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Adenosine Receptor Antagonists and Behavioral Activation in NF- κ B p50 Subunit Knockout Mice

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Abstract

Aims—Our previous work revealed that mice lacking the p50 subunit of transcription factor nuclear factor kappa B (NF- κ B) (p50 KO mice) and genetically intact F2 mice have similar locomotion under basal conditions, yet p50 KO mice show greater locomotor activation after caffeine ingestion. In this report, we test whether KO mice display altered caffeine pharmacokinetics or increased caffeine-induced DA turnover relative to F2 mice, and evaluate the impact of intraperitoneal administration of specific adenosine and DA receptor antagonists on locomotor activity.

Main methods—Concentrations of DA and caffeine were measured using high performance liquid chromatography. DA turnover was measured after treatment of mice with an inhibitor of tyrosine hydroxylase. Locomotor activity was measured using telemetry.

Key findings—The data reveal that 1) caffeine concentrations in blood and brain are similar in KO and F2 mice after oral or intraperitoneal administration; 2) KO mice show greater DA turnover under basal conditions, but turnover is similar in both strains after caffeine administration; 3) the specific A2AAR antagonist SCH 58261 induces greater locomotion in KO versus F2 mice; and 4) the activating effect of SCH 58261 in KO mice is prevented by prior treatment with the D2R antagonist raclopride.

Significance—These findings support the conclusions that 1) A2AAR has a major impact on behavioral activation of p50 KO mice, and 2) D2R mediated neurotransmission is critical to this effect.

Keywords

caffeine; dopamine; striatum; turnover

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Introduction

The striatum incorporates two major locomotor-regulatory circuits, the direct and indirect pathways, which together modulate cortical output that determines locomotor activity. At the postsynaptic level, type A1 adenosine receptors (A1AR) and type D1 dopamine receptors (D1R) form heterodimers on striatal neurons of the direct pathway, whereas heterodimers of A2A adenosine receptors (A2AAR) and D2 dopamine receptors (D2R) form on neurons of the indirect pathway (reviewed in (Ferre, 2008; Ferre et al., 2008; Xie et al., 2007b)). Inhibitory interactions both within and between these heterodimeric receptors modulate striatal neuronal excitability and neurotransmission.

In vitro and in vivo studies indicate that the transcription factor NF- κ B (nuclear factor kappa B) promotes the expression of A1AR and D2R, but suppresses the expression of A2AAR and D1R, thereby participating in the regulation of receptor density in the striatum. As compared with genetically intact mice, mice with genetic deletion of the p50 subunit of NF- κ B (p50 knock-out, KO) show greater striatal expression of A2AAR, but reduced expression of A1AR and D2R (Xie et al., 2007a). These changes are paralleled by quantitative changes in the G proteins that are coupled to these receptors (Xie et al., 2007a). Based on the function of these receptors in striatal neurotransmission, the combination of increased A2AAR and reduced D2R should logically be associated with less locomotor activity in p50 KO mice both under basal conditions and after treatment with the non-specific adenosine receptor antagonist caffeine. However, in contrast to this expected outcome, genetically intact F2 and p50 KO mice have similar amounts of locomotor activity under basal conditions, yet p50 KO mice show greater behavioral activation in response to caffeine ingestion (Xie et al., 2007a).

In this report, we evaluate possible reasons for this effect. We hypothesized that differences in caffeine pharmacokinetics, striatal dopamine (DA) turnover, and/or levels of the DA transporter (DAT) could contribute to these behavioral effects in p50 KO mice. The experiments reported here test these hypotheses by measuring caffeine concentrations in serum and brain after oral or intraperitoneal administration of caffeine, striatal concentrations of DA and dihydroxyphenylacetic acid (DOPAC) under basal conditions and after administration of caffeine, and protein levels of the DA transporter (DAT) in F2 and p50 KO mice. In addition, we characterized behavioral changes induced in F2 and p50 KO mice after intraperitoneal administration of selective adenosine and DA receptor antagonists.

Material and Methods

Experimental animals

Four-week-old male B6;129P2-Nfkb1(tm1bal) mice (p50 KO) and B6129PF2/J mice (F2, the recommended control strain) were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed by strain in groups of 2 or 4 per cage and were used experimentally at approximately 8 weeks of age. Cages were maintained at $22 \pm 1^\circ\text{C}$ on a 12:12 hour light:dark cycle. Food and water were available ad libitum. All animal use in this study was approved by the Laboratory Animal Care and Use Committee of the Southern Illinois University School of Medicine.

Behavioral evaluation

To permit telemetric monitoring of locomotor activity and core temperature, mice were surgically implanted with an intraperitoneal transmitter (Transoma Medical, St. Paul, MN). Anesthesia was achieved using a subcutaneous injection of a mixture of ketamine (50 mg/kg) and xylazine (50 mg/kg), with additional anesthetic administered if necessary during surgery. A bland ophthalmic ointment was applied to the eyes before surgery to retard desiccation. All

surgery was performed using aseptic techniques. After surgery, one ml of sterile saline solution was injected into intraperitoneal cavity to maintain hydration. Ibuprofen was provided in drinking water from 1 day before through 5 days after surgery to provide analgesia. After surgery, mice were housed in individual cages in a sound-shielded, temperature-controlled chamber under a 12:12 h light:dark cycle (9:00 am to 9:00 pm) at $22 \pm 1^\circ \text{C}$, and were allowed at least 14 days for post-surgical recovery prior to experimental use.

