Time-Dependent Changes in Receptor/G-Protein Coupling in Rat Brain following Chronic Monoamine Transporter Blockade

Kerry A. O’Connor, Linda J. Porrino, Huw M. L. Davies, and Steven R. Childers

Department of Physiology/Pharmacology, Center for the Neurobiological Investigation of Drug Abuse, Wake Forest University Health Sciences, Winston-Salem, North Carolina (K.A.O., L.J.P., S.R.C.); and Department of Chemistry, State University of New York at Buffalo, Buffalo, New York (H.M.L.D.)

Received September 28, 2004; accepted January 10, 2005

ABSTRACT

The potent tropane analog, WF-23 [2β-propanoyl-3β-(2-naphthyl) tropane], blocks dopamine, serotonin, and norepinephrine transporters with high affinity in vitro and blocks transporters for at least 2 days following a single in vivo administration. Previous studies demonstrated desensitization of monoamine receptor-coupled G-proteins in brain following chronic treatment of rats with WF-23. The current study sought to determine the time course of this desensitization and the behavioral effects of receptor desensitization. Rats were treated with 1 mg/kg WF-23 and injected i.p. every 48 h for 1 to 21 days. Receptor activation of G-proteins was determined by guanosine 5′-O-(3-[35S]thiotriphosphate) ([35S]GTPγS) binding in brain sections for monoamine receptors, as well as for opioid receptors as a nonmonoamine receptor control. Chronic treatment with WF-23 produced significant reductions in D2, 5-hydroxytryptamine 1A, and α2-adrenergic receptor-stimulated [35S]GTPγS binding; however, the time course of desensitization varied with different receptors. There was no effect of WF-23 treatment on μ opioid-stimulated [35S]GTPγS binding at any time point. Consistent with previous studies, there was no effect of WF-23 treatment on D2 receptor binding, as determined by [3H]spiperone autoradiography. Locomotor activity was significantly increased for up to 48 h following acute administration of WF-23, demonstrated by increased photocell beam interruptions. WF-23-induced increases in locomotor activity occurred following repeated administration, as above, for up to 7 days. Following 7 days of treatment, there was a significant decrease in WF-23-increased locomotor activity. This reduction occurred at the same time point as the decrease in D2 receptor/G-protein coupling, suggesting a role of D2 desensitization in producing tolerance to WF-23-mediated behavior.

Actions of the biogenic amines are terminated via reuptake by specific transporters. Cocaine and many antidepressants exert their actions via blockade of this process (Ritz et al., 1987). Although most studies suggest the reinforcing effects of cocaine are mediated by inhibition of dopamine transporters, cocaine inhibits dopamine, serotonin, and norepinephrine transporters (DAT, SERT, and NET, respectively) with approximately the same affinity (Ritz et al., 1987). Studies involving DAT knockout mice also support the involvement of SERT and NET in the mechanisms of behavioral effects of cocaine (Sora et al., 2001; Mead et al., 2002).

Many laboratories have utilized long-acting biogenic amine transporter blockers (Carroll et al., 1992; Madras et al., 2003; Appell et al., 2004) to investigate the neurobiological actions of cocaine. Davies and colleagues (Davies et al., 1993) have developed a novel series of tropane analogs based on vinyl-carbenoid precursors, which exhibit a greater range of structural modification possibilities and increased metabolic stability. The most potent analog to date, WF-23, binds with high affinity at DAT (K1 = 0.12 nM), SERT (K1 = 0.39 nM), or NET (K1 = 2.9 nM) (Bennett et al., 1995). WF-23 has also been shown to produce increases in locomotor activity for up to 24 h following a single injection (1 mg/kg i.p.); the same treatment with WF-23 also blocked DAT binding in rat striatum by at least 48 h after injection (Daunais et al., 1998). The high affinity for biogenic amine transporters and long duration of action of WF-23 in both behavioral and biochemical assays provides a useful pharmacological tool to investigate the effects of long-term blockade of biogenic amine transporters.

