Development of Behavioral Sensitization to the Cocaine-Like Fungicide Triadimefon Is Prevented by AMPA, NMDA, DA D1 but Not DA D2 RECEPTOR Antagonists

R. Reeves, M. Thiruchelvam, and D. A. Cory-Slechta

Department of Environmental Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642.

Received November 5, 2003; accepted January 15, 2004

Triadimefon (TDF) is a triazole fungicide that blocks the re-uptake of dopamine (DA) and leads to increased locomotor activity levels in mice and rats, effects similar to those of indirect DA agonists such as cocaine. We recently found in mice that intermittent TDF administration led to robust locomotor sensitization, a phenomenon reflecting neuronal plasticity, following challenge with the same TDF dose after a 2-week withdrawal period. The current study sought to determine whether antagonists to DA D1-like receptors (SCH 23390; SCH), DA D2-like receptors (remoxipride; Rem), ionotropic glutamate n-methyl-d-aspartate (NMDA) receptors (CPP), or ionotropic glutamate alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (NBQX) could prevent the development of TDF behavioral sensitization, therefore indicating their mechanistic involvement in TDF sensitization. Mice were treated with either vehicle, SCH (0.015 mg/kg), remoxipride (Rem, 0.3 mg/kg), CPP (2.5 mg/kg) or NBQX (10.0 mg/kg), followed 30 min later by vehicle or 75 mg/kg TDF (TDF), twice a week for 7 weeks, with locomotor activity measured post-dosing once a week. After a 2-week withdrawal period, mice were challenged with 75 mg/kg TDF or vehicle, to test for the presence of behavioral sensitization. Pretreatment with SCH, CPP, or NBQX, but not Rem, blocked the development of behavioral sensitization to TDF specifically for vertical activity. Antagonists that blocked TDF vertical sensitization also attenuated the increase in extracellular DA turnover (homovanillic acid [HVA]/DA) normally associated with this behavioral response. Therefore, DA D1, NMDA and AMPA receptors appear to be necessary for the development of behavioral sensitization to TDF. As such, TDF may be considered an environmental risk factor for behavioral dysfunctions linked to glutamatergic and dopaminergic systems.

Key Words: triadimefon; behavioral sensitization; SCH 23390; remoxipride; NBQX; CPP.
zation to psychostimulants like cocaine, recent findings point to a potential role of glutamatergic systems as well. For example, a cocaine challenge to rats previously exposed to repeated doses of cocaine induces a sensitized release of glutamate in the NA (Pierce et al., 1996a), an effect ascribed, in part, to an increase in excitatory amino acid transmission from prefrontal cortex to the NA (Pierce et al., 1998). Other evidence of glutamatergic involvement is based on studies using intra-medial prefrontal cortex (mPFC), -NA, or -VTA injections of n-methyl-d-aspartate (NMDA) antagonists (Kalivas and Ales-datter, 1993; Karler et al., 1998) or alpha-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid (AMPA) (Pierce et al., 1996a; Zhang et al., 1997), with results indicating that stimulation of these receptor subtypes may be necessary or even sufficient to induce behavioral sensitization.

Because TDF appears to share mechanisms in common with cocaine, it may also share cocaine’s other behavioral and neurochemical properties. Indeed, a recent study from our laboratory demonstrated that intermittent injections of TDF to mice produced both the development and expression of locomotor sensitization after a 2-week withdrawal period (Reeves et al., 2003). The present study sought to determine the potential involvement of dopaminergic and/or glutamatergic mechanisms in these effects using pretreatments with the DA D1-like antagonist SCH 23390 (SCH), the DA D2-like antagonist remoxipride (Rem), the competitive NMDA antagonist CPP, or the AMPA antagonist NBQX, during the development phase of TDF sensitization. These antagonists were chosen based on previous studies showing that pretreatment with these drugs either blocked TDF-induced hyperactivity (MacPhail, 1993) or the development of cocaine sensitization (Aston-Jones et al., 1999; Li et al., 1997). In addition, in order to determine if pretreatment with these antagonists could concurrently modify neurochemical changes associated with TDF sensitization, neurotransmitter levels were measured in striatum, a brain region known to be involved in the neuroadaptations associated with cocaine and amphetamine sensitization, 8 h after the 2-week post-sensitization TDF challenge.

