidate together with the SSRI fluoxetine on gene regulation of the striatal neuropeptides substance P, dynorphin and enkephalin. These neuropeptides are

cell type markers for the direct and indirect output pathways (i.e., striatonigral and striatopallidal projection neurons, respectively), but they also serve as neurotransmitters in these projection neurons (Steiner and Gerfen 1998) and are thought to participate in addiction processes (e.g., Shippenberg *et al.* 2007; see below). We here demonstrate that the SSRI fluoxetine potentiates methylphenidate-induced substance P expression. In addition, while methylphenidate alone in the present dose-range did not alter dynorphin mRNA levels, the combination of methylphenidate + fluoxetine increased the expression of this opioid peptide. In contrast, enkephalin expression was not affected by these drug treatments. We conclude that changes in neuropeptide regulation produced by methylphenidate are exacerbated by fluoxetine and that this potentiation selectively occurs in direct pathway neurons.

Fluoxetine potentiates molecular effects of methylphenidate in the striatum: neuropeptides vs. immediate-early genes

The effects of psychostimulants such as cocaine and amphetamine on striatal gene regulation are well established (see Yano and Steiner 2007; Steiner and Van Waes 2012). For example, acute and repeated administration of cocaine increases the expression of substance P, dynorphin and enkephalin (Yano and Steiner 2007). While the effects on substance P and dynorphin expression are robust, those on enkephalin are more modest (see Yano and Steiner 2005b). We recently showed that, similar to cocaine, acute methylphenidate treatment (5–10 mg/kg) also robustly enhanced substance P expression (Yano and Steiner 2005b). In contrast, methylphenidate in this dose range had minimal or no effects on dynorphin and enkephalin expression (Yano and Steiner 2005b). Studies using repeated methylphenidate treatments confirmed such differential effects. For example, a once-daily treatment with 10 mg/kg for 7 days (Brandon and Steiner 2003) resulted in significant blunting (repression) of substance P inducibility (similar to cocaine; Steiner and Gerfen 1993) and produced a moderate increase in dynorphin mRNA levels, while leaving enkephalin expression unchanged (Brandon and Steiner 2003). An increase in dynorphin peptide levels in both the striatum and the target region of the direct pathway, the substantia nigra, was found after a more aggressive methylphenidate treatment (4 injections of 10 mg/kg over 6h; Alburges *et al.* 2011).

In the present study, we confirmed and extended our earlier findings and addressed the mechanisms underlying the neuropeptide regulation by psychostimulants. Similar to our previous results (Yano and Steiner 2005b), 5 mg/kg (but not 2 mg/kg) of methylphenidate significantly increased substance P expression in the striatum. This gene induction was amplified by co-administration of fluoxetine (5 mg/kg), which by itself had no effect. A similar potentiation of gene regulation was seen for dynorphin. While a single methylphenidate injection (5 mg/kg) failed to produce significant changes in dynorphin expression, the addition of fluoxetine to methylphenidate resulted in a robust increase in dynorphin mRNA levels on all three rostrocaudal levels of the striatum. These effects thus mirror the previously described fluoxetine potentiation of methylphenidate-induced immediate-early gene expression (Steiner *et al.* 2010; Van Waes *et al.* 2010). Together, these findings indicate that enhancing serotonin action (SSRI) in conjunction with dopamine (methylphenidate) produces more cocaine-like gene regulation (dynorphin effects; see Fig. 1). These observations are in line with previous results showing that, while dopamine is critical, serotonin contributes significantly to cocaine-induced regulation of neuropeptide (dynorphin) and immediate-early gene expression in the striatum (e.g., Bhat and Baraban 1993; Horner *et al.* 2005).

Fluoxetine potentiation of striatal neuropeptide expression: most robust in sensorimotor striatum and selective for direct pathway

The regional distribution of methylphenidate-induced gene regulation in the striatum is similar to that induced by cocaine and amphetamine; gene regulation preferentially occurs in the sensorimotor striatum (Yano and Steiner 2005a; Cotterly *et al.* 2007). Our previous study demonstrated that the same is true for fluoxetine potentiation of methylphenidate-induced immediate-early gene expression (zif 268, c-fos) (Van Waes *et al.* 2010). Our present results extend this finding to the neuropeptides substance P and dynorphin. While again not restricted to the sensorimotor striatum, this potentiation was most pronounced in sensorimotor sectors. The potential functional significance of this effect is addressed below.