Blockade of DAT, SERT, and NET by cocaine or other

ABBREVIATIONS: DAT, dopamine transporter; SERT, serotonin transporter; NET, norepinephrine transporter; WF-23, 2β-propanoyl-3β-(2-naphthyl) tropane; [35S]GTPγS, guanosine 5′-O-(3-[35S]thiotriphosphate); 5-HT1A, 5-hydroxytryptamine 1A; DAMGO, [d-Ala2,N-Me-Phe4,Gly5-ol]-enkephalin; NPA, (R)-propyl-norapomorphine; 8-OH-DPAT, 8-hydroxy-2- dipropylaminotetralin; [125I]RTI-55, 3β-(4-[125I]iodophenyl)-tropane-2-carboxylic acid methyl ester.
inhibitors produces increased levels of monoamines in the synaptic cleft and a prolonged exposure of receptors to these neurotransmitters (Hemby et al., 1995). Both receptor desensitization, as defined by prolonged uncoupling of receptors from G-proteins, and down-regulation, defined as a decrease in receptor number, occur in response to chronic agonist exposure (Lefkowitz et al., 1990). One of the advantages of a potent long-acting monoamine transporter inhibitor like WF-23 is the opportunity to examine monoamine receptor desensitization that occurs following chronic increases in extracellular monoamines.

Monoamines exert their actions by binding to specific receptors, most of which belong to the superfamily of seven transmembrane-spanning receptors that couple to G-proteins (Neve, 1997). The receptor G-protein activation cycle has been well characterized to show that binding of agonists to G-protein-coupled receptors dramatically increases the affinity of Gα subunits for GTP (Birnbaumer et al., 1990). This change in Gα can be detected by agonist-stimulated [35S]GTPγS binding, which can be assayed both in brain membranes (Lorenzen et al., 1993; Traylor and Nahorski, 1995) and in brain sections by autoradiography (Sim et al., 1995). Specifically, activation of [35S]GTPγS binding in brain has been specifically measured for monoamine receptors, specifically dopamine D2 (Rinken et al., 1999; O’Connor et al., 2004), α2-adrenergic (Happe et al., 2000), and 5-HT1A receptors (Newman-Tancredi et al., 1996; Sim-Selley et al., 2000b). Agonist-stimulated [35S]GTPγS binding provides an excellent method to determine effects of chronic drug treatment on receptor/G-protein desensitization, i.e., the loss of activation of Gα subunits by receptor agonists (Breivogel et al., 1997a, 1999; Sim-Selley et al., 2000a).

It is clear that chronic treatment with cocaine and other monoamine transporter inhibitors may affect receptor-mediated signal transduction by long-term increases in extrasynaptic monoamines. For example, alterations in several signal transduction mechanisms have been demonstrated following chronic administration of cocaine (Beitner-Johnson et al., 1992; Unterwald et al., 1996; Kushner and Unterwald, 2001). In a previous study (O'Connor et al., 2004), we showed that 1992; Unterwald et al., 1996; Kushner and Unterwald, 2001). Transduction mechanisms have been demonstrated following receptor desensitization that occurs following chronic increases in extracellular monoamines. For example, alterations in several signal transduction mechanisms have been demonstrated following chronic administration of cocaine (Beitner-Johnson et al., 1992; Unterwald et al., 1996; Kushner and Unterwald, 2001). In a previous study (O’Connor et al., 2004), we showed that 1992; Unterwald et al., 1996; Kushner and Unterwald, 2001). Transduction mechanisms have been demonstrated following receptor desensitization that occurs following chronic increases in extracellular monoamines. For example, alterations in several signal transduction mechanisms have been demonstrated following chronic administration of cocaine (Beitner-Johnson et al., 1992; Unterwald et al., 1996; Kushner and Unterwald, 2001). In a previous study (O’Connor et al., 2004), we showed that

Materials and Methods

Materials. WF-23 was synthesized as previously described (Davies et al., 1994) and was dissolved in phosphate-buffered saline, which served as the vehicle. [35S]GTPγS (1150–1395 Ci/μmol), [125I]RTI-55 (2200 Ci/mol), and [3H]spiperone (15.7 Ci/μmol), Kodak BioMax MS Films, Kodak BioMax HE TranScreens, and TR tritium sensitive phosphor screens were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). DAMGO, 8-cyclopentyl-1,3-dipropylxanthine, fluoxetine, GDP, ketanserin, norepinephrine, RTI-5-(242 × 30 cm). locomotion was

8-OH-DPAT were obtained from Sigma-Aldrich (St. Louis, MO). X-ray films were obtained from Phenix Research (Hayward, CA).

Animals. Male Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing 200 to 300 g at the time of the experiment, were used in all studies. Rats were treated with i.p. injections of WF-23 (1 mg/kg) or saline every 48 h for 3 to 21 days (n = 6 per group). The dose and treatment period were based on previous studies (Daunais et al., 1998) that demonstrated a robust behavioral response and a 50% decrease in [125I]RTI-55 binding 48 h after a single injection of WF-23 (1 mg/kg i.p.). Animals were pair-housed in a climate-controlled room with a 12-h light/dark cycle. Food and water were available ad libitum. All animals were adapted to vivarium conditions for 5 days before testing. Injections were given so that all testing occurred during the light phase of the cycle (7:30 AM–4:30 PM). All procedures were carried out in accordance with established practices as described in the National Institutes of Health Guide for Care and Use of Laboratory Animals. In addition, all procedures were reviewed and approved by the Animal Care and Use Committee of Wake Forest University.