MATERIALS AND METHODS

Animals. Male C57BL/6 mice (6–8 weeks old) were purchased from Taconic Farms (Germantown, NY). Animals were individually housed, with food and water available ad libitum, in a room maintained under constant temperature and humidity conditions with a 12:12 light–dark cycle. All mice were habituated for 1 week in the vivarium prior to commencement of experiments. All animals were cared for and treated in accord with NIH and University of Rochester Animal Care and Use Committee guidelines.

Chemicals. All solvents for high performance liquid chromatography with electrochemical detection (HPLC-EC) were purchased from Sigma (St. Louis, MO). TDF was purchased from Chem Service (West Chester, PA). The DA D1-like antagonist SCH 23390 (SCH) and the competitive NMDA antagonist CPP (1R)-3-(2-carboxypyridazin-4-yl)propanephosphonic acid were purchased from Sigma (St. Louis, MO). The AMPA antagonist NBQX (2,3-dihydroxy-6-nitro-7-sulfamoylbenzof[1,4]oxazine) was purchased from AG Scientific, Inc. (San Diego, CA), and the DA D2-like antagonist remoxipride (Rem) was purchased from Tocris (Avonmouth, UK). All other chemicals were at least analytical grade and were purchased from Sigma, unless otherwise noted.

Experimental design. A total of 200 C57BL/6 male mice were injected ip with vehicle (veh, corn oil, 0.2 ml), and locomotor activity was measured for 3 consecutive days for habitation to the test environment. The next day mice were split into 10 groups (n = 14–24/group; see Table 1) and injected ip with: vehicle (veh, corn oil), SCH 23390 (SCH, 0.015 mg/kg), CPP (2.5 mg/kg), NBQX (10.0 mg/kg), or remoxipride (Rem; 0.3 mg/kg). Doses of antagonists were based on preliminary acute ip dose-response studies in C57BL/6 male mice (data not shown). Doses were selected that were behaviorally relevant, meaning those resulting in at least modest, or at best statistically significant, decreases in locomotor activity compared to vehicle controls. Modest doses were needed, as it was important that the dose had the potential to block expression of TDF sensitization, rather than simply acutely reducing TDF-induced hyperactivity. Therefore we attempted to choose doses that had minimal effect on TDF-induced vertical or ambulatory activity.

This pretreatment was followed 30 minutes later by ip injection with either veh or 75 mg/kg TDF (TDF), after which mice were placed in activity chambers for 45 minutes. These treatments were administered twice a week for 7 weeks for a total of fourteen doses, and locomotor activity was measured immediately after injections once a week. Injection volumes were based on body weight and ranged from 0.22 to 0.30 ml. Exactly 13 days after the last TDF dose, all mice were injected with veh and placed in locomotor chambers for 75 minutes to test for spontaneous activity and any conditioning to the test environment per se. The next day, exactly 2 weeks after the fourteenth TDF dose, the same mice were split into 20 groups (n = 7–12 per group), challenged ip with either veh or 75 mg/kg TDF (TDF), and immediately placed in locomotor chambers for 75 minutes to test for expression of behavioral sensitization. Brains were collected 8 h after this 2-week TDF challenge for determination of neurotransmitter levels.

### Table 1

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<th>Group name (n = 7–12)</th>
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Locomotor activity. Automated chambers (Opto-Varimex Minor, Columbus Instruments International Corporation, Columbus, OH) were used to quantify locomotor activity. Each chamber was equipped with infrared photobeams (3 mm in diameter) separated by 24.4 mm on a horizontal plane 39 mm from the floor of the chamber. A second set of photobeams that divided the chamber vertically was located 57 mm above the horizontal photobeams. Photobeam breaks were recorded each minute for 45 min (developmental phase) or 75 min (expression phase or challenge) for horizontal (a single break in the bottom set of photobeams), vertical (rearing on hind legs; breaks in the second set of photobeams), and ambulatory movements (breaking more than one consecutive photobeam in the bottom set of photobeams). Mice were initially habituated to the locomotor activity chambers in three sessions occurring on consecutive days, each preceded by a vehicle injection. After the third such habituation session, the antagonist + TDF exposure protocols were implemented, and effects on locomotor activity assessed immediately after each weekly injection, with activity counts totaled in 3- or 5-min blocks across the session. While all three behaviors, vertical, ambulatory, and horizontal, were measured, statistical analyses revealed that treatment-related changes in horizontal and ambulatory activity were very comparable. Therefore, only vertical and ambulatory activities are presented here.