The frequencies emitted by the surgical-implanted intraperitoneal transmitter were received by a DSI receiver (RPC-1) installed under each individual animal cage. Collected signals were processed through a DSI analog converter. Signals were sampled and processed by DQ3 software (Transoma Medical, St. Paul, MN) for analysis. Locomotor activity was recorded across the entire period of observation and then summarized and averaged to obtain mean values for 10 minute or 2 hour intervals, depending on the requirements of each experiment. Core body temperatures were recorded simultaneously and analyzed in the same manner.

Measurement of caffeine in serum and brain

F2 and p50 KO mice were given caffeine (Sigma-Aldrich, St. Louis, MO) by intraperitoneal injection or gavage. The drug was administered immediately prior to dark onset at a dose of 15 mg/kg. F2 mice underwent euthanasia at 1, 5, 15, and 30 minutes after drug administration. Based on data from the F2 mice, p50 KO mice were tested only at the 5 minute time point due to difficulty in obtaining large numbers of these mice. At the appropriate time after caffeine administration, blood was collected by cardiac puncture under isoflurane anesthesia. Mice then underwent cervical dislocation and decapitation without recovery from anesthesia. The heads were immersed into liquid nitrogen for 6 seconds. Brains were then rapidly removed from the skull, placed in a capped plastic container, quickly frozen in liquid nitrogen, and transferred to -80°C pending further processing. Blood was placed in capped containers and remained at room temperature until clotting occurred (approximately 30 minutes). Samples were then centrifuged, and sera (supernatant) were removed and stored at -80°C until further processing.

Methylxanthines in sera were measured directly without extraction. Caffeine was extracted from brain according to a published method (Kaplan et al., 1989). Brains were weighed, placed in centrifuge tubes, and homogenized for 30 seconds in 0.1 M HCl (1:5) at room temperature using a Con-torque power unit (Eberbach Corporation, Ann Arbor, MI). β -hydroxypropyl theophylline (0.4 mg per 10 ml of final volume) was then added to the homogenate as an internal standard to monitor extraction. The mixture was transferred to a glass extraction tube, 5 ml of chloroform were added, and samples were vortexed for 60 seconds, followed by centrifugation at $400 \times g$ for 10 minutes. The lower phase was carefully aspirated and transferred to a collection glass tube. This extraction procedure was performed for two additional times. The three organic phases were pooled (total volume of about 15 ml) and evaporated by heating to $55\text{--}58^\circ \text{C}$ in water bath under aeration with nitrogen gas in a chemical fume hood. Residues remaining in the tubes were suspended in 300 μl of mobile phase (a mixture of methanol and 1% acetic acid at a ratio of 17:83) for analysis by high performance liquid chromatography (HPLC) according to published methods (Jeppesen et al., 1996; Kaplan et al., 1989). Prior to analysis by HPLC, serum samples were diluted at ratio of 1:5 with mobile phase. Serum samples and re-suspended brain extract were both filtered through 0.2 μm syringe filters. Samples (100 μl) were then assayed for caffeine by HPLC using a Shimadzu SCL-10A VP HPLC system with a Discovery RP-Amide C16 Supelguard pre-filter cartridge (Supelco Cat# 505080) and a Discovery RP-Amide C16 column (Supelco Cat# 505013, 15cm $\times$ 4.6 mm). The flow rate was 1ml/minute under a pressure of 760 mmHg. The run time for each sample was 15 minutes. A set of standards containing both caffeine and 7- β -hydroxytheophylline was assayed with each set of samples. Data from these standards were used to calculate areas under the curve, which were used to construct a standard curve that was used to calculate the amount of caffeine and 7- β -

hydroxytheophylline (internal standard) in the sera and brain samples. Final caffeine concentrations are expressed as $\mu\text{g/ml}$ of serum and ng/g of gross brain weight.

Western blot analysis of dopamine transporter (DAT) in mouse striatum

F2 and p50 KO mice (8 each) were euthanized by cervical dislocation under isoflurane anesthesia. Heads were immediately removed and immersed into liquid nitrogen for 6 seconds. Brains were then removed, the striata were rapidly dissected out on an ice-cold surface, immediately snap-frozen in liquid nitrogen, and stored -80°C until processing. For Western blot analysis, striata were sonicated in 750 μL of RIPA buffer (20mM Tris, pH 7.5, 150mM NaCl, 1% nonidet P-40, 0.5% sodium deoxycholate, 1mM EDTA, 0.1% SDS) containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO.). Homogenates were centrifuged at $10,000 \times g$ for 10 min. at 4°C , and protein concentration of supernatants determined using the bicinchoninic acid (BCA) assay (Thermo-Fisher Scientific, Rockford, IL). Equal amounts of protein (30 μg) from each sample were loaded onto 10% polyacrylamide gels. The proteins were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Towbin et al., 1979). DAT protein was detected using a 1:1000 dilution of a rabbit polyclonal antibody (PhosphoSolutions, Aurora, CO.). A mouse monoclonal anti-actin (diluted 1:5000; Sigma-Aldrich) was used to estimate the total amount of protein loaded on the gel. Antibody binding was revealed using goat anti-rabbit or rabbit anti-mouse HRP-linked IgG (diluted 1:5000) and the ECL immunoblotting detection system (GE Healthcare Life Sciences, Pittsburgh, PA). Chemiluminescence was detected using the LAS-4000 and image analysis was done using Multi Gauge V3.0 software (FujiFilm Life Science, Woodbridge, CT). The molecular weight markers were much darker than the bands for DAT and were therefore removed from the gel prior to exposure for DAT (i.e., a 10 sec exposure was used for the molecular weight ladder, whereas a 600 sec exposure was used for DAT). The DAT and β -actin shown are taken from the same membrane; DAT bands were also less intense than β -actin bands.