[35S]GTPγS Autoradiography. Receptor/G-protein coupling was assayed in rat brain sections using agonist-stimulated [35S]GTPγS autoradiography (Sim et al., 1995; Rinken et al., 1999). Saline- and WF-23-treated animals were euthanized by rapid decapitation 48 h after the last injection on days 3, 7, 15, and 21 (n = 6 per time point). Brains were removed and prepared for sectioning. Rat brain sections were preincubated for 10 min in TME buffer (50 mM Tris-HCl, 3 mM MgCl2, 0.2 mM EGTA, 100 mM NaCl, pH 7.4), then 15 min with 1 to 2 mM GDP and 1 μM 6-cyclopentyl-1,3-dipropylxanthine at 25°C. Sections were incubated for 90 min at 30°C for D2 and 120 min at 25°C for μ, α2, and 5-HT1A. Agonists included: 300 μM norepinephrine (α1), 10 μM NPA (D2), 3 μM DAMGO (μ opioid), and 3 μM 8-OH-DPAT (5-HT1A). The sections were then washed, exposed to X-ray film, and analyzed as described previously (Sim et al., 1995). Agonist-stimulated activity was calculated by subtracting the optical density in basal sections (GDP only) from that of agonist-stimulated sections, and results are expressed as percent stimulation over basal activity. For each agonist, triplicate sections of brain from at least four animals were used.

[3H]Spiperone Autoradiography. D2 receptor binding was assayed in rat brain sections using [3H]spiperone autoradiography (Palacios et al., 1981; Araki et al., 1997). Sections of rat brain at the level of the caudate/putamen from saline- and WF-23-treated animals were prepared as described above. Rat brain sections were preincubated for 10 min in Tris buffer (50 mM Tris-HCl, 1 mM MgCl2, pH 7.6) at 25°C. Sections were incubated in Tris buffer with 0.6 nM [3H]spiperone and 100 nM ketanserin for 60 min at 25°C. Nonspecific binding was assayed in the presence of unlabeled spiperone (0.2 μM). Sections were then washed twice in Tris and once in H2O at 4°C. Sections were then dried and exposed to TR tritium sensitive storage phosphor screens (PerkinElmer Life and Analytical Sciences) for 3 weeks. The Cyclone Storage Phosphor System with OptiQuant image analysis software (version 03.10) was used to scan images from storage phosphor screens. Images were then imported and analyzed in NIH Image J (version 1.30 for MacIntosh). Specific binding was determined by subtracting the optical density in basal sections (GDP only) from that of agonist-stimulated sections, and results are expressed as percent stimulation over basal activity. For each agonist, triplicate sections of brain from at least four animals were used.

[125I]RTI-55 Autoradiography. DAT binding was performed using [125I]RTI-55 autoradiography (Boja et al., 1992; Yoshiyuki and Tsunehiko, 1997) to explore occupancy of DAT by WF-23. Brain sections were incubated in buffer (10 mM sodium phosphate, 0.32 M sucrose, pH 7.4) with 30 nM fluoxetine and 10 μM [125I]RTI-55 (2200 Ci/mmol) at 25°C for 60 min. Nonspecific binding was assayed with 1 μM WF-23. The sections were then washed, exposed to X-ray film (Kodak BioMax MS Film with BioMax HE TranScreen) at –80°C, and analyzed as described previously (Sim et al., 1995). Preincubations of tissue were excluded to minimize washout of bound WF-23.

Behavioral Testing. Locomotor activity was assessed in openfield clear plastic test chambers (42 × 42 × 30 cm). Locomotion was

Downloaded from jpet.aspetjournals.org at ASPECT Journals on September 23, 2017
measured by electronic counters that detected interruptions of eight independent photocell beams (Omnitech, Columbus, OH). The following measures were recorded and stored in 10-min intervals: horizontal activity (the total number of horizontal beam interruptions) and forward locomotor or ambulatory activity, vertical activity or rearing and stereotypy (the total number of consecutive breaks of the same beam or two adjacent beams).