Biochemical analysis. Striatal and frontol cortical (FC) sections were dissected 8 h after the 2-week TDF challenge, following cervical dislocation without anesthesia, and sections were placed in 0.5 ml 0.1 N perchloric acid. Tissues were sonicated and centrifuged for 20 min at 10,000 rpm. Supernatants were stored at –80°C until analyzed for the concentration of DA and its metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and for serotonin (5HT) by HPLC-EC methods used in our laboratory as described in detail elsewhere (Thiruchelvam et al., 2000). The final concentration of neurotransmitters was expressed in terms of ng/mg protein. DA turnover was expressed as the ratio of DOPAC/DA (intracellular DA turnover) or HVA/DA (extracellular DA turnover). Pellets were later digested in 1 ml (striatum) and 0.25 ml (FC) of 0.5 N NaOH for measurements of protein concentration using Bio-Rad assay reagents.

The 8-h post-TDF expression time point for measurement of neurotransmitter changes was chosen based on a preliminary time-course experiment in which male C57BL/6 mice were injected with TDF75 or veh twice a week for 7 weeks, followed by a 2-week challenge of either TDF75 or veh, and then sacrificed at 1, 4, 8, 24, 48 h, 7 days, or 1 month after the 2-week challenge for striatal HPLC analysis (data not shown). The 8-h time point (shown in Figs. 2, 4, 6, and 8) revealed increases in extracellular DA turnover (HVA/DA) selectively selectively in animals that expressed TDF sensitization or the TDF-TDF group. Thus, the 8-h time point was employed in the present study with the intention of determining, if an antagonist was successful in blocking behavioral sensitization, whether it could also block the neurochemical changes that are specific to that behavioral response (or increase in striatal extracellular DA turnover).

Data and statistical analysis. Overall effects of treatment on ambulatory and vertical activity were first analyzed by a 2-within, 1-between repeated measures analysis of variance (RMANOVA; SuperAnova) with blocks of time (across a single session) and TDF dose number as the within-group factors and treatment group as the between-groups factor. If main effects of treatment or interactions were confirmed, a subsequent RMANOVA was performed with treatment as a between-groups factor and dose as the within-group factor. If significant main effects or interactions were again confirmed, individual one-way ANOVAs or Fishers PLSD post hoc tests were then used to further assess the nature of the effect. Evaluation of locomotor activity changes across blocks of time within a session were also analyzed by RMANOVA using 5-min blocks as a within-group factor and treatment as a between-groups factor, with subsequent one-way ANOVAs at each time point as appropriate.

Several statistical comparisons were utilized to determine the efficacy of antagonist treatment.

(1) To determine if antagonist pretreatment was successful in blocking the development of TDF sensitization, locomotor activity levels in response to TDF challenge after 2-week withdrawal were compared in groups that received antagonist before each of the fourteen intermittent TDF doses (ant + TDF - TDF) to those receiving fourteen intermittent doses of TDF alone (veh + TDF - TDF). While the ability of antagonist pretreatment to modify the acute hyperactive effects of TDF across the fourteen intermittent doses (ant + TDF vs. veh + TDF) was evaluated, it was not considered the ultimate assessment of altered development of TDF sensitization per se.

(2) Since it is possible that antagonist pretreatment during the development period could result in sustained effects on locomotor activity and, thereby, block sensitization at the 2-week TDF challenge, the effects of fourteen intermittent doses of the antagonist alone followed by veh at the 2-week challenge (ant + veh - veh) were statistically compared to effects of vehicle treatment and challenge (veh + veh - veh).

(3) The possibility that repeated antagonist treatment per se could cross-sensitize to a TDF challenge was also considered. Cross-sensitization occurs when repeated treatment with one drug leads to a sensitized locomotor response following challenge with another drug; cross-sensitization studies of agonists with drugs of abuse are frequently used to determine whether the receptor is sufficient for the neuroadaptations that lead to the development of sensitization. The most common method of verifying behavioral sensitization has been to compare locomotor response to drug challenge following the repeated drug treatment regimen (here, veh + TDF - TDF), to vehicle treatment followed by drug challenge (veh + veh - TDF). Therefore, to rule out the possibility of cross-sensitization, the relevant comparison would be activity levels in response to TDF challenge following administration of antagonist alone (ant + veh - TDF) versus vehicle alone (veh + veh - TDF); higher activity levels following TDF challenge in the former group would be consistent with cross-sensitization.

