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Fluoxetine potentiates methylphenidate-induced behavioral responses: Enhanced locomotion or stereotypies and facilitated acquisition of cocaine self-administration

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Abstract

The medical psychostimulant methylphenidate (MP) is used to treat attention-deficit hyperactivity disorder and recreationally as a “cognitive enhancer”. MP is a dopamine reuptake inhibitor, but does not affect serotonin. Serotonin contributes to addiction-related gene regulation and behavior. Previously, we showed that enhancing serotonin action by adding a selective serotonin reuptake inhibitor, fluoxetine (FLX), to MP potentiates MP-induced gene regulation in striatum and nucleus accumbens, mimicking cocaine effects. Here, we investigated the behavioral consequences of MP+FLX treatment. Young adult male rats received MP (5 mg/kg, i.p.) or MP+FLX (5 mg/kg each) daily for 6-8 days. Behavioral effects were assessed in an open-field test during the repeated treatment. Two weeks later the motor response to a cocaine challenge (25 mg/kg) and the rate of acquisition of cocaine self-administration behavior were determined. Our results demonstrate that FLX potentiates effects of MP on open-field behavior. However, we found differential behavioral responses to MP+FLX treatment, as approximately half of the rats developed high rates of focal stereotypies (termed “MP+FLX/high reactivity” group), whereas the other half did not, and only showed increased locomotion (“MP+FLX/low reactivity” group). Two weeks later, cocaine-induced locomotion and stereotypies were positively correlated with MP+FLX-induced behavior seen at the end of the repeated MP+FLX treatment. Moreover, the MP+FLX/high reactivity group, but not the low reactivity group, showed facilitated acquisition of cocaine self-administration.

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Author contributions

Conceptualization: HS, MM; Data curation: LL, JAB, MM, HS; Formal analysis: HS, MM; Funding acquisition: HS, MM; Project administration and supervision: HS, MM. HS wrote the original draft, and LL, JAB, MM and HS reviewed and edited the report.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

These results demonstrate that repeated MP+FLX treatment can facilitate subsequent cocaine taking behavior in a subpopulation of rats. These findings suggest that MP+FLX exposure in some individuals may increase the risk for psychostimulant use later in life.

Keywords

Psychostimulant; Serotonin reuptake inhibitor; Dopamine; Serotonin; Stereotypies; Addiction; Substance abuse

1. Introduction

Findings in preclinical studies demonstrate that exposure to psychotropic drugs can produce long-lasting neurobehavioral changes that may facilitate addiction and other psychiatric disorders later in life [1–3]. Increasing numbers of individuals are being exposed to psychotropic drugs such as the medical psychostimulant methylphenidate (MP, Ritalin) for the treatment of certain mental disorders or as a "cognitive enhancer" [4,5]. For example, in 2008 approximately 3 million children between 4 and 17 years of age were treated with psychostimulants for attention-deficit hyperactivity disorder (ADHD) in the US alone [6]. In addition, there is increasing misuse of MP for recreational purposes or as a cognitive enhancer by children, students and adults [4,5,7,8]. According to surveys, up to 20 % of college students indicated such nonmedical use to improve concentration, stay awake to study, or party (e.g., [4,8,9]). The 2014 National Survey on Drug Use and Health found that more than 1 million individuals age 12 and older in the US admitted to current nonmedical use of prescription stimulants, continuing a decade-long trend [10]. Recreational use is more problematic, as it often involves high-level exposure due to intravenous administration, snorting etc. [4,11].

Co-exposure to more than one psychotropic drug also frequently occurs. For example, combinations of MP and a selective serotonin reuptake inhibitor (SSRI) are used to treat comorbid ADHD/major depressive and anxiety disorders [12–14]. These treatments typically continue into adulthood. MP+SSRI concomitant treatments are also indicated for a number of other conditions in adults, including augmentation therapy in major depressive disorder [15–17], augmentation of partial response to stimulants [18], reversal of stimulant-induced insomnia [19] and others. Aside from depression, SSRIs are also used to treat several other disorders, including post-traumatic stress disorder, obsessive-compulsive disorder, phobias, anxiety, and eating disorders. Unintended co-exposure thus occurs in patients taking SSRIs who use MP recreationally or as a cognitive enhancer [4,20].

Long-term neurobehavioral changes induced by psychotropic drugs are typically mediated by altered gene regulation. A host of research over the last decade has demonstrated that MP produces changes in the expression of hundreds of genes in rodents (for reviews, see [11,21]), although such gene regulation tends to be more limited than that induced by illicit psychostimulants such as cocaine [21]. These differential molecular effects are likely related to the differential neurochemical effects of these psychostimulants. Cocaine blocks the reuptake of dopamine and serotonin, while MP only blocks the reuptake of dopamine, but not of serotonin (e.g., [22]; see [21]). While dopamine is critical, serotonin contributes

to the impact of cocaine on gene regulation [23–25] and behavior (e.g., [26]). Interactions between dopamine and serotonin also occur with MP+SSRI combinations. Our findings from a series of studies show that SSRIs [fluoxetine (FLX) or citalopram], in doses that by themselves have no effect on gene regulation, potentiate MP-induced gene regulation in the rat striatum (for review, see [27]). In as far as the affected genes are considered part of the molecular basis for addiction [11], these results suggest an increased addiction liability for MP+SSRI combinations.

Less is known on the behavioral effects of MP+SSRI combinations in rodents. One early study found that acute treatment with FLX increased the locomotor stimulant effects of MP in rats [28]. Further work demonstrated that repeated MP+FLX treatment in juvenile rats facilitated cocaine-induced place preference conditioning when these rats were adults [29]. Our previous studies showed that adding FLX to MP treatment produced increased levels of behavioral stereotypies in an open-field test [30,31]. This same treatment had no overall effect on subsequent cocaine self-administration, but we observed variable responses between rats, whereby some showed heightened self-administration behavior and others not [32], indicating that MP+FLX treatment could differentially affect subpopulations of rats. Together with the facilitated place preference conditioning [29], these findings suggest that MP+FLX combinations may enhance cocaine-induced motivated behavior.

In the present study, we further investigated the behavioral effects of MP+FLX combinations. We examined behavioral changes in the open-field (locomotion, stereotypies) during the repeated MP+FLX treatment, as well as subsequent behavioral responses to cocaine, either in an open-field test or in the cocaine self-administration model. We assessed the effects of intermittent treatment (once daily, 6–8 days) with MP and FLX in doses of 5 mg/kg each, which produces robust changes in addiction-related gene regulation [27]. The present behavioral analysis was performed in young adult rats, as patients in this age group are most often exposed to MP+FLX combinations and have access to illicit psychostimulants such as cocaine [10,33].

2. Methods

2.1. Subjects

Male Sprague–Dawley rats [300–330 g (~10 weeks old) at the beginning of the drug treatment; Harlan, Madison, WI, USA] were housed 2–3 per cage under standard laboratory conditions (12:12 h light/dark cycle; lights on at 07:00 h) with food and water available ad libitum. Experiments were performed between 13:00 and 17:00 h. Prior to the drug treatment, 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.

2.2. Repeated drug treatments

Experiment 1.—Open-field behavior: The repeated drug treatment consisted of an intraperitoneal (i.p.) injection of vehicle (V), methylphenidate HCl (5 mg/kg, MP; in 0.02 % ascorbic acid, 1 ml/kg; Sigma, St. Louis, MO, USA), or methylphenidate plus fluoxetine

(5 mg/kg, FLX; Sigma) (MP+FLX) [30], once daily for 6 days (Fig. 1). This FLX dose is in the clinically relevant range [34,35], while the MP dose is at the upper limit or above of doses used clinically and is thus likely more relevant for MP abuse (see [11]). This treatment was performed in a behavioral laboratory adjacent to the housing room. Drug-induced behavior was measured with a Truscan activity monitoring system (arena, 43 × 43 cm; light beam spacing, 1.3 cm; sampling rate, 500 msec; Coulbourn Instruments, Allentown, PA, USA). Locomotion ("ambulation counts") and local repetitive movements (stereotypies; "stereotypy 1 counts") were assessed. For control purposes, behavior was also video-taped. Before the drug injection, the rat was placed in the open-field arena for a 40-min habituation period. After the injection, the drug-induced behavior was measured for 90 min. Two weeks after the last treatment (Fig. 1), all rats received a cocaine challenge (25 mg/kg, C; cocaine HCl, Sigma) (groups V/C, MP/C, MP+FLX/C; n=7–11) to assess their behavioral response to cocaine. After the cocaine injection, the rat was again placed in the open-field apparatus, and ambulation and stereotypy counts were measured for 40 min.

Experiment 2.—Cocaine self-administration: The drug treatment, once daily for 8 days (Fig. 1), was identical to that in experiment 1, except that behavior (ambulation) was measured with an SDI photobeam activity system (San Diego Instruments, San Diego, CA, USA). Again, the rats were first placed in the activity cages (47 × 25 cm) for a 40-min habituation period and then received an injection of V, MP (5 mg/kg), FLX (5 mg/kg) or MP+FLX (n=6–16), and ambulation was recorded for another 90 min.