Animals were habituated to the chamber for 4 consecutive days before testing, for 60 min each day. On the last 2 days of habituation, animals received saline injections. On the 5th day (test day 1), the animals were placed in the chamber for 30 min where preinjection data were obtained. They were then given a single i.p. injection of WF-23 (1 mg/kg) or saline and returned to the locomotor chamber where their activity was recorded for an additional 2.5 h. This procedure was repeated on days 3, 5, 7, and 15. At each time point, locomotor activity was assessed in treatment groups consisting of WF-23 (n = 8) and saline (n = 8). Animals received injections every 48 h throughout the study. Locomotor activity was assessed on day 21 in the absence of drug for 30 min.

**Data Analysis.** Unless otherwise indicated, autoradiography data are reported as mean values ± S.E.M. of at least three separate experiments, each of which were performed in triplicate. Net-stimulated [35S]GTPγS binding is defined as stimulated binding minus basal binding. Percent decrease is defined as (100 − [net-stimulated binding in saline-treated animals]/[net-stimulated binding in WF-23-treated animals]) × 100%. Statistical significance of all data was determined by one-way analysis of variance with repeated measures, followed by Tukey-Kramer test for multiple comparisons to compare WF-23 to vehicle-treated groups (O’Connor et al., 2004). Densitometric analysis of these results confirmed these time-dependent reductions in agonist-stimulated [35S]GTPγS binding for all three monoamine receptor systems (Table 1).

Quantification of autoradiograms at the level of the caudate/putamen showed that D2-stimulated [35S]GTPγS binding was not affected after 3 days of chronic WF-23 treatment, but was significantly reduced (by 23 ± 4% and 34 ± 7%) following 7 and 15 days of treatment, respectively, and further reduced (by 72 ± 10%) following 21 days of treatment. α2-Adrenergic-stimulated [35S]GTPγS binding in amygdala was significantly reduced following 7 days of treatment (by 21 ± 7%), with further reductions occurring with longer treatment (46 ± 9% after 15 days; 61 ± 4% after 21 days). Reductions in α2-adrenergic-stimulated activity in amygdala were observed in some animals after 3 days of WF-23 treatment, but were not significant in the whole treated group. Densitometric analysis of 5-HT1A receptor-stimulated [35S]GTPγS binding in hippocampus revealed significant reduction following a dosing regimen of 10 μM NPA (caudate/putamen), μ opioid using 3 μM DAMGO (caudate/putamen), α2-adrenergic using 300 μM norepinephrine (amygdala), and 5-HT1A using 3 μM 8-OH-DPAT (hippocampus).

**Results**

**Effects of Chronic WF-23 Treatment on Receptor-Activated G-Proteins.** The dose of WF-23 and treatment period were based on previous studies (Daunais et al., 1998) that evaluated the effects of a single administration of WF-23 on horizontal locomotor activity and [125I]RTI-55 binding in caudate/putamen. These studies had demonstrated a 50% decrease in [125I]RTI-55 binding 48 h after a single injection of WF-23 (1 mg/kg i.p.). More recent results showed that chronic treatment with WF-23 (injected every 48 h for 15 days) produced significant desensitization of monoamine receptor-activated G-proteins (O’Connor et al., 2004). Therefore, in our study, injections were given every 48 h and brains removed 48 h following the last injection after various durations of treatment with WF-23.

The time course effects of chronic WF-23 treatment on receptor-activated G-proteins were examined for three monoamine receptors, D2, α2-adrenergic, and 5-HT1A, as well as μ opioid receptors as a nonmonoamine control. Figure 1 compares typical autoradiograms from saline-treated rats and from rats treated with WF-23 for 3 and 21 days with D2- and μ opioid-stimulated [35S]GTPγS binding in caudate/putamen, α2-adrenergic activity in amygdala, and 5-HT1A activity in hippocampus. In caudate, a robust decrease in D2-stimulated binding was observed after 21 days of chronic WF-23 treatment with no effect observed after only 3 days of treatment. In contrast, there was no effect on μ opioid-stimulated [35S]GTPγS binding in caudate after either 3 or 21 days of WF-23 treatment. In amygdala, chronic WF-23 administration produced some reduction in α2-adrenergic-stimulated [35S]GTPγS binding after 3 days of treatment, with even further reduction evident after 21 days. Similarly, in hippocampus, chronic WF-23 administration also produced reduction in 5-HT1A receptor-stimulated [35S]GTPγS binding after 3 days of treatment, with even further reductions after 21 days (Fig. 1).

Densitometric analysis of these results confirmed these time-dependent reductions in agonist-stimulated [35S]GTPγS binding for all three monoamine receptor systems (Table 1).
single injection of WF-23 (reduced by 33 ± 6%), with further reductions following longer treatment with WF-23 (reduced by 59 ± 6% following 21 days). Although 5-HT1A-stimulated [35S]GTPγS binding was also observed in septum and dorsal raphe, no significant effect of chronic WF-23 was observed in either region (data not shown). In contrast, there was no effect of chronic WF-23 treatment on μ opioid-stimulated [35S]GTPγS binding in caudate/putamen at any time point.