Assessment of striatal DA and DA turnover

F2 and p50 KO mice were randomly allocated to receive either caffeine by oral gavage, injection of α -methylparatyrosine (α -MPT; Sigma-Aldrich, St. Louis, MO), or caffeine injection followed immediately by α -MPT injection. α -MPT and caffeine were dissolved in 0.9% sterile saline and administered respectively at 250 mg/kg and 15 mg/kg body weight via intraperitoneal injection (caffeine and/or α -MPT) or gavage (caffeine). At scheduled time points after treatment, mice were euthanized by cervical dislocation under isoflurane anesthesia. Heads were immediately removed and immersed into liquid nitrogen for 6 seconds. Brains were then removed and placed on an ice-cold surface. Striata were quickly dissected, placed in screw-capped containers, and immediately frozen by inserting the tube into liquid nitrogen. Samples were then transferred to and stored -80°C until further processing.

For measurement of DA and DOPAC concentrations, striata were homogenized in 500 μL of 0.1 N perchloric acid by ultrasonic disruption (Sonic dismembrator Model 100, Fisher Scientific; 3 sec at pulse intensity setting 2.5). Homogenates were then centrifuged at $10,000 \times g$ for 15 min at 4°C . Supernatants were removed and frozen at -80°C until assayed. Pellets were re-suspended in 100 μL of 0.5 N NaOH by continuous rotation overnight at room temperature. 400 μL of 10 mM PBS was then added to solubilized protein pellet, and the Bio-Rad protein assay was performed to measure protein concentration. These protein values were used to normalize values obtained from the HPLC assay. HPLC assays were performed using a Bioanalytical HPLC System with an electrochemical detector. Separation of the different catecholamines in the sample was accomplished by a reverse phase C18 ODS column (maintained at 32°C by heating) and a citrate-phosphate buffer, pH 2.78 consisting of 30 mM citric acid, 50 mM dibasic sodium phosphate, 0.1 mM EDTA, 15% methanol, 325 mg/L 1-

octane sulfonic acid. The buffer was pumped at a rate of 1.0 ml/min. Sets of standard solutions of DA and DOPAC were assayed to create standard curves. Samples were pre-diluted at a ratio of 1:5 in 0.1 N perchloric acid, and 100 μ l aliquots were injected onto the column for separation. Each sample was run in duplicate for DOPAC and DA. The elution times were approximately 8.12 min for DOPAC and 9.76 min for DA. The effluent from the column was passed across a glassy carbon electrode set at +0.75V to oxidize the hydroxyls on the catechol ring. The current was detected at sensitivities of 5 nA for DOPAC and 20 nA for DA. The area under the curve obtained for samples was compared to the area under the curve for the standards. The final DA and DOPAC data were corrected for dilution and normalized against the protein content of striatal samples to express DA and DOPAC concentrations as ng/mg protein.

Assessment of locomotor activity after systemic administration of caffeine

F2 and p50 KO mice were evaluated for the behavioral effects of caffeine (15 mg/kg body weight) administered by intraperitoneal injection at light onset (n = 6 per strain) and dark onset (n = 7 per strain). Activity counts were recorded at 10 minutes intervals for 2 hours after injection. Basal activity data were obtained from untreated mice during the same diurnal interval (9.00 AM to 11.00 AM) 24 hours before caffeine treatment.

Assessment of locomotor activity after sequential drug treatments

Because of difficulty in obtaining adequate numbers of p50 KO mice, drugs were tested sequentially in both F2 and p50 KO mice. Each mouse received intraperitoneal injections of the following drugs in this sequence: 1) SCH58261 (10 mg/kg); 2) DPCPX (5 mg/kg); 3) raclopride (0.5 mg/kg); 4) raclopride (0.5 mg/kg) followed 15 minutes later by SCH 58261 (10 mg/kg); and 5) simultaneous injection of DPCPX and SCH 58261 (doses as above). Mice were permitted no fewer than 7 days between treatments to allow elimination of the drug. Locomotion was recorded for 24 hours before each drug treatment in all mice. When an individual mouse was tested sequentially with different drugs, this 24-hour pre-injection period was used to provide basal measures of activity for evaluation of the effects of the subsequent drug treatment.

Drugs were dissolved either in 0.9% pyrogen-free saline (caffeine and raclopride) or DMSO (SCH 58261 and DPCPX) and were administered in a volume of 0.3 to 0.6 ml of saline solution or 50 μ l of DMSO, based on doses calculated according to the individual body weight of each mouse. A separate series of experiments evaluated the behavioral effects of vehicle injections using the same protocol as described below for drug treatment. Sterile normal saline (used to prepare caffeine and raclopride) was injected at dose of 1 ml/kg body weight (n = 9 for F2 mice and 7 for p50 KO mice, respectively). Dimethyl sulfoxide (DMSO, used to prepare SCH 58261 and DPCPX) was injected at dose of 50 μ l per mouse (n = 4 for F2 mice and n = 3 for p50 KO mice). All drug solutions were freshly prepared prior to injection, which was performed immediately prior to dark onset at 9.00 AM. Locomotion was recorded at 10 minute intervals for 2 hours after injection.