2.3. Cocaine self-administration procedures

One week after the last treatment, rats received surgery under isoflurane anesthesia, to implant an intravenous Silastic catheter for subsequent intravenous cocaine self-administration. Briefly, the catheter was inserted in the right external jugular vein and passed subcutaneously to exit the mid-scapular region [36]. After one week of recovery (i.e., 2 weeks after the last drug treatment), these animals started cocaine self-administration training (Fig. 1).

Self-administration training occurred for 10 consecutive days (2h/day) in operant chambers (41 × 24 cm floor area, 21 cm high; MED Associates) equipped with two nose-poke holes on opposing walls. Nose pokes in the "active" hole delivered one infusion of cocaine (fixed-ratio 1, FR1; 150 μg/100 μl/kg). Nose poking in the "inactive" hole was recorded but had no consequences. A successful infusion was signaled by illumination of a light inside the active hole and a timeout period (10–30 s) during which additional nose pokes were recorded but had no consequences.

2.4. Statistics

Treatment effects were determined by one- and two-factor ANOVA. Newman-Keuls post hoc tests were used to describe differences between individual groups (Statistica, StatSoft, Tulsa, OK, USA). Behavioral outcomes were also compared by Pearson correlations.

3. Results

3.1. Experiment 1: open-field behavior

3.1.1. Open-field behavior during repeated MP+FLX treatment ("sensitization")—

On each of the 6 days of repeated treatment, ambulation and stereotypies were measured for 90 min after the drug injection. Time courses from days 1, 3 and 5 are displayed in Fig. 2. Analysis of these behavioral effects showed that, for rats treated with MP+FLX, there were two subgroups that responded differently (Figs. 2 and 3). Rats of one subgroup (termed "MP+FLX/high reactivity" group; n=5) displayed maximally elevated ambulation counts on days 1–2 (compared with MP alone) and increasingly high levels of focal stereotypies from day 3 onwards (Fig. 3); during the periods of high stereotypy counts, ambulation was much reduced (Fig. 2). The other subgroup ("MP+FLX/low reactivity" group; n=6) expressed high levels of ambulatory activity from day 2 onwards and showed some stereotypies only towards the end of the 6-day treatment (Fig. 3). These differences between the two subgroups were most pronounced during min 6–40 after drug administration, when focal stereotypies in the "high reactivity" group peaked and ambulation was suppressed (Fig. 2). Later in the session, as the stereotypies in this group receded, ambulatory activity recovered (Fig. 2). The statistical analysis (Fig. 3) thus focused on the period min 6–40. In contrast to the MP+FLX-treated rats, the MP-treated rats did not show two subgroups.

The statistical analysis revealed the following (Fig. 3).

Ambulation: For ambulation over 6 days (Fig. 3, top left), there were significant main effects of treatment [$F(3,24)=44.27$; $P<0.0001$] and days [$F(5,120)= 5.11$; $P<0.001$] and a significant treatment x days interaction [$F(15,120)=7.16$; $P<0.0001$]. On days 1–2 pooled (Fig. 3, right), the treatment effect [$F(3,24)=30.01$; $P<0.0001$] was produced by significantly increased ambulation counts in all drug-treated groups compared with V controls (all $P<0.001$), as well as by higher ambulation in MP+FLX/high reactivity ($P<0.01$) and MP+FLX/low reactivity ($P<0.05$) groups compared with the MP group. On days 3–4 [$F(3,24)=27.08$; $P<0.0001$], all drug-treated groups displayed significantly more ambulation than V controls ($P<0.01$–0.001), but the MP+FLX/high reactivity group showed significantly lower ($P<0.01$) and the MP+FLX/low reactivity group showed significantly higher counts ($P<0.05$) than the MP group. The MP+FLX/low reactivity group also displayed more ambulation than the MP+FLX/high reactivity group ($P<0.001$). Similarly, on days 5–6 [$F(3,24)=28.89$; $P<0.0001$], the MP group ($P<0.001$) and the MP+FLX/low reactivity group ($P<0.001$), but not the MP+FLX/high reactivity group ($P>0.05$), displayed more ambulation than V controls. The MP+FLX/high reactivity group showed significantly lower counts than the MP group ($P<0.001$), but the MP+FLX/low reactivity group was not different from the MP group ($P>0.05$). Again, the MP+FLX/low reactivity group displayed more ambulation than the MP+FLX/high reactivity group ($P<0.001$).

Stereotypies: For stereotypies over 6 days (Fig. 3, bottom left), there were significant main effects of treatment [$F(3,24)=29.13$; $P<0.0001$] and days [$F(5,120)=10.72$; $P<0.0001$] and a significant treatment x days interaction [$F(15,120)=13.54$; $P<0.0001$]. However, on

days 1–2 (Fig. 3, right), there was no treatment effect [$F(3,24)=0.55$; $P>0.05$]. On days 3–4 [$F(3,24)=51.49$; $P<0.0001$], the MP+FLX/high reactivity group displayed significantly more stereotypies than all the other groups (all $P<0.001$). No other differences were seen. Similarly, on days 5–6 [$F(3,24)=22.59$; $P<0.0001$], the MP+FLX/high reactivity group showed significantly higher stereotypy counts than all other groups (all $P<0.001$). In addition, the MP+FLX/low reactivity group also displayed more stereotypies than V controls ($P<0.05$).

Correlations between ambulation and stereotypies: We used correlation analysis to further compare ambulation and stereotypy counts during the 6-day treatment, for MP+FLX/high reactivity (Fig. 4, red) and MP+FLX/low reactivity (blue) groups pooled. For the total counts in the 90-min sessions over the 6 days, there was a significant negative correlation between ambulation and stereotypies ($r=-0.798$, $P<0.005$) (Fig. 4, left). When considering only the time period with very high stereotypy levels, min 6–40 of the test session, over the 6 days, this correlation was more robust (total 6 days: $r=-0.832$, $P<0.002$) (data not shown). When considering only days 3–4 or 5–6 (highest stereotypy levels), this correlation improved further [days 3–4: 90 min, $r=-0.803$, $P<0.005$; min 6–40, $r=-0.851$, $P<0.001$ (Fig. 4, right); days 5–6: 90 min, $r=-0.827$, $P<0.002$; min 6–40, $r=-0.858$, $P<0.001$] (not shown).

In summary, the two MP+FLX-treated groups differed most from each other and from the MP only-treated group on days 3-4 of the repeated treatment. The MP+FLX/high reactivity group showed significantly more stereotypies than the other groups and less locomotion than the MP+FLX/low reactivity group and the MP group.

3.1.2. Open-field behavior during cocaine challenge—Two weeks after the repeated drug treatment, these rats received an acute cocaine (25 mg/kg) challenge in order to assess the effects of the MP and MP+FLX pretreatments on subsequent cocaine-induced open-field behavior. The behavior (ambulation and stereotypies) was measured for 40 min after cocaine injection, and total counts are presented (Fig. 5). There was a significant main effect of treatment on cocaine-induced ambulation [$F(3,24)=4.44$; $P<0.05$] (Fig. 5, top). This effect reflected significantly lower ambulation counts in the MP+FLX/high reactivity/C group compared with all other groups [vs. V/C ($P<0.01$), vs. MP/C ($P<0.05$) and vs. MP+FLX/low reactivity/C ($P<0.05$)] (Fig. 5, top). There was also a significant main effect of treatment on stereotypies [$F(3,24)=9.50$; $P<0.001$] (Fig 5, bottom). This effect was produced by significantly higher counts in the MP+FLX/high reactivity/C group compared with the V/C ($P<0.001$) and MP/C ($P<0.001$) groups, but not the MP+FLX/low reactivity/C ($P=0.055$) group. The MP+FLX/low reactivity/C group also showed more stereotypies than the V/C ($P<0.05$) and MP/C ($P<0.05$) groups (Fig. 5, bottom). For groups MP+FLX/high reactivity/C and MP+FLX/low reactivity/C pooled, there was again a significant negative correlation between ambulation and stereotypies during the cocaine challenge test ($r=-671$, $P<0.05$) (data not shown).

3.1.3. Relationship between MP+FLX-induced and cocaine challenge-induced open-field behavior—To determine whether and when behavioral responses during the repeated MP+FLX treatment (sensitization) in individual rats predicted the behavioral responses to the subsequent cocaine challenge two weeks later, we used

correlation analysis. The results showed that, for every comparison, behavior during min 6–40 of the repeated treatment sessions (sensitization) better predicted behavior during the cocaine challenge than total counts during the whole 90-min sensitization sessions, and thus only the results for min 6–40 are presented (Fig. 6).