**Effects of Chronic WF-23 Treatment on D2 Receptor and DAT Binding in Caudate/Putamen.** These experiments were designed to determine whether chronic WF-23 treatment affected D2 receptor binding and to use DAT binding in brains from WF-23-treated animals as a measure of occupancy of DAT by WF-23 after chronic treatment. The observed decrease in receptor-stimulated [35S]GTPγS binding after chronic WF-23 treatment may be attributable to a functional uncoupling of the receptor-G-protein unit, a reduction in D2 receptor number, or a combination of both mechanisms. We have previously demonstrated that chronic WF-23 treatment produced desensitization of D2 receptor-activated G-proteins in the absence of down-regulation of D2 receptors following 15 days of WF-23 treatment (O’Connor et al., 2004). However, it is possible that the longer treatment with WF-23 in the current study may result in alterations in receptor number due to the prolonged period of transporter blockade.

Therefore, [3H]spiperone binding was performed following 21 days of WF-23 treatment to assess D2 receptor density. Representative autoradiograms of saline and WF-23-treated animals (Fig. 2, left) show significant binding of [3H]spiperone in caudate/putamen, with no discernible effect of chronic WF-23 treatment. Densitometric analysis of saline- and WF-23-treated sections (n = 6 per group) revealed no significant difference between groups (WF-23 treated animals = 100 ± 3% of saline values). Therefore the decrease in D2-activated G-proteins occurred in the absence of down-regulation of D2 receptors in caudate/putamen following 15 to 21 days of WF-23 treatment.

In addition, occupancy of DAT by WF-23 during the chronic treatment paradigm was assessed in striatal sections from rats treated chronically with saline or WF-23 using [125I]RTI-55 autoradiography. Preliminary experiments using naive animals demonstrated that addition of 30 nM flupentixol was sufficient to block binding of [125I]RTI-55 to SERT (data not shown). Reductions in [125I]RTI-55 binding were observed following a single injection of WF-23 as assessed on day 3, and this reduction appeared to be relatively constant regardless of the duration of treatment (Fig. 2, right). Densitometric analysis showed that [125I]RTI-55 binding to DAT was decreased on average by 32 ± 5% in caudate/putamen of rats chronically treated with WF-23 up to 48 h after each administration when compared with controls (Table 2).

**Effects of WF-23 on Locomotor Activity.** Daunais et al. (1998) showed that a single dose (1 mg/kg) of WF-23 produced robust increases in locomotor activity, beginning as early as 60 min after injection of WF-23 and lasting 24 h. In the current study, locomotor activity was measured to determine changes occurring after chronic treatment with WF-23. Data were collected in both pre- and postinjection sessions to assess the locomotor activity 48 h after each injection of drug (Fig. 3). In the preinjection phase, where animals were tested for 30 min before injection of drug, data were used to assess the locomotor activity 48 h after each injection, as well as to establish a baseline on each injection day. After injection of drug, postinjection data were collected for 150 min. These

### Table 1

Effect of chronic WF-23 treatment on receptor-stimulated [35S]GTPγS binding

<table>
<thead>
<tr>
<th>Region</th>
<th>Agonist</th>
<th>Net-Stimulated [35S]GTPγS Binding (nCi/g, % Saline)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Saline Day 3 Day 7 Day 15 Day 21</td>
</tr>
<tr>
<td>CPu</td>
<td>NPA</td>
<td>252 ± 18 227 ± 18 186 ± 10* 166 ± 17* 70 ± 24*</td>
</tr>
<tr>
<td></td>
<td>(100 ± 7%)</td>
<td>(90 ± 7%) (77 ± 4%) (66 ± 7%) (28 ± 10%)</td>
</tr>
<tr>
<td>CPu</td>
<td>DAMGO</td>
<td>225 ± 18 218 ± 27 207 ± 22 205 ± 35 200 ± 14</td>
</tr>
<tr>
<td></td>
<td>(100 ± 8%)</td>
<td>(100 ± 12%) (92 ± 10%) (106 ± 15%) (93 ± 6%)</td>
</tr>
<tr>
<td>Amyg</td>
<td>NE</td>
<td>271 ± 14 219 ± 38* 180 ± 18* 161 ± 35* 78 ± 11*</td>
</tr>
<tr>
<td></td>
<td>(100 ± 5%)</td>
<td>(89 ± 13%) (79 ± 7%) (54 ± 9%) (39 ± 4%)</td>
</tr>
<tr>
<td>Hippo</td>
<td>8-OH-DPAT</td>
<td>342 ± 33 189 ± 20* 181 ± 19* 173 ± 11* 163 ± 21*</td>
</tr>
<tr>
<td></td>
<td>(100 ± 10%)</td>
<td>(67 ± 6%) (52 ± 6%) (42 ± 3%) (41 ± 6%)</td>
</tr>
</tbody>
</table>

CPu, caudate/putamen; Amyg, amygdala; Hippo, hippocampus.