Changes in levels of neurotransmitters were analyzed by one-factor ANOVAs with treatment as the between-group factor, followed by Fishers PLSD post hoc where appropriate. In all cases, p values ≤ 0.05 were considered statistically significant.

RESULTS

Body Weight

There were no significant treatment-related effects on body weight, with all groups gaining an average of 7 g over the experiment, as measured at the final TDF challenge 2 weeks post withdrawal.

SCH 23390: Effect on Locomotor Activity Across Intermittent TDF Dosing

Ambulatory activity. Figure 1A shows group mean ± SE total ambulatory activity levels expressed as a percent of control for injections across the developmental phase of TDF sensitization (or the fourteen intermittent doses), for the 13-day post-withdrawal veh challenge, and for the expression phase (i.e., the 2-week post-withdrawal TDF challenge). RMANOVA across the fourteen induction doses revealed a significant main effect of treatment (F13.4 = 11.653, p < 0.0001), as well as an interaction of treatment by dose (F12.136 = 2.291, p = 0.011). [Data for control groups (veh + veh) and groups treated with TDF alone (veh + TDF) are the same for Figs. 1A, 2A, 3A, and 4A]. TDF induced significantly higher ambulatory activity levels across doses, effects that were reduced but not eliminated by pretreatment with SCH prior to TDF (SCH + TDF), with statistically significant differences at
doses 12 ($p = 0.014$) and 14 ($p < 0.0001$), and a similar trend at dose 8. Treatment with SCH alone (SCH+veh) did not alter ambulatory activity levels as compared to control.

**Vertical activity.** Figure 1B shows corresponding changes in vertical activity. [Data for the control group (veh+veh) and that treated with TDF alone (veh+TDF) are the same for figures 1B, 2B, 3B, and 4B]. TDF produced significant increases in vertical activity at each of the fourteen doses. SCH alone decreased vertical activity, effects that were significant at doses 12 ($p = 0.045$) and 14 ($p = 0.027$) and ranged from 30 to 40%. Pretreatment with SCH prior to TDF (SCH+TDF) reduced the increases in vertical activity associated with TDF at doses 1 ($p = 0.005$), 12 ($p = 0.034$), and 14 ($p = 0.029$), with similar trends at doses 4 and 8.

**SCH 23390: Effect on TDF Locomotor Sensitization Upon 2-Week Post-Withdrawal Challenge**

**Ambulatory activity.** Activity levels of the veh+TDF group were slightly but significantly higher than control at the 13-day vehicle challenge (Fig. 1A; $p = 0.003$). Mice were split into eight groups at day 14 and tested for expression of behavioral sensitization. [Data for control groups (veh+veh–veh) and groups treated with TDF alone (veh+TDF–veh, veh+veh–
TDF, and veh+TDF–TDF) are the same for Figs. 2A, 3A, and 4A. Ambulatory activity levels of mice sensitized with TDF and then challenged with TDF exceeded those of controls acutely challenged with TDF by 70% (veh/H11001 and then challenged with TDF exceeded those of controls 4A]. Ambulatory activity levels of mice sensitized with TDF and veh/H11001 TDF–TDF) are the same for Figs. 2A, 3A, and 4A. In contrast to ambulatory activity, pretreatment with SCH led to a significant reduction in the development of vertical sensitization to TDF, as evidenced by the lack of difference between the SCH+TDF–TDF and the veh+TDF–TDF groups. Furthermore, SCH alone did not cross-sensitize to TDF; that is there were no significant differences in ambulatory activity levels between the SCH+veh–TDF and the veh+veh–TDF groups. Residual increases in spontaneous ambulatory activity were found at the 2-week challenge in the SCH+TDF–veh group, as compared to control (p = 0.043).