For total activity (min 6–40) over the 6 repeated treatment sessions (sensitization), there were positive correlations between counts during the sensitization sessions and counts during the subsequent cocaine challenge session, for ambulation ($r=0.917$, $P<0.001$) and for stereotypies ($r=0.651$, $P<0.05$) (not shown). However, these effects were dependent on the treatment duration, as the strength of the correlations increased across the repeated treatment sessions. Thus, for counts on days 1–2 of the repeated treatment, there were no significant correlations (sensitization x challenge; ambulation: $r=0.372$, $P>0.05$; stereotypies: $r=0.464$, $P>0.05$) (not shown). For days 3–4, there was a positive correlation for ambulation ($r=0.750$, $P<0.01$), but not for stereotypies ($r=0.477$, $P>0.05$) (Fig. 6, top). For days 5–6, there was a positive correlation for both ambulation ($r=0.825$, $P<0.002$) and stereotypies ($r=0.683$, $P<0.05$) (not shown). This effect was maximal at the end (day 6) of the repeated treatment (ambulation: $r=0.828$, $P<0.002$; stereotypies: $r=0.766$, $P<0.01$) (Fig. 6, bottom). Fig. 6 also shows that, both on day 6 of sensitization (but not days 3–4) and during the cocaine challenge, two animals of the MP+FLX/low reactivity group (blue) displayed high levels of stereotypies, similar to the MP+FLX/high reactivity group (red) (Fig. 6, bottom right).

Overall, therefore, for both ambulation and stereotypies, the behavior shown at the end of the repeated MP+FLX treatment (sensitization period) best predicted the subsequent behavior in the cocaine challenge test 2 weeks later.

3.2. Experiment 2: cocaine self-administration

3.2.1. Open-field behavior during repeated MP+FLX treatment—

In experiment 2, rats were repeatedly treated with V, MP, FLX or MP+FLX for 8 days, and ambulation was assessed for 90 min after each drug administration (stereotypies could not be measured with the SDI activity system used in the self-administration facility). Again, ambulation during min 6–40 of the repeated treatment sessions (sensitization) differentiated best between the treatment groups (Fig. 7). Similar to experiment 1, there were two subgroups in MP+FLX-treated animals, but not with any other treatment. In the MP+FLX/high reactivity subgroup ($n=6$), ambulation was maximal on days 1 or 2 and strongly declined thereafter (Fig. 7, left). In contrast, in the MP+FLX/low reactivity subgroup ($n=9$), ambulation peaked on days 2–3 or later, and leveled off thereafter.

Statistical analysis revealed the following. Over the 8 repeated treatment days, there were significant main effects of treatment [$F(4,42)=48.73$; $P<0.0001$] and days [$F(7,294)=5.66$; $P<0.0001$] and a significant treatment x days interaction [$F(28,294)=5.69$; $P<0.0001$] (Fig. 7, left). On days 1–2 (Fig. 7, right), there was a significant effect of treatment [$F(4,42)=51.85$; $P<0.0001$] that reflected increased ambulation counts (vs. V or FLX controls) in the MP, MP+FLX/high reactivity and MP+FLX/low reactivity groups (all $P<0.001$) (FLX vs. V, $P>0.05$). The MP+FLX/high reactivity and MP+FLX/low reactivity groups also displayed more ambulation than the MP group ($P<0.001$). On days 3–4 [$F(4,42)=52.45$; $P<0.0001$], the MP ($P<0.001$), MP+FLX/high reactivity ($P<0.05$) and MP+FLX/low reactivity groups

(P<0.001), but not FLX, showed more ambulation than V controls. However, the MP+FLX/high reactivity group displayed less ambulation (P<0.05) and the MP+FLX/low reactivity group displayed more ambulation (P<0.001) than the MP group. The MP+FLX/high reactivity group also showed less ambulation than the MP+FLX/low reactivity group (P<0.001). At the end of the treatment (days 7–8) [F(4,42)=13.25; P<0.0001], the MP and MP+FLX/low reactivity groups (P<0.001), but not the FLX or MP+FLX/high reactivity groups, showed more ambulation than V controls. Again, the MP+FLX/high reactivity (but not MP+FLX/low reactivity) groups displayed less ambulation than the MP group (P<0.001). The MP+FLX/high reactivity group also showed less than the MP+FLX/low reactivity group (P<0.001).

In summary, again the two MP+FLX-treated groups differed most from each other and from the MP-treated group on days 3–4. That is, the MP+FLX/high reactivity group showed significantly less locomotion than the MP+FLX/low reactivity group and the MP group, and the low reactivity group showed significantly more locomotion than the MP group.

3.2.2. Acquisition of cocaine self-administration—Two weeks after the repeated drug treatment, the cocaine self-administration training began. This training consisted of 10 sessions (once daily) with 2-h access to cocaine.

Our statistical analysis (Fig. 8) showed that, for total number of cocaine infusions in 2 h over the 10 days, there was no significant main effect of treatment [F(4,42)=1.53; P=0.21], but a significant effect of days [F(9,378)=34.79; P<0.0001] and a significant treatment x days interaction [F(36,378)=1.90; P<0.002] (Fig. 8, top left). Inspection of these data (Fig. 8, top left) indicated that animals in the MP+FLX/high reactivity group tended to acquire cocaine self-administration faster, as their number of infusions reached a maximal level already during training days 4–6 and leveled off thereafter, while the other groups hardly approached this level by the end of the training. Thus, when the 2-h total for days 4–6 was considered, there was a significant effect of treatment [F(4,42)=2.77; P<0.05] (Fig. 8, top right). This effect reflected significantly higher numbers of infusions in the MP+FLX/high reactivity group compared with all the other groups (all P<0.05). All the other groups showed similar infusion rates.

Post-hoc inspection of these data further showed that the infusion rates at the beginning of each training session differed most. When the number of infusions during the first 10 min of the 2-h sessions was considered, over the 10 days, there was a robust tendency for a treatment effect [F(4,42)=2.42; P=0.06], a significant effect of days [F (9,378)=26.15; P<0.0001] and a significant treatment x days interaction [F(36,378)=2.01; P<0.001] (Fig. 8, bottom left). Again, this effect was significant for days 4–6 [F(4,42)=4.33; P<0.01] (Fig. 8, bottom right) and reflected a higher number of infusions in the MP+FLX/high reactivity group than in the other groups (all P<0.01) (MP+FLX/high reactivity, 233 % of V controls).

These findings show that the MP+FLX/high reactivity group acquired the cocaine self-administration behavior faster than the MP+FLX/low reactivity and other groups, and that this difference was most robust at the beginning of the 2-h training sessions.

3.2.3. Relationship between MP+FLX-induced behavior and subsequent cocaine self-administration—We assessed whether the behavior during the repeated MP+FLX treatment (sensitization sessions) predicted the subsequent acquisition of cocaine self-administration. Our results demonstrate that the total number of cocaine infusions during the first 10 min of training days 4–6 (Fig. 9), followed by those in 120 min of days 4–6 (not shown) (i.e., during the training periods when these groups differed most, see above), was best predicted by ambulation during the repeated MP+FLX treatment (sensitization). The results for ambulation counts from min 6–40 of the sensitization sessions are presented in Fig. 9. The same pattern was obtained when the total ambulation counts over 90 min were considered (not shown).

This correlation analysis showed that ambulation during min 6–40 on treatment days 1–2 (sensitization) did not correlate with cocaine infusions on days 4–6 for the first 10 min of training ($r=-0.033$, $P>0.05$) or for the total 120 min of training ($r=-0.079$, $P>0.05$) (not shown). In contrast, the ambulation on treatment days 3–4 best predicted subsequent cocaine intake. Thus, ambulation during min 6–40 on treatment days 3–4 was negatively correlated with the number of infusions on days 4–6 for the first 10 min of training ($r=-0.574$, $P<0.05$) (Fig. 9, top) and for the total 120 min of training ($r=-0.519$, $P<0.05$) (not shown). Conversely, there were no significant correlations between ambulation on treatment days 5–6 ($r=-0.353$; $r=-0.281$; both $P>0.05$), days 7–8 ($r=-0.466$; $r=-0.467$; both $P>0.05$) (all not shown), or day 8 [$r=-0.464$, $P>0.05$ (Fig. 9, bottom); $r=-0.474$, $P>0.05$ (not shown)] and subsequent cocaine intake. As can be seen in Fig. 9, bottom, for treatment day 8, a possible relationship between MP+FLX-induced ambulation and subsequent cocaine infusions was negated by a few animals in the MP+FLX/low reactivity group (blue dots) that displayed very low ambulation counts (likely due to high stereotypy levels) at the end of the repeated MP+FLX treatment.

In summary, these findings demonstrate that, in contrast to cocaine-induced ambulation/stereotypies, which were best correlated with behavior at the end of the repeated MP+FLX treatment (sensitization) (experiment 1), the cocaine self-administration behavior was best predicted by the pattern of ambulation/stereotypies on days 3–4 of the repeated MP+FLX treatment.