For each drug treatment, procedures were as follows: Data recording began in untreated mice one day before drug administration. The next day, drug was administered by intraperitoneal injection to mice during the 8 min period prior to dark onset (i.e., 9.00 AM). Preliminary analysis revealed that the peak locomotor effects occurred during the initial 60 min after injection, declined thereafter, and were near basal values at 100–120 min after drug administration. We therefore analyze and report locomotion data at 10 minute intervals during the initial 120 min after drug administration (i.e., 9.00 AM to 11.00 AM). Tests of all drugs used identical procedures, and data obtained after drug administration were compared with values obtained for each mouse during the same diurnal interval on the preceding day. Mice were euthanized after completion of the entire series of injections.

Statistical analysis

SPSS for windows software (SPSS Inc., Chicago, IL) was used for data analysis. One-way analysis of variance (ANOVA) was performed to assess between-strain DAT band density data and concentrations of caffeine and dopamine. Multi-factor repeated-measures ANOVA was performed to evaluate locomotion data. The between-subject factor was mouse strain, and within-subject factors were activity in 2 h or 10 min intervals before and after treatment. Both types of ANOVAs were followed by Bonferroni corrections for multiple comparisons. Data were summarized as mean $\pm$ standard error (SEM), and statistically significant effects were defined as $p < 0.05$.

Results

Caffeine concentration in sera and brain (Fig. 1)

Caffeine concentrations were measured in blood and brain at 1, 5, 15 and 30 minutes after intraperitoneal injection of caffeine in F2 mice. In serum, concentrations of caffeine did not vary significantly among the time points tested (11.7 ± 2.3 , 12.9 ± 1.1 , 12.7 ± 0.1 , and 10.9 ± 0.3 $\mu\text{g/ml}$ sera at 1, 5, 15, and 30 minutes, respectively, $n = 3, 6, 3, 3$, respectively). Serum caffeine concentrations were measured in p50 KO mice at 5 minutes after caffeine administration. The concentration in serum was 10.8 ± 0.5 $\mu\text{g/ml}$ ($n = 6$), which did not differ significantly from the concentration in F2 mice at that time point.

In brain of F2 mice, caffeine concentrations peaked at 5 minutes after intraperitoneal injection and did not change significantly at the 15 and 30 minute time points (0.1 ± 0.1 , 9.6 ± 2.2 , 7.7 ± 1.1 , and 6.3 ± 1.6 $\mu\text{g/mg}$ protein, respectively; $n = 3, 6, 3, 3$). Thus, caffeine concentrations rose more gradually in brain as compared with serum, requiring between 1 and 5 minutes to reach peak levels in brain as compared with less than 1 minute in serum. The concentration measured in brain of KO mice at 5 minutes after injection (12.4 ± 1.1 , $\mu\text{g/mg}$ protein; $n = 6$) was not significantly different from F2 mice at that time point.

Serum concentrations of caffeine at 1 and 4 hours after oral administration (15 mg/kg by gavage), were 6.5 ± 0.2 and 7.2 ± 1.2 $\mu\text{g/ml}$ for F2 mice and 2.0 ± 0.1 and 1.8 ± 0.2 $\mu\text{g/ml}$ for KO mice ($n = 4$ for F2 mice and 3 for KO mice). Caffeine concentration was significantly higher at the 1 hour versus 4 hour time point ($F=69.066$; $df=1,5$; $p<.001$), but the two strains were not significantly different from each other at either time point.

Caffeine concentrations in brain at 1 and 4 hours after oral administration were 6.4 ± 0.4 and 1.9 ± 0.2 $\mu\text{g/mg}$ protein in F2 mice and 7.2 ± 1.2 and 1.9 ± 0.3 $\mu\text{g/mg}$ protein in KO mice (sample size as above). Brain caffeine concentrations were significantly higher at the 1 hour versus 4 hour time point ($F=62.007$; $df=1,5$; $p=.001$), but the two strains did not differ significantly from each other at either time point.

DA and DOPAC concentrations, DA transporter, and DA turnover in striata of F2 and KO mice

DA and DOPAC concentrations after oral administration of caffeine (Fig. 2)—DA and DOPAC concentrations were measured in striata of F2 and p50 KO mice before and at 1 and 4 hours after administration of caffeine (15 mg/kg) by gavage. Striatal DA and DOPAC concentrations in untreated mice and at 1 or 4 hours after treatment did not vary significantly between strains ($n = 6$ per group). The DOPAC/DA ratio data showed a significant groups $\times$ time interaction ($F=4.62$; $df=2,20$; $p=.022$). In p50 KO mice, the ratios of DOPAC to DA were lower at 1 and 4 hours after treatment as compared with basal values, but significance was not retained after Bonferroni correction.

DA transporter in F2 and KO mice (Fig. 3)—The DA transporter, as measured by Western blot analysis, was present in equivalent levels in striata of F2 and p50 KO mice (n = 8 per strain).

DA depletion after α -MPT treatment (Fig. 4)—DA turnover was measured based on depletion of DA after administration of the tyrosine hydroxylase inhibitor α -MPT. In F2 mice, striatal DA concentrations did not vary as a function of time after injection. In contrast, in p50 KO mice, striatal DA concentration dropped significantly within 15 minutes after α -MPT treatment ($F=14.493$; $df=1,19$; $p=.001$) and remained lower at 60 minutes ($F=5.78$; $df=1,18$; $p=.027$).

Striatal DA concentrations were also measured at 15 or 60 minutes after simultaneous administration of α -MPT and caffeine. At 15 and 60 minutes after the combined treatment, DA values were not significantly different between strains. In p50 KO mice, combined treatment did not significantly alter values as compared with α -MPT alone, whereas in F2 mice, the co-administration of caffeine and α -MPT caused a significant reduction in DA concentrations as compared with α -MPT alone ($F=9.849$; $df=1,27$; $p = 0.004$). Thus, p50 KO mice appear to have a marginally more rapid turnover of striatal DA than do F2 mice under basal conditions, and administration of caffeine increases DA turnover in F2 but not p50 KO mice.