Vertical activity. No significant treatment-related differences in spontaneous vertical activity were evident at the 13-day veh challenge (Fig. 1B). The 2-week post-withdrawal challenge revealed robust expression in the TDF-sensitized group, with activity levels of the veh+TDF–TDF group 200% greater than control (veh+veh–TDF; p = 0.025). [Data for control groups (veh+veh–veh) and groups treated with TDF alone (veh+TDF–veh, veh+veh–TDF, and veh+TDF–TDF) are the same for Figs 2B, 3B, and 4B]. In contrast to ambulatory activity, pretreatment with SCH led to a significant reduction in the development of vertical sensitization to TDF challenge (cf. SCH+TDF–TDF vs. veh+TDF–TDF; p = 0.012). SCH alone did not produce residual decreases in spontaneous activity (cf. SCH+veh–veh vs. veh+veh–veh), and no cross-sensitization between SCH and TDF was detected (cf. SCH+veh–TDF vs. veh+veh–TDF).

SCH 23290: Effect on Striatal Neurochemistry

Neurochemical effects specific to TDF sensitization. In order to determine whether antagonists that blocked TDF behavioral sensitization also reversed the associated neurochemical changes, brains were collected 8 h after the 2-week challenge for striatal and cortical neurochemical determinations. The 8-h post-TDF expression time point for measurement of neurotransmitter changes was chosen based on preliminary time-course experiments showing increases in extracellular DA turnover (HVA/DA) selectively in animals that expressed TDF sensitization (described in methods). In the present experiments, no treatment-related changes in frontal cortical neurotransmitter levels were observed; therefore, only striatal results are shown.

TDF sensitization following TDF challenge increased striatal extracellular DA turnover (HVA/DA) relative to vehicle (Fig. 2; p = 0.042 vs. veh+veh–veh) and relative to acute TDF challenge (p = 0.019 vs. veh+veh–TDF). These effects served as the basis for determining whether antagonist treatments reversed the neurochemical changes associated with TDF sensitization.

Figure 2 shows that repeated treatment with SCH alone (SCH+veh–veh) did not lead to any long-term changes in the neurotransmitters measured here. TDF challenge produced significant decreases, on the order of approximately 35–50%, in DA (p = 0.023) and DOPAC (p = 0.034) following repeated SCH treatment (cf. SCH+veh–TDF vs. veh+veh–TDF).

TDF challenge in the antagonist pretreated sensitized group (SCH+TDF–TDF) revealed dichotomous effects on intracellular DA turnover (DOPAC/DA; p = 0.046), and 5HT levels (p = 0.021) compared to the TDF-sensitized group (veh+TDF–TDF). However, the most critical comparison was considered to be in extracellular DA turnover (HVA/DA), since this was the only neurotransmitter change specifically associated with TDF sensitization. Indeed, SCH pretreatment not only blocked the development of vertical sensitization to TDF, but also fully reversed the neurochemical changes associated with TDF sensitization.
reversed the increase in extracellular DA turnover normally associated with this behavioral response ($p = 0.009$).

**Remoxipride: Effect on Locomotor Activity Across Intermittent TDF Dosing**

**Ambulatory activity.** Figure 3A shows group mean ± SE total ambulatory activity levels as a percent of control for Rem antagonism. RMANOVA revealed a significant main effect of treatment ($F_{3,34} = 8.968, p = 0.0002$) as well as an interaction of treatment by dose ($F_{12,116} = 1.881, p = 0.042$). Subsequent post hoc analysis indicated that Rem alone did not influence ambulatory activity levels (Rem+veh), nor did pretreatment with Rem (Rem+TDF) alter the increases in ambulatory activity produced by TDF alone.

**Vertical Activity.** Figure 3B shows corresponding data for group mean total vertical activity levels. RMANOVA revealed a significant main effect of treatment ($F_{3,34} = 3.19, p = 0.021$) but no interaction of treatment by dose. Rem alone (Rem+veh) did not influence vertical activity levels relative to control, and
pretreatment with Rem did not systematically alter TDF-induced increases in vertical activity.

**Remoxipride: Effect on TDF Locomotor Sensitization Upon 2-Week Post-Withdrawal Challenge**

**Ambulatory activity.** Pretreatment with Rem did not block the development of ambulatory sensitization to TDF (Fig. 3A; cf. Rem+TDF–TDF vs. veh+TDF–TDF). Cross-sensitization between Rem and TDF (cf. Rem+veh–TDF vs. veh+veh–TDF) was not observed, nor were there conditioning effects or residual changes in spontaneous activity due to pretreatment with Rem alone or with TDF (cf. Rem+veh–veh and Rem+TDF–veh vs. control).