4. Discussion

This study investigated the behavioral consequences of repeated MP+FLX co-exposure. Our most important findings include the following. (1) Treatment with FLX (5 mg/kg) alone did not produce significant changes in open-field behavior (locomotion, stereotypies), but this FLX dose potentiated MP-induced behavior during the course of the repeated treatment. (2) However, subgroups of rats showed differential drug effects on this behavior. Approximately, 40–50 % of the rats (designated MP+FLX/high reactivity group) displayed maximally increased locomotion during the first 2 days of the repeated MP+FLX treatment, followed by fast increasing levels of focal stereotypies and consequently suppressed locomotion. In contrast, the other half of the rats (MP+FLX/low reactivity group) displayed a delayed increase in locomotion, with some focal stereotypies in a few individuals emerging only towards the end of the treatment. (3) This differential

behavioral responsiveness during the repeated MP+FLX treatment was associated with a differential response to cocaine 2 weeks later, for both locomotion/stereotypies and cocaine self-administration behavior. For one, locomotion and stereotypy rates during MP+FLX pretreatment were positively correlated with those during the subsequent cocaine challenge. (4) Importantly, when assessed in the cocaine self-administration procedure, the MP+FLX/high reactivity group, but not the MP+FLX/low reactivity group or MP-only-treated group, showed faster acquisition of cocaine self-administration behavior than controls.

4.1. Effects of MP+FLX on open-field behavior: locomotion vs. stereotypies

It has long been known that acute and repeated treatment with psychostimulants (dopamine agonists) produce behavioral stereotypies, especially with higher doses and/or extended treatment duration [37–39]. Stereotypies refer to highly repetitive behaviors that are executed over and over without apparent purpose [40]. The specific behaviors emitted are species-, drug- and dose-dependent. For example, low doses may only induce locomotor activation, while higher doses will often also produce (more or less focal) stereotypies, including repetitive head bobbing, sniffing and licking. During the course of a repeated drug treatment, episodes with focal stereotypies typically become more prominent (e.g., [41]). The stereotypies induced by MP and MP+FLX mostly consist of repetitive sniffing/whisking and head/neck movements (head bobbing) [30,31,42].

The temporal sequence of behavioral activation after administration of psychostimulants or dopamine agonists typically starts with an increase in locomotor activity (ambulation), which is followed by a period of stereotypies. When the drug effect wears off, stereotypies give way to a second period of enhanced locomotion (triphasic motor response; [41]). During intense, focal stereotypies, locomotion typically ceases [41,43]. Our results show that there was a strong negative correlation between locomotor counts and stereotypy counts also with repeated MP+FLX treatment. In the present study, the above temporal pattern emerged from treatment day 3 onwards in approximately half of the rats, the MP+FLX/high reactivity group. In the rest, the MP+FLX/low reactivity group, this pattern only appeared in a few animals towards the end of the treatment (hence the distinction “high” vs. “low reactivity”).

Stereotypies are produced by deficient functioning of basal ganglia circuits [40,41,44] and, in the case of treatment with dopamine agonists, are associated with specific patterns of gene regulation in the striatum [41,45]. This is consistent with our previous findings demonstrating that acute and chronic treatment with MP+FLX produce potentiated gene regulation (compared with MP-only treatment) in the striatum. This effect was demonstrated for a range of genes, including those encoding transcription factors, neuropeptides and neurotransmitter receptors (for review, see [11,27]). Future studies will have to determine whether some of these genes are differentially affected in MP+FLX/high vs. low reactivity groups.

4.2. Effects of MP+FLX pretreatment on cocaine-induced behavior and cocaine self-administration

We also investigated whether these drug treatments modified subsequent cocaine self-administration behavior. Our findings demonstrate that the MP+FLX/high reactivity group showed facilitated acquisition of cocaine self-administration when tested starting two weeks after the repeated MP+FLX treatment. The time course indicates that rats in this group were similar to those in the other groups on the first two days of cocaine self-administration. However, they displayed increased rates of cocaine taking from day 3 onwards, and their intake leveled off after day 5 of the 10-day self-administration course, whereas rats in the other groups showed steady increases that developed more slowly, approaching the rates seen in the MP+FLX/high reactivity group only towards the end of the 10-day course.

These findings after intermittent i.p. MP+FLX administration for 8 days are consistent with recent results showing that prolonged oral administration (8h/day, 4 weeks) of MP+FLX in drinking water, in doses that produced clinically relevant drug plasma levels [46], resulted in increased cocaine taking in the self-administration procedure when tested 2 weeks later [47]. In that study, MP pretreatment alone produced facilitated acquisition of cocaine intake during the first week of self-administration, whereas MP+FLX pretreatment potentiated cocaine intake from week 2 on [47]. This longer pretreatment regimen with oral MP+FLX also induced more robust potentiation of striatal gene regulation [48] and was associated with a variety of other behavioral changes, including enhanced sucrose consumption and altered anxiolytic- and antidepressant-like effects in MP+FLX-treated rats [49]. The present findings show that even short-term intermittent MP+FLX exposure can result in facilitated cocaine taking.

In our present study, the shorter intermittent pretreatment with MP alone did not facilitate acquisition of cocaine self-administration compared with vehicle controls. Previous studies investigating the effects of MP pretreatment on subsequent cocaine self-administration yielded equivocal results, with some reporting facilitation (e.g., [50–53]), while others did not [53–55]. Overall, these findings underscore the importance of variables such as age of treatment (younger animals seem to be more sensitive; [36,50]), treatment duration, drug doses, etc. for the emergence of facilitated cocaine taking.

4.3. Are different subtypes of responders to MP+FLX at differential risk for subsequent cocaine taking?

In our previous studies, we had observed a considerable variance in the behavioral responses to MP+FLX (or to cocaine after repeated MP+FLX treatment; [31,32]). Our present detailed behavioral analysis revealed that in the early phase of the repeated MP+FLX treatment rats tended to respond either with an early increase in locomotion followed soon by emerging strong stereotypies (MP+FLX/high reactivity group) or with a delayed increase in locomotor activity and some stereotypies only by the end of the treatment (low reactivity group). Our findings demonstrate that the MP+FLX/high reactivity group then showed facilitated acquisition of cocaine self-administration two weeks after the repeated MP+FLX treatment.

Not surprisingly, given the neurochemical and molecular similarities between MP+FLX and cocaine treatment [21], for both groups pooled, the locomotor and stereotypy rates during the cocaine challenge 2 weeks later correlated best with locomotor and stereotypy rates measured at the end of the repeated MP+FLX pretreatment (sensitization period). In contrast, the rate of cocaine intake during the self-administration sessions correlated best with locomotion/stereotypies during days 3–4 of the sensitization period, when the two subgroups differed most, and not with behavior at the end of the sensitization period (day 8). These findings indicate that the differential cocaine intake did not merely reflect differential sensitization by MP+FLX. Rather, the different cocaine intake rates seem to reflect different behavioral endophenotypes [56]: individuals responding early with stereotypies to MP+FLX treatment subsequently acquired cocaine self-administration faster. In rodents, several different behavioral endophenotypes have been described that predict cocaine taking [56], and these are thought to be associated with different gene expression profiles [56]. Our results indicate that stereotypies can be added to the list of such behavioral markers for identifying individuals with an enhanced vulnerability for psychostimulant taking.

4.4. Potential mechanisms

The mechanisms mediating these differential behavioral responses to MP+FLX treatment demonstrated here are unclear. Based on our previous work and that of others, it can be speculated that differences in dopamine (or serotonin) receptor signaling may be involved. MP-induced gene regulation in the striatum is mediated by striatal D1 dopamine receptors [57,58] and occurs predominantly but not exclusively in D1 receptor-expressing striatal projection neurons [11]. Moreover, the MP+FLX-induced behavioral stereotypies [30,31] are associated with a potentiation of MP-induced gene regulation, again mostly in striatal D1 neurons [27]. While widespread throughout the dorsal and ventral striatum, these molecular changes are most prominent in the dorsolateral (sensorimotor) striatum [27]. Striatal dopamine receptors and the sensorimotor striatum are instrumental for dopamine agonist-induced stereotypies [40,44] and are also important for psychostimulant abuse/addiction (e.g., for drug-habit formation, relapse; [59–62]). It is interesting to note that at least one endophenotype [56] prone to enhanced cocaine taking (“sign trackers”) differs in striatal dopamine receptor expression, with higher levels of D1 receptors at the beginning of the training and lower levels of D2 receptors after repeated training, compared with a control phenotype (“goal trackers”; [63]).

On the other hand, there is evidence that serotonin (5-HT) receptors may also be involved (e.g., [26]). For example, the 5-HT1B receptor subtype is implicated in psychostimulant action. Thus, the 5-HT1B receptor has been shown to facilitate cocaine effects on striatal gene regulation and behavior [24,64], and it has been proposed that this facilitation reflects disinhibition of dopamine input to the striatum by 5-HT1B action in the midbrain [64]. Stimulation of the 5-HT1B receptor indeed also facilitates MP-induced behavior [28] and striatal gene regulation [65,66], mimicking FLX effects. Moreover, MP treatment increases 5-HT1B receptor expression in striatal projection neurons [65], an effect that is also potentiated by co-administration of FLX [65]. However, given the many serotonin receptor subtypes expressed in the brain [67] and the complex interactions between the serotonin and dopamine systems [26], other serotonin receptors (e.g., 5-HT1A; [68]) may also be

involved in these effects. Future work will have to investigate whether differential dopamine or serotonin receptor function may underlie the differential development of stereotypies and cocaine self-administration demonstrated in the present study.