*p ≤ 0.05, significantly different from control by Tukey-Kramer t test for multiple comparisions.

![Fig. 2](https://i.imgur.com/3dWF23.png) Effects of chronic WF-23 treatment on D2 receptor and DAT binding in caudate/putamen. Shown are representative autoradiograms of coronal sections of rat brain at the level of the striatum following 21 days of saline (top), 3 days of WF-23 (middle), and 21 days of WF-23 treatment (bottom). D2 receptor binding was assessed using [3H]spiperone (left), and occupancy of dopamine transporters by WF-23 was measured using [125I]RTI-55 binding (right).
data reflect both the direct behavioral response to WF-23, as well as the cumulative effects of WF-23 following chronic treatment for up to 21 days. Preinjection testing at day 21 occurred 48 h after the final WF-23 injection so that this group matched the treatment paradigm of animals for biochemical testing; this accounts for the fact that preinjection data were analyzed for 21 days, whereas postinjection data were analyzed for only 15 days of WF-23 treatment.

Preinjection data (Fig. 4, top) showed that WF-23 produced significant elevations (215 ± 16%) in horizontal activity for up to 48 h after a single injection when compared with saline-treated rats. However, 48 h after the second injection of WF-23 (day 5), preinjection values were reduced (180 ± 18%). Following 7 days of treatment, these preinjection increases were no longer significant (119 ± 9%).

Postinjection data (Fig. 4, bottom) showed that the first injection of WF-23 produced significant peak elevations in locomotor activity (690 ± 10%). Subsequent injections on day 3, 5, and 7 continued to produce increases in locomotor activity (531 ± 10%, 584 ± 35%, and 518 ± 50%). In contrast, WF-23-produced increases in locomotor activity were severely attenuated after 15 days (327 ± 13%), with levels significantly lower (40% of day 1 levels) than levels observed on day 1.

Figure 5 shows the time course of locomotor activity within each session after 1, 5, 7, and 15 days of treatment. Peak activity levels occurred 60 min after the first injection, consistent with the findings of Daunais et al. (1998). These levels decreased slightly after 60 min, but remained significantly elevated for the duration of the session. Following subsequent injections on days 3 and 7, the peak increase in activity occurred more rapidly (usually within 20 min) and also remained elevated for the duration of the session. In contrast, on day 15, locomotor activity was elevated for only 70 min, followed by a reduction in activity to levels not significantly different from saline after 80 min which remained low for the duration of the session. Moreover, the peak of activity (which occurred at 50–60 min) was significantly lower after 15 days of treatment compared with 1, 3, and 7 days.

### Discussion

Previous results showing that a single administration of WF-23 produced long-term blockade of both DAT and SERT binding (Daunais et al., 1998) suggested that this tropane analog would be useful in producing long-term increases in biogenic amines after chronic administration. Furthermore, our previous studies (O'Connor et al., 2004) showed that chronic WF-23 treatment reduced monoamine receptor/G-protein-coupling in specific areas of the brain. Although desensitization of G-protein-coupled receptors in response to chronic agonist exposure has been extensively documented, these previous studies (O'Connor et al., 2004) were the first to demonstrate similar effects in response to chronic transporter blockade. The current study shows that transporter blockade-mediated desensitization of dopamine receptors occurs with specific regional and temporal patterns that closely parallel the time course of changes in locomotor activity.

### Table 2
Effects of chronic WF-23 treatment on [125I]RTI-55 binding

<table>
<thead>
<tr>
<th>Specific Binding of [125I]RTI-55 (ROD, % Saline)</th>
<th>Saline</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 15</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>64 ± 5</td>
<td>53 ± 4</td>
<td>42 ± 2</td>
<td>31 ± 1</td>
<td>50 ± 3</td>
</tr>
<tr>
<td></td>
<td>(100 ± 8%)</td>
<td>(72 ± 6%)</td>
<td>(63 ± 3%)</td>
<td>(62 ± 2%)</td>
<td>(72 ± 5%)</td>
</tr>
</tbody>
</table>