**Vertical activity.** Pretreatment with Rem did not block the development of vertical sensitization to TDF (Fig. 3B; cf. Rem+TDF–TDF vs. veh+TDF–TDF). There was no cross-sensitization between Rem and TDF (cf. Rem+veh–TDF vs. veh+veh–TDF), nor were there residual effects on spontaneous activity due to pretreatment with Rem alone or with TDF (cf. Rem+veh–veh or Rem+TDF–veh vs. veh+veh–veh).

**Remoxipride: Effect on Striatal Neurochemistry**

Intermittent treatment with remoxipride alone resulted in long-term decreases of approximately 35% (2 weeks after last Rem dose) in DA ($p = 0.047$) and increases of more than 50% in both intracellular DA turnover (DOPAC/DA; $p = 0.02$) and extracellular DA turnover (HVA/DA; $p = 0.012$; cf. veh+veh–veh vs. Rem+veh–veh, Fig. 4). A TDF challenge resulted in more pronounced decreases in DOPAC in mice pretreated with Rem as compared to veh (cf. veh+veh–TDF vs. Rem+veh–TDF, $p = 0.045$). Rem pretreatment did not block the increase in extracellular DA turnover associated with TDF sensitization, and it significantly increased 5HT levels, an effect opposite that occurring in the sensitized group (cf. veh+TDF–TDF vs. Rem+TDF–TDF, $p = 0.007$).

**CPP: Effect on Locomotor Activity Across Intermittent TDF Dosing**

**Ambulatory activity.** Figure 5A shows group mean ± SE total ambulatory activity levels for the determination of CPP antagonism. RMANOVA revealed a significant main effect of treatment ($F_{3,34} = 10.415$, $p < 0.0001$) but no interaction of treatment by dose. Subsequent post hoc analyses revealed that CPP alone did not influence ambulatory activity (cf. CPP+veh vs. veh+veh), nor did pretreatment with CPP (CPP+TDF) reduce the stimulatory effects of TDF.

**Vertical activity.** Figure 5B shows corresponding group mean ± SE total vertical activity counts. RMANOVA revealed a significant main effect of treatment ($F_{3,34} = 10.488$, $p < 0.0001$), but no interaction of treatment by dose. CPP alone generally decreased vertical activity levels, effects that were statistically significant only at doses 4 ($p = 0.022$) and 8 ($p = 0.037$). With the exception of dose 12, pretreatment with CPP (CPP+TDF) significantly and dramatically reversed the acute effect of TDF on vertical activity, with reductions generally of greater magnitude than those associated with CPP alone.

**CPP: Effect on TDF Locomotor Sensitization Upon 2-Week Post-Withdrawal Challenge**

**Ambulatory activity.** Pretreatment with CPP did not attenuate the development of ambulatory sensitization to TDF, since activity levels of the group pretreated with CPP were not significantly different from those of the TDF-sensitized group (Fig. 5A; cf. CPP+TDF–TDF vs. veh+TDF–TDF). CPP did cross-sensitize to TDF, with ambulatory activity levels of the CPP+veh–TDF group 73% higher than those of the
FIG. 5. Effect of pretreatment with 2.5 mg/kg CPP during the development phase on TDF behavioral sensitization. Total ambulatory (A) and vertical (B) locomotor activity counts measured immediately after injection as a percentage change from the veh+veh and veh+veh–veh groups. Each data point represents a group mean ± S.E. (n = 14–24 for dose 1–14 and 2-week veh, n = 7–12 for 2-week challenge), across 14 intermittent injections, challenge with veh 2 weeks after last of 14 injections, and after 2-week challenge with vehicle or TDF. For explanation of treatment groups, see Table 1. Following RMANOVA, subsequent Fischer post-hocs were used to compare treatment groups with the respective control value for each dose. *p ≤ 0.05 compared to veh+veh or veh+veh–veh groups, −p ≤ 0.05 compared to veh+TDF group, and #p ≤ 0.05 compared to veh+veh–TDF group.

veh+veh–TDF group (p = 0.021). Also, residual increases in spontaneous ambulatory activity were seen at the 2-week challenge in the CPP+TDF–veh group, compared to control (p = 0.023).