4.5. Clinical considerations and conclusions

MP+FLX combinations are indicated for several medical conditions. These include ADHD/depression comorbidity, which is seen with up to 40 % prevalence in pediatric ADHD [69,70], as well as other conditions (e.g., [15,16,71]). Dual exposure to these medications also occurs as a result of using MP as a cognitive enhancer [4] by patients taking SSRIs. Our previous gene regulation studies show that combining FLX with MP treatment potentiates MP-induced regulation of addiction-related genes [27], which occurs preferentially in the sensorimotor striatum [27] that mediates habit forming and compulsive aspects of addiction [72–74]. While prolonged oral treatment with MP+FLX resulted in more robust facilitation of subsequent cocaine self-administration [47], our present results show that even after short-term intermittent MP+FLX treatment a subgroup of rats displayed facilitated acquisition of cocaine taking, suggesting a potentially increased risk for substance use disorder produced by exposure to MP+FLX combinations, at least in some subjects. This is consistent with the finding that drug-induced addiction-related neuroplasticity in lateral striatal circuits is modulated by several neurotransmitters including dopamine and serotonin [74]. Our present findings also indicate that some individuals are more susceptible to this facilitating effect of MP+FLX, and that this subgroup can be identified by their responding to MP+FLX treatment with focal stereotypies. Stereotypies (repetitive behaviors) reflect aberrant striatal function and are associated with various neuropsychiatric disorders (cf. [40,41]). Drug-induced stereotypies may thus serve as a marker for individual susceptibility to substance use disorder.

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Data availability

Data will be made available on reasonable request.

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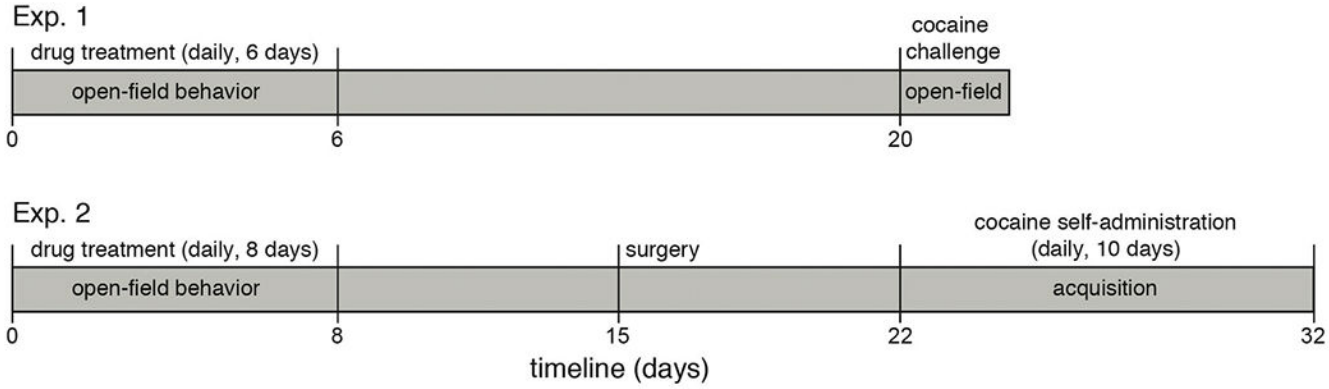

Fig. 1.

Timeline of drug treatments and behavioral assessments.

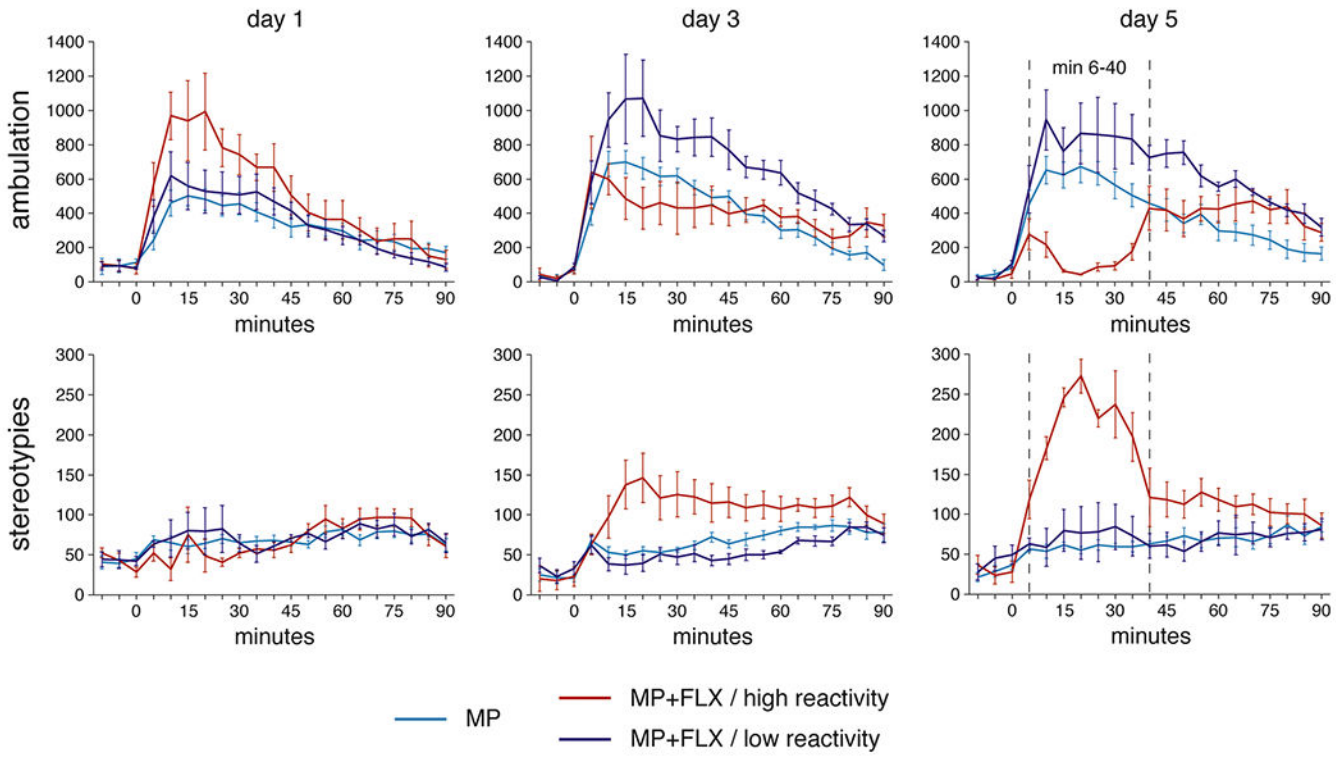

Fig. 2.

Changes in open-field behavior during repeated methylphenidate plus fluoxetine (MP+FLX) treatment. Rats were treated daily for 6 days with MP (5 mg/kg; n=10) or MP+FLX (5 mg/kg each; n=11), and behavior was recorded with a Truscan activity monitoring system for a total of 130 min each day. Drugs were administered after a 40-min habituation period. Time courses for locomotion (ambulation counts) (mean±SEM) (**top**) and stereotypy counts (**bottom**) during 15 min before and 90 min after the drug injection are presented for treatment days 1, 3 and 5. Analysis showed that the MP+FLX-treated rats exhibited two different behavioral profiles. Approximately half of the rats displayed maximally increased ambulation on days 1-2, followed by increasing stereotypy levels and reduced ambulation during days 3-6 (denoted as “MP+FLX/high reactivity” group, n=5). The other half (“MP+FLX/low reactivity” group, n=6) showed high levels of ambulation from day 2 onwards and some emerging stereotypies with decreasing ambulation only towards the end of the treatment (day 6; see Fig. 3). Stereotypy counts typically peaked during min 6-40 of the test sessions, during which time period ambulation was largely suppressed (see day 5, right). When stereotypies receded, locomotion reemerged. Such differential behavioral effects were not seen in the MP only-treated group.