Regarding the cell types affected, our previous findings (Brandon and Steiner 2003; Yano and Steiner 2005a, 2005b) indicated that methylphenidate-induced gene regulation occurs selectively in neurons of the direct striatal output pathway (substance P, dynorphin; see above). This is in agreement with results by others showing that repeated methylphenidate treatment increases FosB expression in direct, but not in indirect, pathway neurons (Kim *et al.* 2009). This was demonstrated in transgenic mice that had these neurons tagged by bacterial artificial chromosome-driven expression of enhanced green fluorescent protein under the control of D1 (direct pathway) or D2 receptor promoters (indirect pathway) (Kim *et al.* 2009).

Our present study investigated which striatal output pathway is affected by the methylphenidate + fluoxetine combination treatment. We assessed drug effects on the neuropeptide markers substance P, dynorphin and enkephalin. While a segregation of these neuropeptides between the two pathways is not absolute, a principal colocalization of substance P/dynorphin and enkephalin mRNAs with molecules selectively contained in, respectively, direct and indirect pathway neurons (D1 and D2 receptors, retrograde tracers) has been demonstrated by a variety of molecular techniques (Gerfen and Young 1988; Gerfen *et al.* 1990; Gerfen *et al.* 1991; Le Moine and Bloch 1995; Surmeier *et al.* 1996; Heiman *et al.* 2008; see also Reiner and Anderson 1990; Steiner 2010).

Our results suggest that, based on these neuropeptide markers, the methylphenidate + fluoxetine effects are direct pathway-selective. Potentiation of substance P and dynorphin induction was found in nine of the 23 striatal sectors. In contrast, none of these 23 sectors showed a drug effect on enkephalin, the indirect pathway marker. This is contrary to cocaine and amphetamine, which have significant (if more moderate) effects also on enkephalin expression (e.g., Hurd and Herkenham 1993; Steiner and Gerfen 1993; Wang and McGinty 1996; Spangler *et al.* 1997). The lack of a methylphenidate + fluoxetine effect on enkephalin expression is likely not related to the temporal characteristics of enkephalin induction; enkephalin expression is robustly enhanced by various acute drug treatments, for example, by D2 receptor blockers (e.g., Steiner and Gerfen 1999), within the same time frame. These findings thus indicate that a SSRI given in conjunction with methylphenidate does not change this pathway selectivity. Therefore, given that cocaine affects both pathways (see above), fluoxetine, at least at the dose tested here, does not seem to render methylphenidate effects more cocaine-like in this regard.

What are the mechanisms underlying these differential pathway effects for cocaine vs. methylphenidate + fluoxetine? Gene regulation in the striatum is mediated by complex interactions between multiple neurotransmitter systems (Keefe and Horner 2010), including dopamine, serotonin and glutamate (input from the cortex, which is regulated by norepinephrine) (see Steiner 2010). The above drugs vary in their absolute and relative affinities for their targets (i.e., dopamine, serotonin, norepinephrine transporters). For example, cocaine and methylphenidate have different relative affinities for the dopamine vs.

norepinephrine transporters (cocaine is more dopamine transporter-selective than methylphenidate; see Yano and Steiner 2007). Thus, even with overall similar neurochemical profiles, to some degree differential molecular effects of these drugs are not surprising.