Drug effects on locomotor activity

Basal conditions and vehicle administration—F2 (n = 15) and p50 KO (n = 11) mice were evaluated for locomotion and core temperature for 24 hours under baseline conditions (Fig. 5). Significant differences were not detected between the two mouse strains regardless of whether the data were evaluated based on 2-hour averages obtained over the entire 24 hour interval (Fig. 5) or as 10 minute averages during the initial 2 hour interval after dark onset (data not shown).

Administration of saline to F2 (n = 9) and p50 KO (n = 7) mice immediately before dark onset caused a significant increase in activity during the initial 20 minutes after injection ($F=42.513$; $df=1,14$; $p < 0.001$) (Fig. 6). However, the pattern and magnitude of the effect was not influenced by strain. Similarly, administration of the vehicle DMSO to F2 (n = 4) and p50 KO (n = 3) mice immediately before dark onset caused a significant increase in activity in both strains ($F=6.831$; $df=1,5$; $p = 0.047$), but this effect also did not vary as a function of strain (Fig. 6).

Caffeine—Intraperitoneal injection of caffeine (15 mg/kg) at either light onset (n = 6 per strain) or dark onset (n = 7 per strain) increased activity in both F2 and p50 KO mice, as compared with activity during the corresponding basal period ($F=62.165$; $df=1,31$; $p < 0.01$) (Fig. 7). Activity was marginally higher in p50 KO mice as compared with F2 mice, as indicated by a significant time by strain interaction ($F=4.144$; $df=1,31$; $p=.05$). However, the main effect of strain was not significant.

SCH 58261—Intraperitoneal injection of F2 and p50 KO mice (n = 7 per strain) with SCH 58261 (a selective A2AAR antagonist; 10 mg/kg) immediately prior to dark onset increased locomotor activity in both strains relative to the basal values ($F=75.828$; $df=1,12$; $p < 0.001$), with no significant interaction of time and strain (Fig. 8). When the treatment phase was evaluated separately, a significant strain effect was detected ($F=7.844$; $df=1,12$; $p = 0.016$).

DPCPX—Intraperitoneal injection of F2 and p50 KO mice (n = 7 per strain) with DPCPX (a selective A1AR antagonist; 5 mg/kg) immediately prior to dark onset increased activity in both strains relative to the basal values ($F=28.977$; $df=1,12$; $p < 0.001$) (Fig. 8). This effect was not

strain-dependent ($p = 0.072$). The DPCPX effect was similar in magnitude to the effect of treatment with vehicle alone (Fig. 6) and was markedly smaller in magnitude than the effect of SCH 58261.

Raclopride prior to SCH 58261—Intraperitoneal injection of raclopride (0.5 mg/kg) immediately before dark onset did not significantly alter locomotor activity in either strain of mice ($n = 7$ per strain) (data not shown). Administration of raclopride 15 minutes before administration of SCH 58261 (10 mg/kg) was associated with a significant increase in activity as compared with the basal period ($n = 7$ F2 and 7 KO mice; $F=87.684$; $df=1,12$; $p < 0.001$) (Fig. 9), but the effect was less robust than that of SCH 58261 alone (Fig. 8). The effect varied significantly as a function of strain, with F2 mice showing more activity than did p50 KO mice ($F=7.957$; $df=1,12$; $p = 0.015$). Analysis of treatment data alone ($n=12$ F2 and 13 KO mice) revealed a significant effect of time ($F=65.268$; $df=1,23$; $p < 0.001$) and strain ($F=8.663$; $df=1,23$; $p=.007$) but no interaction.

SCH 58261 and DPCPX—The effect of joint administration of SCH58261 and DPCPX on locomotor activity was evaluated in F2 ($n = 6$) and p50 KO ($n = 5$) mice (Fig. 9). The combined drug treatments increased activity as compared with basal conditions ($F=48.841$; $df=1,9$; $p<0.001$), but this effect was not influenced by mouse strain ($p = 0.111$). Analysis of treatment data alone revealed a significant effect of time ($F=33.014$; $df=1,9$; $p < 0.001$), and strain ($F=6.037$; $df=1,9$; $p = 0.036$) but no interaction of time with strain.

Discussion

Caffeine pharmacokinetics

The data reported here show that concentrations of caffeine in serum and brain do not differ significantly between p50 KO and genetically intact F2 mice after intraperitoneal or oral administration. These findings suggest that the two strains have similar caffeine absorption from gastrointestinal tract and peritoneal cavity, drug penetration across blood-brain barrier, and metabolism and excretion of caffeine. The differential sensitivity of mice to locomotor stimulation after caffeine ingestion (Xie et al., 2007a) is therefore not likely to be due to differences in caffeine pharmacokinetics in p50 KO mice as compared with the genetically intact F2 mice.

In rats given intraperitoneal injections of caffeine, caffeine concentrations in peripheral blood closely parallel those measured in striatum (Heppert and Davies, 1999). In F2 mice in our study, caffeine concentrations peaked in brain at 5 minutes after intraperitoneal injection, and, consistent with previous findings (Kaplan et al., 1989), decreased more rapidly in brain than in blood, with no significant between-strain differences at the common time point (5 minutes after administration). These data support the conclusion that p50 KO and F2 mice have similar rates of caffeine clearance from blood and brain. Although caffeine concentrations peaked in brain at 5 minutes after injection, the maximal behavioral change occurred at 10 minutes after treatment (see below). This temporal difference could be due to the time required for receptor-triggered intracellular events that impact neuronal activity.