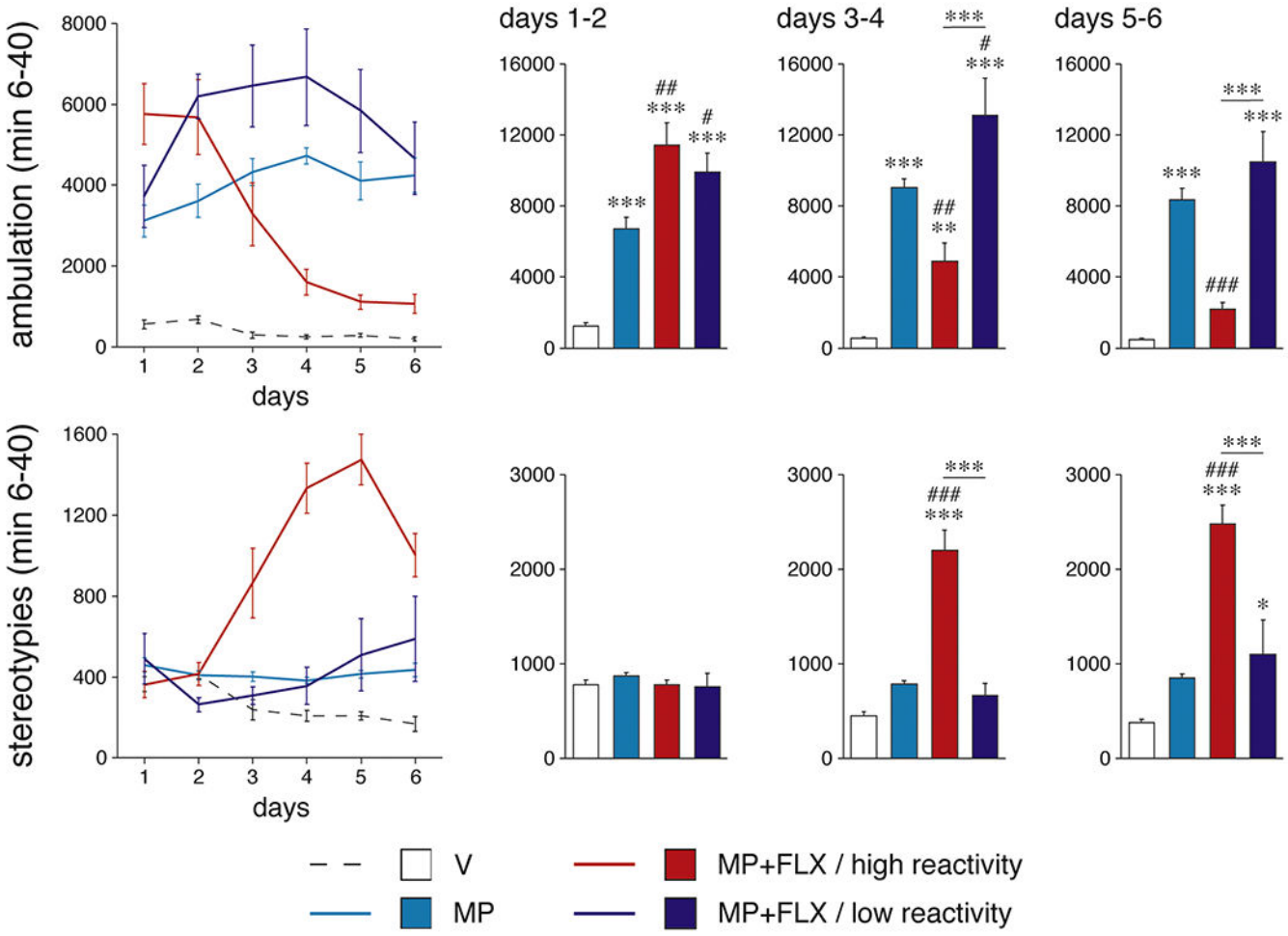

Fig. 3.

Effects of repeated MP+FLX treatment on open-field behavior. Ambulation counts (mean±SEM) (**top**) and stereotypy counts (**bottom**) are presented for rats that received vehicle (V; n=7), MP (5 mg/kg; n=10) or MP+FLX (5 mg/kg each; high reactivity group, n=5; low reactivity group, n=6) once daily for 6 days. Time courses for total counts (during min 6-40 of the session) for days 1-6 (left) and counts pooled for days 1-2, 3-4 and 5-6 (**right**) are shown. The MP+FLX/high reactivity group showed the highest ambulation levels on days 1-2, followed by increasing stereotypy levels and suppressed ambulation. In contrast, the MP+FLX/low reactivity group displayed high ambulation levels from day 2 onwards and some stereotypies (with declining ambulation) on days 5-6. *** P<0.001, ** P<0.01, * P<0.05, vs. V or as indicated; $^{###}$ P<0.001, $^{##}$ P<0.01, $^{#}$P<0.05, vs. MP.

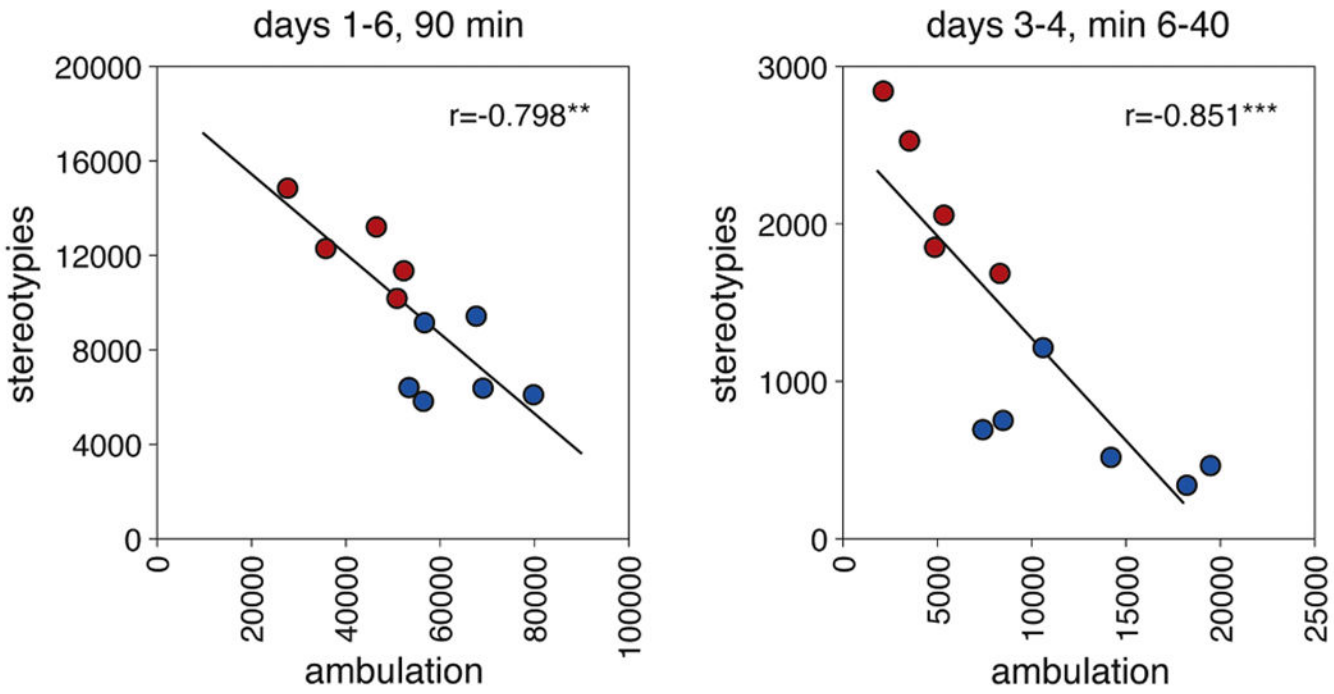

Fig. 4.

Relationship between ambulation counts and stereotypy counts. Scatter plots depict the negative correlation between total ambulation counts and total stereotypy counts during the 90-min sessions on days 1-6 ($r=-0.798$, $P<0.005$) (**left**) and between ambulation counts and stereotypy counts during min 6-40 of the sessions on days 3-4 ($r=-0.851$, $P<0.001$) (**right**). Individual animals of the high reactivity group are shown in red, those of the low reactivity group are in blue.