Differential gene regulation between direct and indirect pathway neurons could also reflect differential receptor expression by these neurons. For example, it is well established that dopamine has opposite effects on gene expression in these neurons, based on differential expression of D1 and D2 receptor subtypes (Gerfen *et al.* 1990; Gerfen *et al.* 1991). Thus, psychostimulant-induced dopamine action facilitates gene expression in direct pathway neurons (via stimulation of D1 receptors), but inhibits gene expression in indirect pathway neurons (via D2 receptors) (for review, see Steiner 2010). On the other hand, glutamate (cortical input) facilitates gene expression in both pathways. Therefore, in direct pathway neurons, glutamate input acts synergistically with dopamine input (D1 receptor stimulation) to increase gene expression, whereas in indirect pathway neurons, the outcome reflects the balance between facilitation by glutamate and inhibition by dopamine via D2 receptor stimulation (Steiner 2010). This antagonistic regulation between glutamate and dopamine (D2) is probably the reason for the comparatively minor molecular effects of psychostimulants in indirect pathway neurons (enkephalin).

The above scenario highlights the importance of cortical input for striatal gene regulation by dopamine/psychostimulants (e.g., Cenci and Björklund 1993; Hanson *et al.* 1995; Ferguson and Robinson 2004). Similar to cocaine, methylphenidate induces immediate-early gene expression also throughout the cortex, and this cortical response is correlated with induction in striatal target sectors of individual cortical regions (Yano and Steiner 2005a; Cotterly *et al.* 2007; Van Waes *et al.* 2010), which probably reflects (some) increased activity in these corticostriatal circuits. Interestingly, in our previous study, we observed a dissociation between cortical and striatal gene regulation after the SSRI treatment. Thus, the robust SSRI potentiation of striatal immediate-early gene expression was not accompanied by any potentiation of cortical immediate-early gene expression (Van Waes *et al.* 2010). This finding suggested that the SSRI potentiation in the striatum was not produced by enhanced corticostriatal input. Given the critical importance of cortical input for increasing gene expression in indirect pathway neurons (see above), the present finding of a lack of effects on enkephalin expression is consistent with a lack of enhanced cortical input after SSRI administration.

What then drives the potentiated gene induction in direct pathway neurons? Serotonin is known to enhance activity in the mesostriatal and mesolimbic/cortical dopamine pathways, by complex interactions in both the dopamine terminal regions and the somatodendritic areas in the midbrain (for reviews, see Muller and Huston 2006; Weikop *et al.* 2007; Bubar and Cunningham 2008). Consistent with these findings, a facilitatory role for serotonin in dopamine-mediated gene regulation in the striatum has been shown before (Bhat and Baraban 1993; Guerra *et al.* 1998; Gardier *et al.* 2000; Horner *et al.* 2005). Therefore, the SSRI potentiation of methylphenidate-induced gene regulation in the direct pathway could reflect potentiated dopamine action mediated by serotonin receptors in the striatum and/or other brain areas. Future studies will have to clarify the mechanisms that mediate this SSRI potentiation.

Relevance for addiction liability of methylphenidate?

As mentioned above, changes in gene regulation produced by cocaine and amphetamine, as well as by methylphenidate and methylphenidate + fluoxetine, are most robust in the sensorimotor striatum. Sensorimotor corticostriatal circuits are critical for stimulus-response (habit) learning, and drug-induced molecular changes in these circuits are implicated in

aberrant habit formation and compulsive behavior in drug addiction (Berke and Hyman 2000; Everitt and Robbins 2005). For example, it has been shown that the sensorimotor striatum mediates relapse to drug seeking in animal models (Vanderschuren *et al.* 2005; Fuchs *et al.* 2006; See *et al.* 2007). Methylphenidate-induced molecular changes in these circuits may thus contribute to the previously observed facilitation of psychostimulant seeking and taking (Brandon *et al.* 2001). Our findings suggest that fluoxetine may enhance such effects of methylphenidate.

Regarding the underlying mechanisms, substance P and dynorphin are neurotransmitters released from direct pathway neurons. The functional significance of altered substance P expression after psychostimulant treatments in these neurons remains unclear. However, there is evidence that increased dynorphin function in striatal neurons might participate in the development of addiction processes (e.g. see Steiner 2010). Notably, increased dynorphin expression has also been found in human cocaine addicts (Hurd and Herkenham 1993; Frankel *et al.* 2008).