DA turnover

Concentrations of DA and DOPAC, the DOPAC/DA ratio, and protein levels of the DA transporter were not significantly different between F2 and p50 KO mice under basal conditions, suggesting similar basal rates of DA synthesis, uptake, and metabolism in the two strains of mice. However, DA depletion was greater after administration of the competitive tyrosine hydroxylase inhibitor α -MPT in p50 KO mice as compared with F2 mice. After blockade of DA synthesis by α -MPT, a more rapid rate of DA depletion suggests more rapid

metabolism of DA. The strain difference we detected thus suggests relatively faster turnover of DA under basal conditions in the p50 KO mice. This could result in greater synaptic accumulation of DA in the KO mice if release exceeded the capacity of the DAT. More definitive assessment of this possibility would require direct quantification of extracellular DA by using *in vivo* microdialysis to collect samples under the conditions used in the current study. However, this scenario is feasible because the DAT was present to a similar degree in both strains of mice. Greater DA turnover after administration of α -MPT to p50 KO mice may indicate a compensatory mechanism that allows p50 KO mice to maintain normal levels of locomotor activity under basal conditions despite their reduced expression of striatal D2R (Xie et al., 2007a). Another possibility is that the more rapid decline in DA in p50 KO mice treated with α -MPT reflects a more robust and strain-dependent stress response to injection of α -MPT as compared with saline.

Our data did not reveal a between-strain difference in DA turnover after the combined administration of caffeine and α -MPT. This finding could be related to the different A1AR densities of the two strains of mice (Xie et al., 2007a). The relatively lower A1AR expression in p50 KO mice, as compared with F2 mice, may reduce the functional presynaptic target for caffeine. Because caffeine promotes the release of both glutamate and DA in rodent striatum (Borycz et al., 2007; Quarta et al., 2004), a change in the A1AR-A2AAR balance could influence glutamate release after caffeine administration. With less inhibition from A1AR, A2AAR activation might elicit greater glutamate release, which would subsequently stimulate (via binding to metabotropic glutamate receptor type I on DA terminals) more DA release, thus balancing the relatively lower rate of striatal DA turnover in F2 mice.

A single dose of caffeine administered by gavage did not differentially alter striatal DA concentrations as a function of mouse strain at either 1 or 4 hours after treatment, although an earlier or intervening change would not have been captured at our selected time points. Those findings, together with the absence of significant between-strain differences in striatal DAT protein or in DA concentrations after combined treatment with α -MPT and caffeine, suggests that although DA turnover may be higher in p50 KO mice under basal conditions, the differential sensitivity to caffeine may be largely related to postsynaptic mechanisms.

Psychomotor effects of drugs

Spontaneous locomotion in rodents is regulated in basal ganglia by joint effects of the direct and indirect pathways. The neuronal activities of the direct and indirect pathways correlate positively and negatively, respectively, to actual locomotor output. The indirect pathway, rather than the direct pathway, appears to have a greater role in mediating caffeine-induced modulation of locomotion. Our current data show that at the doses tested, the selective A2AAR antagonist SCH 58261 promotes locomotion after intraperitoneal injection, whereas caffeine and the selective A1AR antagonist DPCPX have smaller or non-significant effects. The stimulatory effect of SCH 58261 was marginally greater in p50 KO mice as compared with F2 mice, but the effects of caffeine and DPCPX were not strain-dependent. The locomotor-stimulating effects of both caffeine and DPCPX are greatly attenuated in A2AAR knockout mice (El Yacoubi et al., 2000). In addition, selective A2AAR blockade promotes both D1R- and D2R-induced stimulation of locomotion (Fenu et al., 1997). In contrast, selective A1AR blockade only weakly promotes D1R agonist-induced locomotor activity (Pinna et al., 1996). These findings support a greater role for A2AAR, as opposed to A1AR, in caffeine-induced psychomotor stimulation and may reflect dual-pathway regulation of A2AAR (i.e., modulation by the indirect pathway of the A1AR-modulated direct pathway) (Fig. 10).

SCH 58261 is 53 to 750-fold more selective for A2AAR versus A1AR, depending on category of experimental subjects (Ongini et al., 1997). SCH 58261 increases locomotion in rodents to a degree comparable to caffeine, with the psychostimulant effect attributed to blockade of post-

synaptic A2AAR (Lindskog et al., 2002; Svenningsson et al., 1997). A2AAR antagonism indirectly reduces tonic suppression of D2R and promotes dopaminergic neurotransmission in the indirect pathway (Svenningsson et al., 1997). In the current study, intraperitoneal injection of SCH 58261 significantly increased locomotor activity in both F2 and p50 KO mice as compared with basal conditions, with the activity higher in p50 KO mice than in F2 mice at 30 minutes after injection. Concentrations of SCH 58261 peak in blood of mice at about 15 minutes after systemic administration (Svenningsson et al., 1997). Thus, the significant increase in locomotion at 30 minutes after drug injection could correspond to peak drug concentrations in the brain, with a necessary time lag for activation of intracellular signaling systems.