ROD, relative optical densities.
* indicates p ≤ 0.001, significantly different from control by Tukey-Kramer t test for multiple comparisons.
These effects on receptor/G-protein coupling by chronic WF-23 treatment appeared to be specific reductions to monoamine receptor-stimulated $[^{35}S]$GTP$\gamma$S binding. Chronic treatment with WF-23 did not alter $\mu$ opioid-stimulated $[^{35}S]$GTP$\gamma$S binding in the caudate/putamen at any time point examined in the same animals where significant decreases were observed in $D_2$ receptor-stimulated $[^{35}S]$GTP$\gamma$S binding in the same region. These results are similar to homologous desensitization observed after chronic opioid and cannabinoid treatment (Sim et al., 1996; Breivogel et al., 1999) but in contrast to reports of chronic cocaine treatment, where increases have been shown in both monoamine and nonmonoamine receptor signal transduction (Unterwald et al., 1996; Kushner and Unterwald, 2001; Schroeder et al., 2003). It is unlikely that these differences are due to pharmacological differences in the specificity of cocaine and WF-23, since both drugs block all three monoamine transporters, but may involve pharmacokinetic differences between the two transport inhibitors. The cocaine binge treatment used (Unterwald et al., 1996; Kushner and Unterwald, 2001; Schroeder et al., 2003) by many investigators produces temporary high increases in monoamine levels, whereas the long-acting WF-23 tropane analog may produce sustained increases in DA, 5-HT, and NE. Sustained elevations of these monoamines, in contrast to periodic high concentrations, may result in different responses by the receptors.

Desensitization in the absence of down-regulation of dopamine $D_2$ receptors was previously demonstrated following 15 days of WF-23 treatment (O’Connor et al., 2004). In the present study, no decreases in $D_2$ receptor binding, assessed by $[^{3}H]$spiperone binding in caudate/putamen, were observed following 21 days of treatment. These data demonstrate that uncoupling of the receptor/G-protein complex, or desensitization, occur in the absence of receptor down-regulation following long-term transporter blockade by WF-23. This same result was observed after chronic heroin treatment, where $\mu$ opioid-stimulated $[^{35}S]$GTP$\gamma$S binding was reduced with no change in $\mu$ receptor binding (Sim-Selley et al., 2000a) and in contrast to chronic $\Delta^{9}$-tetrahydrocannabinol treatment, which reduced CB1 receptor number as well as CB1-activated G-proteins (Breivogel et al., 1999).

Chronic administration of WF-23 produced specific time-dependent effects which varied across the three monoamine receptors. For example, although significant reductions in $D_2$ receptor-stimulated $[^{35}S]$GTP$\gamma$S binding in striatum were not observed until 15 days of treatment, significant reductions in 5-HT$_{1A}$ activity (in hippocampus) and $\alpha_2$-adrenergic activity (in amygdala) were observed following a single injection of WF-23. Previous studies have demonstrated similar regional differences in time-dependent desensitization of cannabinoid CB1-stimulated $[^{35}S]$GTP$\gamma$S binding following chronic treatment with $\Delta^{9}$-tetrahydrocannabinol. It has been suggested that regional differences in intracellular regulatory mechanisms of signal transduction may contribute to these effects (Breivogel et al., 1997b).

However, in addition to these potential receptor G-protein regulatory mechanisms, there is an extrasynaptic component mediating desensitization of monoamine receptors following chronic WF-23 treatment. Synaptic levels of monoamines are dependent upon both release and uptake processes. Thus, effects of WF-23 on extrasynaptic levels of monoamines will depend on the level of presynaptic activity and may therefore produce larger reductions in receptor-stimulated $[^{35}S]$GTP$\gamma$S binding in areas of higher monoamine release. Furthermore, monoamine receptors, specifically $D_2$ and 5-HT$_{1A}$ function both post- and presynaptically. For example, $D_2$ autoreceptors may be present in a higher proportion in the caudate/putamen than $D_2$ postsynaptic receptors (Neve, 1997). Although NPA-stimulated $[^{35}S]$GTP$\gamma$S binding cannot differentiate $D_2$ autoreceptors from postsynaptic receptors, it is possible that the relative contribution of autoreceptors to postsynaptic receptors may contribute to the time-dependent differences observed in $D_2$ desensitization compared with 5-HT$_{1A}$ and $\alpha_2$-adrenergic receptors.