Animal studies indicate that dynorphin and enkephalin act, at least in part, as negative feedback systems ('brake') to limit dopamine and glutamate input to striatal neurons and help maintain systems homeostasis (Hyman and Nestler 1996; Steiner and Gerfen 1998; Steiner 2010). Best established is that dynorphin inhibits dopamine release via kappa opioid receptors on dopamine terminals and dendrites/cell bodies (e.g., Di Chiara and Imperato 1988; Spanagel *et al.* 1992; Marinelli *et al.* 1998; see Shippenberg *et al.* 2007, for review). It is thought that increased dynorphin signaling after psychostimulant treatments excessively inhibits such inputs to the striatum (Hyman and Nestler 1996; Steiner and Gerfen 1998; Shippenberg *et al.* 2007). As a consequence, during early withdrawal, increased dynorphin function may contribute to somatic signs of withdrawal, such as dysphoria, anxiety, anhedonia and depression (Nestler and Carlezon 2006; Shippenberg *et al.* 2007). Such effects are thought to contribute to maintenance of drug use and/or to reinstatement of use during the early phase of abstinence (see Shippenberg *et al.* 2007). This is one mechanism by which increased dynorphin function following methylphenidate exposure might contribute to its addiction liability. In this scenario, the addition of fluoxetine to methylphenidate would be expected to increase the addiction liability of methylphenidate.

There is behavioral evidence to support this notion, but the functional consequences of combined methylphenidate + fluoxetine exposure may be more complex. A recent study investigated the long-term effects of juvenile treatment (postnatal days 20 to 34) with a methylphenidate + fluoxetine combination (2.0 and 2.5 mg/kg, respectively; Warren *et al.* 2011). Results showed that these rats, as adults, displayed increased sensitivity to drug (cocaine) and sucrose reward (place preference conditioning), as well as enhanced reactivity to stress- and anxiety-eliciting situations (Warren *et al.* 2011). At the neuronal level, these animals showed various molecular changes involving ERK signaling in the ventral tegmental area, which seemed to be mediating at least some of the altered behavioral responses (stress susceptibility). Future studies will have to determine whether methylphenidate + SSRI combinations also enhance drug-seeking/taking tendencies and/or facilitate relapse, and the role played by molecular changes in striatal circuits.

Conclusions

The present study provides further information on the molecular effects of co-exposure to methylphenidate + fluoxetine, which is increasingly used in the treatment of several mental disorders. Collectively, our findings show that fluoxetine potentiates methylphenidate-induced gene regulation in striatal neurons. This potentiation is not only region specific (predominant in sensorimotor striatum), but appears also neuron specific (direct striatal output pathway). These effects of the methylphenidate + fluoxetine combination mimic

some effects of cocaine and amphetamine (regional distribution, dynorphin expression), but differ in others (pathway selectivity). The potentiated changes in gene regulation suggest that the addition of fluoxetine may enhance the addiction liability of methylphenidate.

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Figure 1.

Fluoxetine potentiates methylphenidate-induced substance P and dynorphin expression in the striatum. Illustrations of film autoradiograms depict expression of (A) substance P and (B) dynorphin in coronal sections from the rostral, middle and caudal striatum in rats that were treated with vehicle (V), fluoxetine (5 mg/kg; FLX), methylphenidate (5 mg/kg; MP5), or methylphenidate + fluoxetine (MP5+FLX) and were killed 90 min after drug administration. (C) For comparison with MP5+FLX (B), dynorphin expression in the middle striatum after acute cocaine administration (25 mg/kg; Brandon and Steiner 2003) is shown. Note the similar regional patterns for the two drug treatments. The maximal hybridization signal is black.

Figure 2.

Topography of fluoxetine-potentiated neuropeptide induction by methylphenidate. Maps depict the distribution of (A) substance P, (B) dynorphin, and (C) enkephalin expression in the rostral, middle and caudal striatum after an injection of methylphenidate (5 mg/kg; MP5) or methylphenidate + fluoxetine (5 mg/kg each; MP5+FLX). The potentiation (POT5) denotes the difference between methylphenidate + fluoxetine and methylphenidate alone. The data are expressed relative to the maximal increase for each neuropeptide (% of max.). Sectors with significant differences vs. respective control group ($P < 0.05$) are coded as indicated. Sectors without significant effects are in white. Abbreviations: caudate-putamen: 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 Yano and Steiner 2005b.