Literature reports of the effects of systemic DPCPX on locomotion vary greatly in their findings. For example, DPCPX reportedly increases the activity of some strains of mice, reduces the activity of other mouse strains, and does not affect the activity of rats (Kuzmin et al., 2006; Svenningsson et al., 1997). This variation in effects is consistent with a previous conclusion that A1AR is not crucial in mediating caffeine-induced behavioral stimulation (Halldner et al., 2000). Administration of DPCPX produces a small but significant increase in locomotor activity of F2 and p50 KO mice as compared with untreated mice. However, the magnitude of the DPCPX effect is similar to that of vehicle administration. Furthermore, the effects of DPCPX are not different in F2 versus p50KO mice.

Theoretically more A2AAR and less D2R in the striata of p50 KO mice would permit greater A2AAR suppression of postsynaptic D2R and thus reduce locomotor activity. However, the similar basal activity of F2 and p50 KO mice suggests that despite the imbalanced receptor densities of KO mice, their postsynaptic activity is nonetheless functionally balanced. As a result, the greater SCH 58261-induced locomotion in p50 KO mice suggests that D2R have greater efficacy in mediating downstream signaling as compared with F2 mice. Our finding that SCH 58261-induced enhancement of locomotion was prevented by pre-treatment of p50 KO mice with raclopride suggests that the behavioral sensitivity of p50 KO mice to SCH 58261, and, by extension, to caffeine, depends critically on D2R-mediated neurotransmission. Furthermore, oral caffeine administration caused a sustained increase in locomotion in p50 KO mice during the dark phase (Xie et al., 2007a), whereas injected SCH 58261 and caffeine caused only a transient enhancement of locomotion in p50 KO mice at the doses tested. These different durations of action could reflect different pharmacokinetic features of systemic and oral drug administration or suggest a pharmacodynamic property of caffeine that occurs via mechanisms other than A2AAR antagonism.

The simultaneous treatment of mice with DPCPX and SCH 58261 increased locomotor activity to a similar degree in both strains. However, the large stimulatory effect seen with SCH 58261 alone was attenuated by the combined treatment. Others have reported that the combined administration of low doses of both DPCPX and SCH 58261 to mice promoted locomotion to a degree similar to caffeine (Kuzmin et al., 2006). However, the doses we used for the combined treatment were the same as those we used in the single drug studies. Furthermore, comparison of the effect of caffeine with that of combined A1AR and A2AAR antagonists is complicated in that the affinity of the non-selective antagonist caffeine for A1AR and A2AAR receptors likely differs from that of DPCPX and SCH 58261, which both have high affinity for their target receptors. Furthermore, caffeine acts via mechanisms other than receptor blockade (e.g., inhibition of phosphodiesterase), which could also contribute to different outcomes in comparison with the administration of two selective adenosine receptor antagonists.

From another perspective, the combined treatments with DPCPX and SCH 58261 could create a situation in which neurons of the indirect pathway generate a suppressive effect sufficient to counteract a stimulatory effect caused by elevated synaptic glutamate on neurons of direct

pathway. Such interactions could occur, for example, via increased glutamate release due to presynaptic A1AR blockade by DPCPX. This increased glutamate in turn increases stimulation of postsynaptic NMDA and metabotropic glutamate 5 receptors (mGluR5), as evidenced by enhanced immediate early gene expression after A1AR antagonism (Dassesse et al., 1999). A2AR, which are increased in p50 KO mice (Xie et al., 2007a), are permissive for and interdependent with mGluR5 (Domenici et al., 2004; Kachroo et al., 2005). This situation would allow strong inhibition from indirect pathway that counteracts stimulation from the direct pathway, ultimately attenuating significant motor stimulation in association with A2AAR selective blockade.

Conclusions

This study investigated the impact of loss of the p50 subunit of NF- κ B on the roles of A2AAR, A1AR, and D2R in striatal regulation of locomotion in mice. These findings, together with the data regarding caffeine pharmacokinetics and striatal DA turnover, allow the following conclusions, as depicted in the model shown in Fig. 10. 1) A2AAR antagonism promotes locomotion to a greater degree than does A1AR antagonism; 2) A2AAR antagonism has a greater stimulatory effect in p50 KO mice, which is perhaps due to increased synaptic DA; and 3) because raclopride pre-treatment prevents SCH 58261-induced locomotor activation, DA content and D2R activity are critical to mediating the stimulatory effect of A2AAR antagonists. Additional *in vivo* studies are warranted to test the proposed changes in synaptic neurotransmitters, to evaluate the role of D2R in mediating downstream signal transduction in p50 KO versus F2 mice, and to determine the basis for the behavioral differences in responses to ingested versus injected caffeine. Such information will further our understanding how these substances interact to regulate receptor expression and patterns of neurotransmission, and thereby impact the behavioral outcomes of striatal locomotor control.

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Figure 1. Caffeine concentration in serum and brain after caffeine administration by injection or gavage

A, Caffeine concentration ($\mu\text{g/ml}$) was measured in serum collected from F2 mice at 1, 5, 15, or 30 minutes after intraperitoneal injection of caffeine (15 mg/kg body weight) ($n = 3, 6, 3$, and 3 for each time point, respectively) and from KO mice at 5 minutes after injection ($n = 6$); **B**, Caffeine concentration ($\mu\text{g/ml}$) was measured in serum collected at 1 or 4 hrs after caffeine administration by gavage (15 mg/kg body weight) (F2, $n = 4, 4$ and KO, $n = 3, 3$ at time points of 1 and 4 hr, respectively); **C**, Caffeine concentration ($\mu\text{g/gram}$ of tissue) was measured in whole brain as in **A**; **D**, Caffeine concentration ($\mu\text{g/gram}$ of tissue) was measured in whole brain as in **B**. Values in all panels are averages $\pm$ SEM.