One of the goals of the present study was to examine the effects of chronic WF-23 treatment on locomotor activity. The cocaine-like behavioral effects of WF-23 have been shown in previous studies, which demonstrated that WF-23 substituted for cocaine in self-administration paradigms (Lile et al., 2003; Roberts et al., 2003) and increased locomotor activity for up to 24 h following a single i.p. injection (Daunais et al., 1998). This long duration of action was in contrast to cocaine, whose effects on locomotor activity lasted only 1 h after a single injection (Daunais et al., 1998). Qualitatively, the stereotypic behaviors elicited by WF-23 were indistinguishable from those elicited by cocaine and included rearing, head-bobbing, increased sniffing, and gnawing.

The locomotor assays in the present study revealed an overall loss of WF-23 effects after chronic administration of this drug compared with its acute effects. These data demonstrate that chronic treatment with long-acting psycho-stimulants produces significant behavioral tolerance, at least in terms of locomotor activity. For example, the preinjection data revealed elevations in horizontal activity 48 h following the previous injection of WF-23 for the first 7 days of treatment, consistent with prolonged blockade of DAT for at least 48 h. However, the preinjection increases in horizontal activity diminished during chronic WF-23 treatment, with increases of $215 \pm 16\%$ (versus controls) on day 3, $182 \pm 18\%$ on day 5, and $119 \pm 9\%$ on day 7 (the last value significantly

![Figure 5](image-url)
lower than the increase on day 3, p ≤ 0.05). Postinjection locomotor data revealed a similar tolerance after chronic WF-23 treatment; horizontal activity measured during the 150-min session after WF-23 injection revealed elevations after each injection until day 15 where the WF-23-induced elevations in activity were severely reduced. These effects were not simply limited to horizontal activity, but were also observed when stereotypic activity was evaluated (not shown).

Furthermore, the time course of behavioral tolerance produced by chronic treatment with WF-23 paralleled the observed desensitization of D2 receptor-coupled G-proteins, suggesting that this tolerance may be mediated, in part, by desensitization of monoamine receptor-coupled G-proteins. As shown in Table 1, D2-stimulated [35S]GTPγS binding in caudate/putamen remained relatively constant throughout the chronic WF-23 treatment until day 7, whereas loss in postinjection effects was observed on day 15. The difference in these two courses may simply reflect the large difference in WF-23 present during the preinjection and postinjection phases, i.e., loss in postinjection effects of WF-23 may be occurring at day 7, but masked by the large effects of the drug itself on locomotor activity.

Therefore, these data demonstrate that chronic treatment with a long-acting psychostimulant produces significant behavioral tolerance and suggest that this tolerance may be mediated, in part, by desensitization of monoamine receptor-coupled G-proteins. This is in contrast to the effects of cocaine, a short-acting psychostimulant, which has been shown to produce sensitization, rather than tolerance, following chronic treatment (Post et al., 1979). Locomotor sensitization has been shown to be produced following specific treatment paradigms (Post et al., 1992). In general, intermittent, rather than continuous, exposure of animals to cocaine has been shown to produce behavioral sensitization (Reith et al., 1987). It is not surprising that WF-23, which produces a sustained blockade of DAT (Fig. 2), results in tolerance and not sensitization to the locomotor effects of this psychostimulant in contrast to cocaine.

**References**


Breivogel CS, Sim LJ, and Childers SR (1997b) Regional differences in cannabinoi-


Hemby SE, Co C, Reboissain D, Davies HM, Dworkin SI, and Smith JE (1995) Comparison of a novel tropane analog of cocaine, β2-propanoyl-3β-(4-toly)tropane (PTT) with cocaine HCI in rats: nucleus accumbens extracellular dopamine concent-


Mead AN, Rocha BA, Donovan DM, and Katz JL (2002) Intravenous cocaine induced-
activity and behavioural sensitization in norepinephrine-, but not dopamine-


Therefore, these data demonstrate that chronic treatment with a long-acting psychostimulant produces significant behavioral tolerance and suggest that this tolerance may be mediated, in part, by desensitization of monoamine receptor-coupled G-proteins. This is in contrast to the effects of cocaine, a short-acting psychostimulant, which has been shown to produce sensitization, rather than tolerance, following chronic treatment (Post et al., 1979). Locomotor sensitization has been shown to be produced following specific treatment paradigms (Post et al., 1992). In general, intermittent, rather than continuous, exposure of animals to cocaine has been shown to produce behavioral sensitization (Reith et al., 1987). It is not surprising that WF-23, which produces a sustained blockade of DAT (Fig. 2), results in tolerance and not sensitization to the locomotor effects of this psychostimulant in contrast to cocaine.


**Address correspondence to:** Steven R. Childers, Department of Physiology and Pharmacology, Wake Forest University Health Sciences, Medical Center Blvd., Winston-Salem, NC 27157. E-mail: childers@wfubmc.edu