Figure 3.

Fluoxetine potentiation of methylphenidate-induced neuropeptide expression. Mean density values (mean±SEM, expressed relative to their respective control group; V or FLX) for substance P, dynorphin, and enkephalin expression in rats that received an injection of vehicle (V), fluoxetine (5 mg/kg; FLX), methylphenidate (2 or 5 mg/kg; MP2, MP5), or methylphenidate + fluoxetine (MP2+FLX, MP5+FLX) (n=5–8 per group) are depicted for 5 middle striatal sectors. Abbreviations: m, medial; d, dorsal; dl, dorsolateral; vl, ventrolateral; c, central; * P<0.05, ** P<0.01, *** P<0.001 vs. respective control group (V or FLX); # P<0.05, ## P<0.01, ### P<0.001, MP+FLX vs. MP (potentiation).

Figure 4.

The potentiation of gene induction for dynorphin and substance P displays a regional distribution throughout the striatum similar to that for c-fos and zif 268 (Van Waes *et al.* 2010). Scatterplots show correlations between (A) c-fos and zif 268 ($r=0.840$), (B) substance P and zif 268 ($r=0.542$), and (C) substance P and dynorphin ($r=0.560$) for 5 mg/kg of methylphenidate (POT5) in the 23 striatal sectors (open diamonds, sensorimotor; full circles, non-sensorimotor). The data are expressed as percentage of the maximal potentiation for each gene. Note that the values for c-fos and zif 268 represent gene regulation at 40 min after drug administration (Van Waes *et al.* 2010), while those for substance P and dynorphin were obtained after a 90 min survival period (present study). Potentiation was most robust in sectors of the sensorimotor striatum (open diamonds) (see Fig. 2). $** P < 0.01$, $*** P < 0.001$.

Effects of fluoxetine, methylphenidate and methylphenidate + fluoxetine on substance P, dynorphin and enkephalin expression in the nucleus accumbens.

Table 1

	V	FLX	MP2	MP2 + FLX	MP5	MP5 + FLX
substance P	mC	100±7.2	100±7.8	99±6.5	93±9.9	103±11.1
	IC	100±3.5	100±10.2	99±9.1	105±9.3	81±11.3
	mS	100±5.8	100±4.0	107±3.2	99±4.8	104±5.4
	vS	100±13.9	100±11.8	84±10.2	99±10.2	73±13.1
	IS	100±4.8	100±4.1	101±7.9	95±4.6	95±5.9
dynorphin	mC	100±4.9	100±4.7	97±2.6	104±5.2	89±3.8
	IC	100±4.4	100±5.7	94±2.3	108±4.2	95±3.8
	mS	100±3.6	100±2.9	96±2.4	100±2.5	98±3.3
	vS	100±3.4	100±6.9	97±4.6	107±4.8	91±6.8
	IS	100±5.0	100±6.9	102±3.2	102±2.4	97±5.7
enkephalin	mC	100±6.4	100±7.5	106±6.5	93±3.9	103±4.6
	IC	100±4.5	100±3.1	102±5.4	95±3.9	97±3.7
	mS	100±6.5	100±3.2	94±4.2	110±3.8	89±6.1
	vS	100±3.2	100±5.8	101±4.4	111±3.2	88±2.8
	IS	100±1.7	100±5.1	102±4.3	101±2.1	99±2.1

Mean density values (mean±SEM, expressed relative to their respective control group) measured in the different sectors of the nucleus accumbens in rats that received an injection of vehicle (V), fluoxetine (5 mg/kg; FLX), methylphenidate (2 or 5 mg/kg; MP2, MP5), or methylphenidate + fluoxetine (MP2+FLX, MP5+FLX).

Abbreviations: mC, medial core; IC, lateral core; mS, medial shell; vS, ventral shell; IS, lateral shell. All P>0.05.