Figure 2. Striatal DA and DOPAC concentrations and DOPAC/DA ratios under basal conditions and after oral administration of caffeine

A single dose of caffeine (15 mg/kg body weight) or an appropriate volume of saline was administered by gavage. Mice were euthanized without treatment (control) or at 1 or 4 hours after gavage ($n = 6$ per time point). Samples were processed for HPLC assay of DA and DOPAC. DA and DOPAC values are expressed as ng/mg protein. All data points represent average values $\pm$ SEM.

Figure 3. Dopamine transporter in striatum of F2 and p50 KO mice

Striata were removed from untreated F2 and KO mice and processed for western blotting. All data were corrected for loading and were then normalized according to the average band density value obtained for F2 mice. The molecular weight markers were much darker than the bands for DAT and were therefore removed from the gel prior to exposure for DAT (i.e., a 10 sec exposure was used for the molecular weight ladder, whereas a 600 sec exposure was used for DAT). However, the DAT bands were of the correct molecular weight of approximately 88 kD (i.e., migration position between the 100 and 75 kD bands). The DAT and β -actin shown are taken from the same membrane; DAT bands were also less intense than β -actin bands.

Figure 4. Dopamine concentration after administration of α -MPT with or without caffeine
 F2 and KO mice were injected with α -MPT (250 mg/kg body weight i.p.), followed immediately by injection of caffeine (15 mg/kg body weight, i.p.). Mice were euthanized before or at 15 or 60 minutes after injection (n = 8 per group). Samples were processed for HPLC assay for DA concentration. Values are expressed as ng/mg protein, and are presented as averages $\pm$ SEM.

Figure 5. Basal locomotor activity and core temperature in F2 and p50 KO mice

Locomotor activity was recorded for 24 hours beginning at dark onset in mice that were well acclimated to the recording conditions and had received no experimental intervention. The bar on the abscissa denotes the dark phase of the diurnal cycle. Values did not vary significantly as a function of mouse strain. Data are shown as average value $\pm$ SEM.

Figure 6. Locomotor activity after administration of drug vehicles to F2 and p50 KO mice
 F2 and p50 KO mice were injected intraperitoneally with either 0.3 ml of sterile saline or 50 μ l of 100% DMSO immediately prior to dark onset. Locomotor activity (counts per minute) was recorded during the next 120 minutes. The basal condition refers to data collected from the mice during the same diurnal interval on the preceding day. Data are shown as averages $\pm$ SEM. The horizontal dashed line represents the maximal average value measured under basal conditions, as shown in Figure 4.

Figure 7. Locomotor activity after administration of caffeine to F2 and p50 KO mice

F2 and p50 KO mice were injected intraperitoneally with caffeine (15 mg/kg body weight at a volume of 10 ml/kg body weight) immediately after light onset ($n = 6$ per strain) or immediately before dark onset ($n = 7$ per strain). Locomotor activity (counts per 10 minutes) was then recorded for 120 minutes. The basal condition refers to data collected from the mice during the same diurnal interval on the preceding day. Data are shown as averages $\pm$ SEM. The horizontal dashed line represents the maximal average value measured under basal conditions, as shown in Figure 4.

Figure 8. Locomotor activity after administration of SCH 58261 or DPCPX in F2 and p50 KO mice
 F2 and p50 KO mice were injected with SCH 58261 (a A_{2A}AR selective antagonist) (10 mg/kg body weight) or DPCPX (a A_{1A}AR selective antagonist) (5 mg/kg body weight). Drugs were freshly prepared in 100% DMSO solution and were injected intraperitoneally at a volume of 1.4 ml/kg body weight immediately before dark onset. Locomotor activity (counts per minute) was recorded during the next 120 minutes. The basal condition refers to data collected from the mice during the same diurnal interval on the preceding day. Data are shown as averages $\pm$ SEM. The horizontal dashed line represents the maximal average value measured under basal conditions, as shown in Figure 4. Asterisks (*) indicate $p < 0.05$.

Figure 9. Locomotor activity after administration of SCH 58261 with raclopride or DPCPX in F2 and p50 KO mice

F2 and p50 KO mice were injected with SCH 58261 (an A_{2A}AR selective antagonist) (10 mg/kg body weight) or DPCPX (an A₁AR selective antagonist) (0.5 mg/kg body weight). Drugs were freshly prepared in 100% DMSO solution and were injected intraperitoneally at a volume of 1.4 ml/kg body weight immediately before dark onset. In the top panel, raclopride was injected 15 min before injection of SCH 58261. In the lower panel, SCH 58261 and DPCPX were injected simultaneously. Locomotor activity (counts per minute) was recorded during the 120 minute period after administration of the second drug. The basal condition refers to data collected from the mice during the same diurnal interval on the preceding day. Data are shown as averages $\pm$ SEM. The horizontal dashed line represents the maximal average value measured under basal conditions, as shown in Figure 5.

Figure 10. Adenosine and dopamine receptor mediated striatal neurotransmission in p50 KO and F2 mice: proposed postsynaptic effects of caffeine

The three panels illustrate a proposed model for the impact of dopamine and adenosine receptor subtypes on dopamine release and postsynaptic signal transduction that impact locomotion. Three situations are illustrated: *left*, normal physiological state in genetically intact mice; *middle*, altered receptor changes and associated compensatory changes in p50 KO mice under basal conditions; *right*, p50 KO mice after administration of caffeine, which causes greater psychomotor stimulation in p50 KO versus F2 mice.