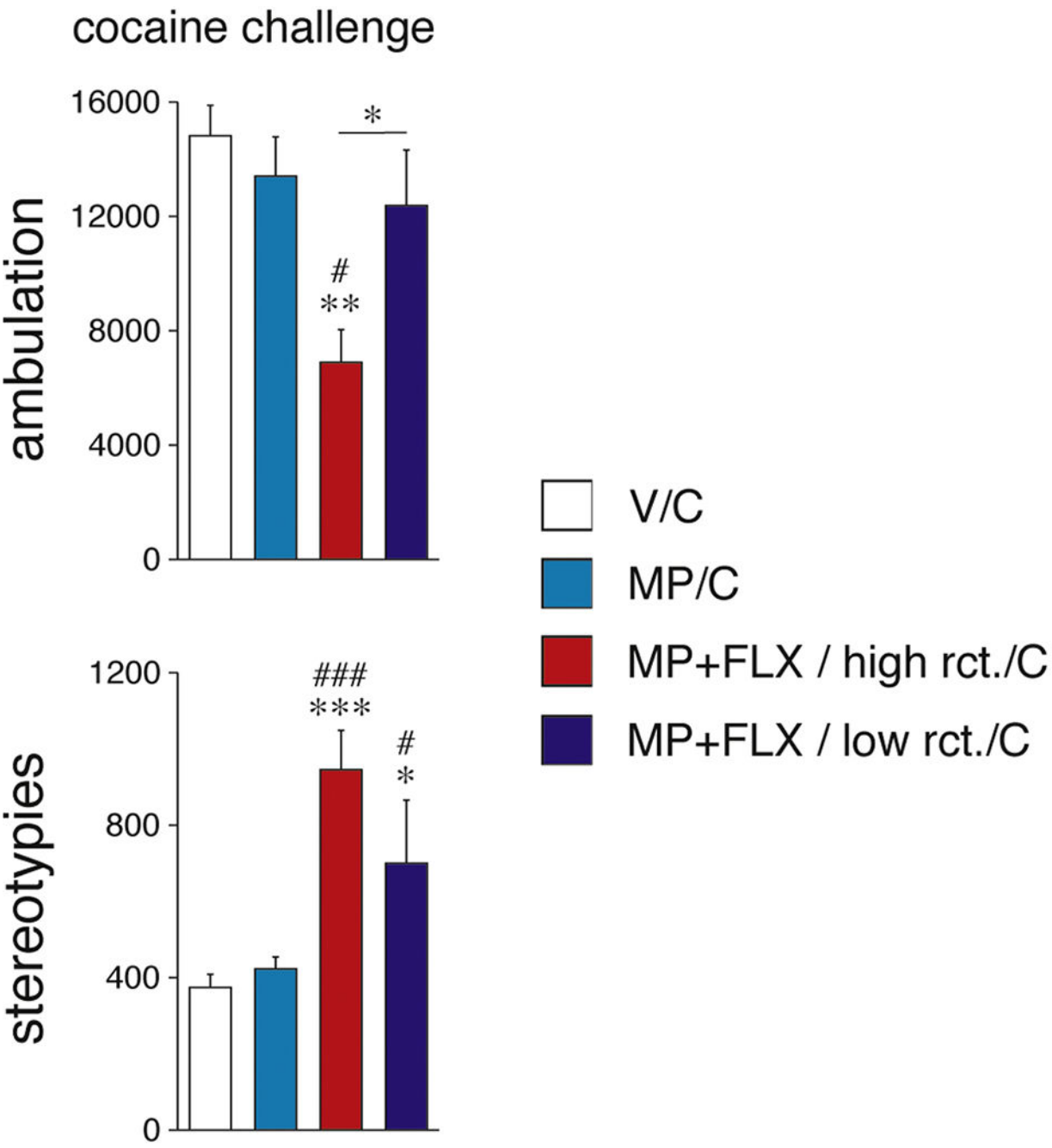

Fig. 5.

Effects of the cocaine challenge on open-field behavior. Ambulation (total counts, mean±SEM) (**top**) and stereotypies (**bottom**) are given for the rats that received vehicle (V), MP (5 mg/kg) or MP+FLX (5 mg/kg each; high reactivity group; low reactivity group) for 6 days, followed by a cocaine challenge (C; 25 mg/kg) two weeks later. Animals were tested in the open-field for 40 min after the cocaine injection. *** P<0.001, ** P<0.01, * P<0.05, vs. V or as indicated; $^{###}$ P<0.001, $^{\#}$P<0.05, vs. MP.

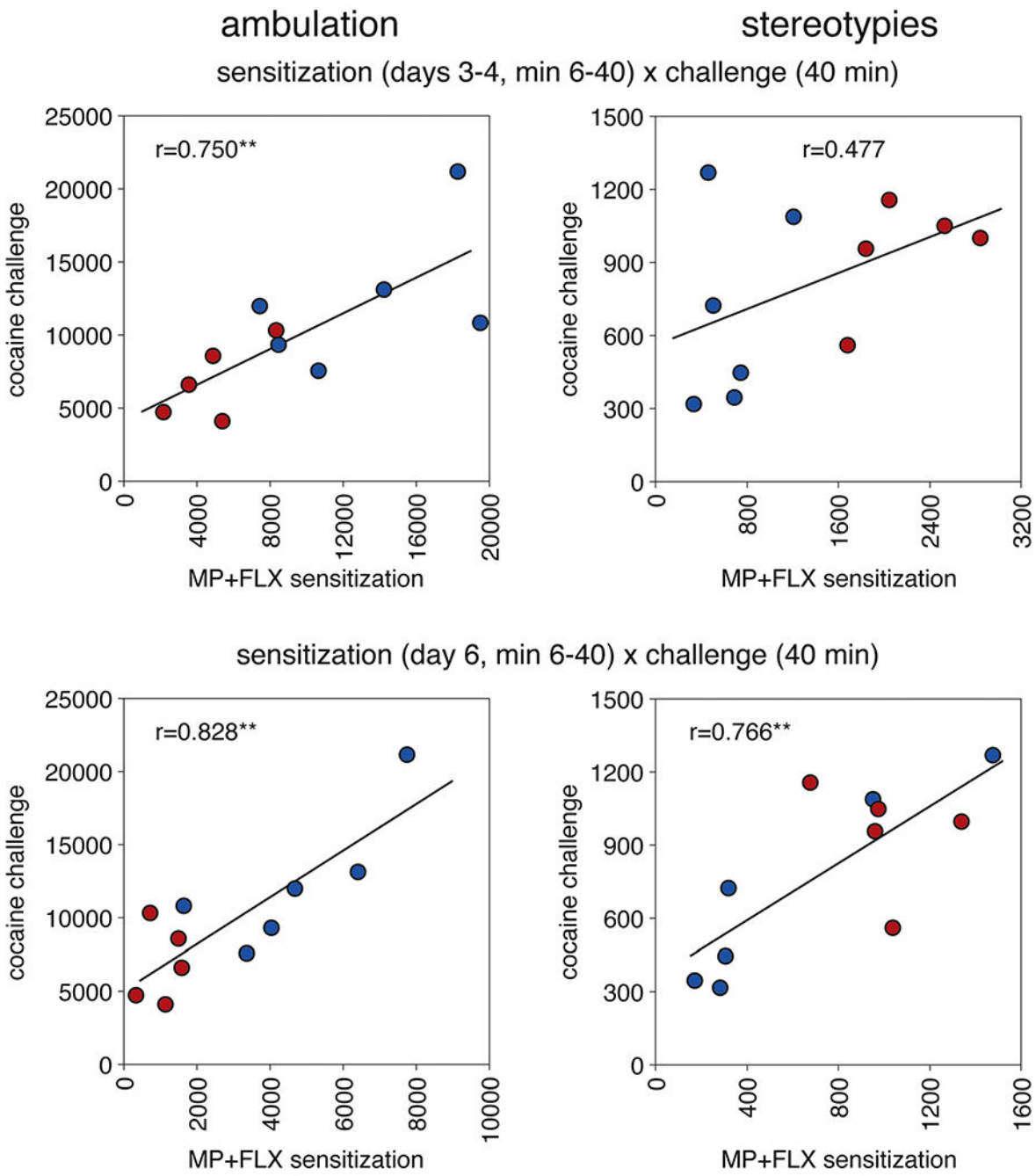

Fig. 6.

Relationship between behavior during the repeated MP+FLX pretreatment ("sensitization") and during the cocaine challenge. Scatter plots depict correlations between cocaine-induced behavior and MP+FLX-induced behavior for ambulation (**left**) and stereotypies (**right**) during days 3-4 (min 6-40) (**top**) and day 6 (min 6-40) (**bottom**) of the repeated MP+FLX treatment. For days 3-4, there was a positive correlation between the two counts for ambulation (r=0.750, P<0.01), but not for stereotypies. There were more robust correlations for day 6 of the sensitization, for ambulation (r=0.828, P<0.01) and for stereotypies

(r=0.766, P<0.01). Therefore, the behavior at the end (day 6) of the repeated MP+FLX treatment (sensitization) best predicted the behavior during the cocaine challenge 2 weeks later.

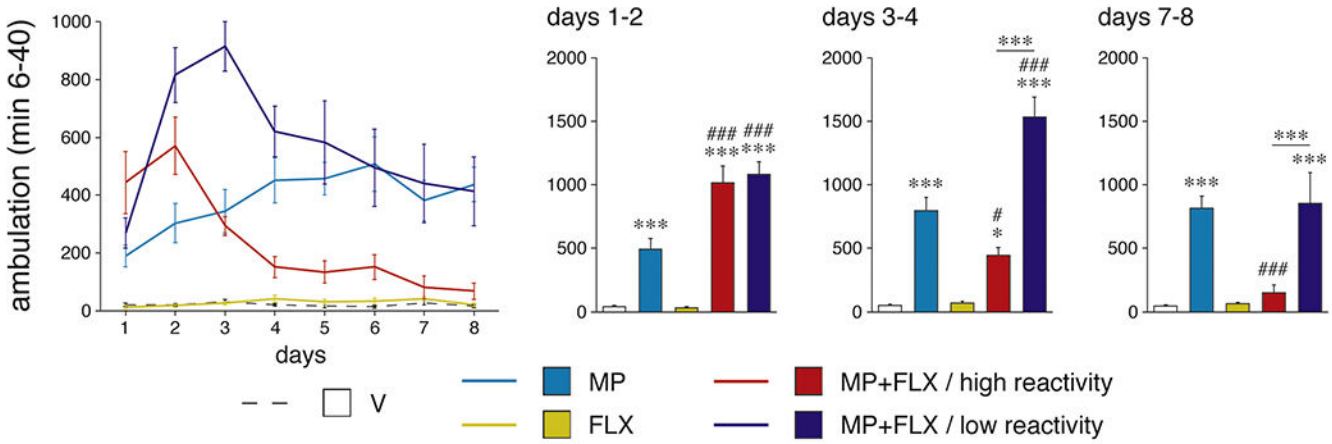

Fig. 7.

Effects of repeated MP+FLX treatment on open-field behavior (measured with SDI activity system; stereotypy counts not available). Time course for ambulation counts (mean±SEM; min 6-40) for days 1-8 (**left**) and counts pooled for days 1-2, 3-4 and 7-8 (**right**) are shown for rats that received vehicle (V; n=16), MP (5 mg/kg; n=10), FLX (5 mg/kg; n=6) or MP+FLX (5 mg/kg each; high reactivity group, n=6; low reactivity group, n=9) once daily for 8 days. As in Exp. 1, the MP+FLX/high reactivity group displayed the highest ambulation levels on days 1-2 of the repeated treatment, followed by rapidly decreasing ambulation counts. In contrast, the MP+FLX/low reactivity group showed the highest ambulation levels on days 2-3, and then somewhat declining levels towards days 7-8. *** P<0.001, * P<0.05, vs. V, FLX or as indicated; ### P<0.001, vs. MP.

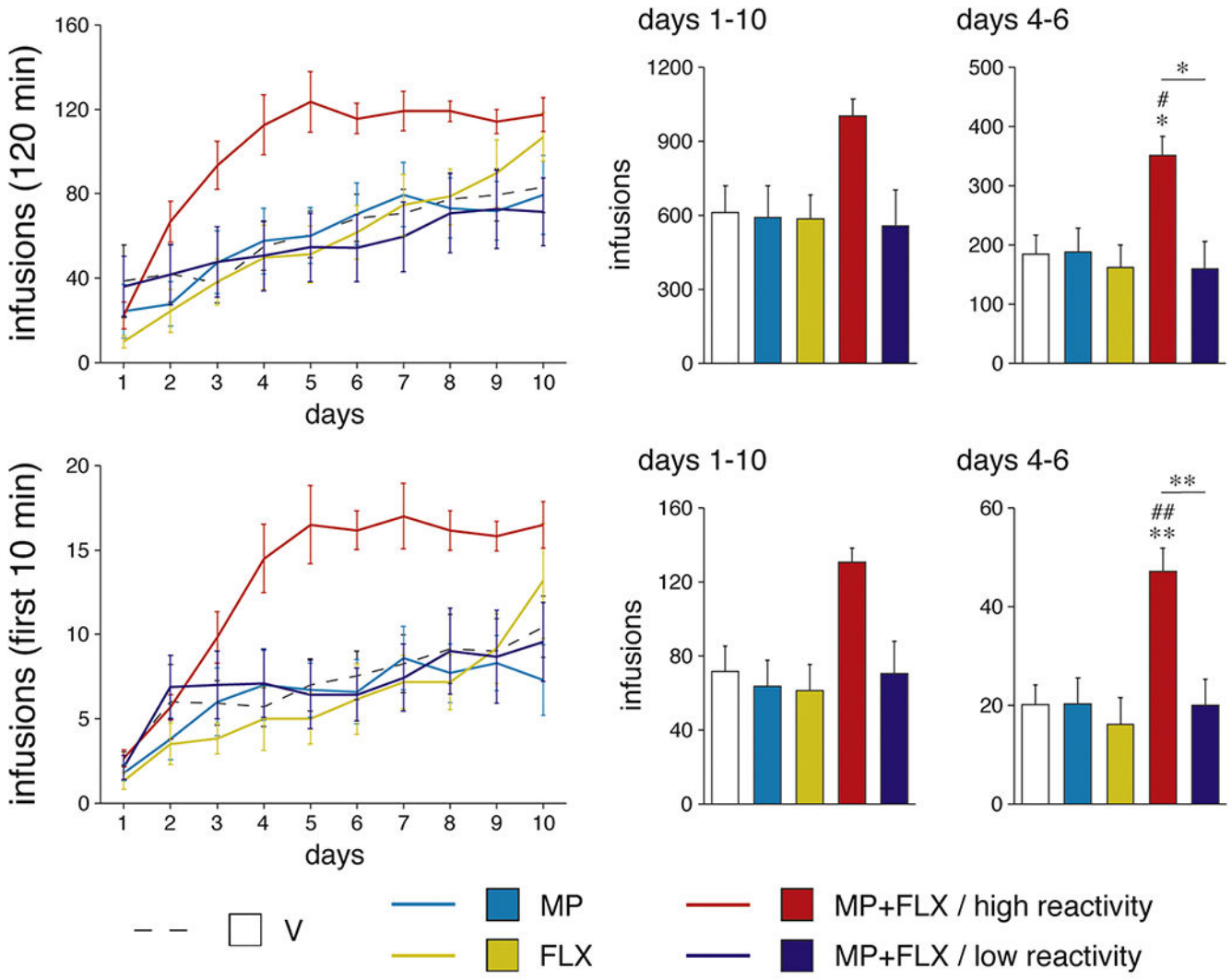

Fig. 8.

Effects of repeated MP+FLX pretreatment on subsequent acquisition of cocaine self-administration 2 weeks later. Rats obtained cocaine infusions by nose poking, with cocaine access for 120 min/day on 10 consecutive days (FR1; 150 μg/100 μl/kg infusions per nose poke). The total number of infusions (mean±SEM) during the 120-min sessions (**top**) or during the first 10 min of the session (**bottom**) are given for rats that were pretreated with vehicle (V; n=16), MP (5 mg/kg; n=10), FLX (5 mg/kg; n=6) or MP+FLX (5 mg/kg each; high reactivity group, n=6; low reactivity group, n=9) once daily for 8 days. The time course for daily infusions on days 1-10 (**left**) and total infusion counts for days 1-10 and days 4-6 (**right**) are presented. The MP+FLX/high reactivity group acquired cocaine self-administration faster than the other groups, as their daily intake already peaked during days 4-6. Notably, the increased cocaine intake in this group was most robust at the beginning of the session (first 10 min). ** P<0.01, * P<0.05, vs. V, FLX or as indicated; $^{\#\#}$ P<0.01, $^{\#}$ P<0.05, vs. MP.

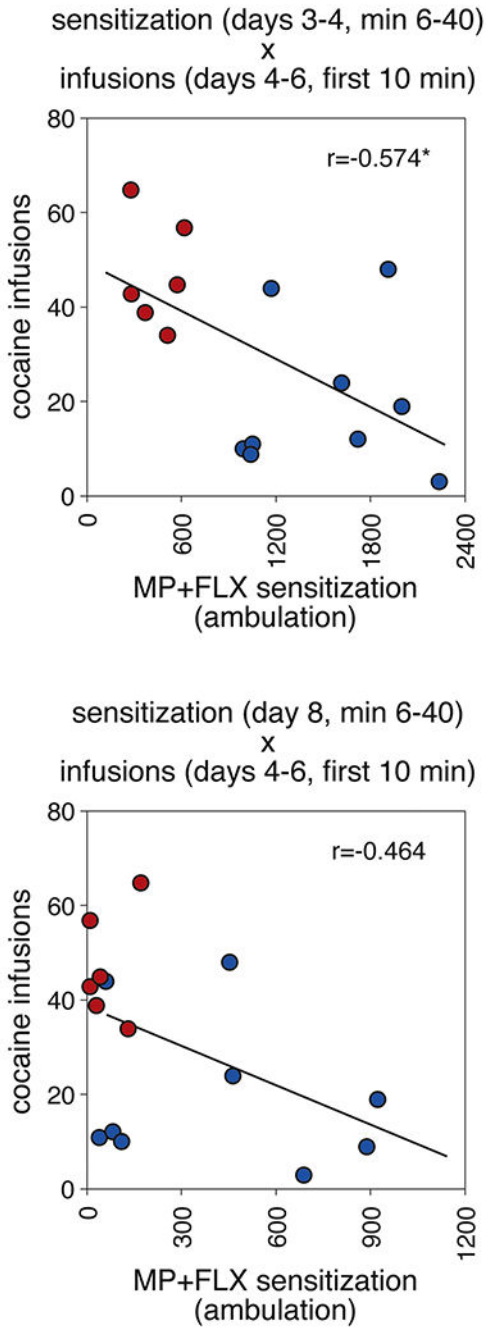

Fig. 9.

Relationship between ambulation counts during repeated MP+FLX pretreatment (sensitization) and cocaine intake during self-administration. Scatter plots show correlations between the rates of cocaine infusions (days 4-6, first 10 min) and ambulation counts during days 3-4 (min 6-40) (**top**) or day 8 (min 6-40) (**bottom**) of the repeated MP+FLX pretreatment. For days 3-4, there was a negative correlation between ambulation counts and cocaine infusions ($r=-0.574$, $P<0.05$). In contrast, there was no significant correlation for

day 8 (r=−0.464, P>0.05). Therefore, the behavior during days 3-4 of the repeated MP+FLX treatment best predicted the rate of cocaine intake 2 weeks later.