**Vertical activity.** In contrast to ambulatory activity, pretreatment with CPP led to a significant reduction of over 50% in the development of vertical sensitization to TDF (cf. CPP+TDF–TDF vs. veh+TDF–TDF; p = 0.002, Fig. 5B, 2-week challenge), while CPP alone produced no residual decreases in vertical activity (cf. CPP+veh–veh vs. veh+veh–veh). The lack of cross-sensitization between CPP and TDF was confirmed by the absence of differences between the CPP+veh–TDF group and the veh+veh–TDF group.

**CPP: Effect on Striatal Neurochemistry**

CPP treatment alone did not lead to any long-term changes in striatal neurochemistry (Fig. 6, cf. veh+veh–veh vs. CPP+veh–veh), nor did repeated CPP alone alter the neurochemical effects of the TDF challenge (cf. veh+veh–TDF vs. CPP+veh–TDF). However, CPP did fully reverse the increase in extracellular DA normally associated with TDF sensitization (cf. CPP+TDF–TDF vs. veh+TDF–TDF, p = 0.032).
NBQX: Effect on Locomotor Activity Across Intermittent TDF Dosing

Ambulatory activity. Figure 7A shows the group mean ± SE total ambulatory activity levels for NBQX antagonism. RMANOVA confirmed a significant main effect of treatment ($F_{3,33} = 13.851, p < 0.0001$) but no interaction of treatment by dose. Subsequent post hoc analysis indicated that NBQX alone (NBQX–veh) did not influence ambulatory activity levels, except at dose 1 ($p = 0.01$) where a reduction was observed. Pretreatment with NBQX (NBQX+TDF) generally decreased the stimulatory effects of TDF, but these reductions were statistically significant only at dose 1 ($p = 0.04$).

Vertical activity. Figure 7B shows the group mean ± SE total vertical activity levels. RMANOVA confirmed a significant main effect of treatment ($F_{3,33} = 3.915, p = 0.017$) but no interaction of treatment by dose. No significant reductions in vertical activity were produced by NBQX alone (NBQX–veh). Pretreatment with NBQX generally, but not fully, attenuated TDF-induced increases in vertical activity, with statistically significant effects at doses 1 ($p = 0.005$), 4 ($p = 0.03$), and 14 ($p = 0.007$) (NBQX+TDF compared to veh+TDF).

NBQX: Effect on TDF Locomotor Sensitization Upon 2-Week Post-Withdrawal Challenge

Ambulatory activity. Pretreatment with NBQX did not block the development of ambulatory sensitization to TDF (Fig. 7A; cf. NBQX+TDF–TDF vs. veh+TDF–TDF) nor did NBQX cross-sensitize with TDF (cf. NBQX+veh–TDF vs. veh+veh–TDF). There were also no conditioning effects or residual changes in spontaneous activity due to treatment with NBQX alone or with NBQX+TDF (cf. NBQX+veh–veh and NBQX+TDF–veh vs. control).

Vertical activity. In contrast to its lack of effects on TDF-induced ambulatory sensitization, pretreatment with NBQX fully blocked the development of vertical sensitization to TDF (cf. NBQX+TDF–TDF vs. veh+TDF–TDF, $p = 0.0012$, Fig. 7B). There was no cross-sensitization between NBQX and TDF (cf. NBQX+veh–TDF vs. veh+veh–TDF), nor were there residual effects on spontaneous activity due to treatment with NBQX alone or NBQX with TDF (cf. NBQX+veh–veh and NBQX+TDF–veh vs. control).

NBQX: Effect on Striatal Neurochemistry

NBQX treatment alone did not lead to any long-term changes in striatal neurochemistry (Fig. 8, cf. veh+veh–veh vs. NBQX+veh–veh), nor did it alter the neurotransmitter changes produced by TDF challenge (cf. veh+veh–TDF vs. NBQX+veh–TDF). However, NBQX fully blocked the increase in extracellular DA normally associated with TDF sensitization (cf. NBQX+TDF–TDF vs. veh+TDF–TDF, $p = 0.018$). NBQX pretreatment also reversed the decreases in 5HT seen in TDF-sensitized mice ($p = 0.015$).

DISCUSSION

These studies sought to elucidate the neurochemical mechanisms necessary for the induction of behavioral sensitization to the fungicide triadimefon. The involvement of both dopaminergic and glutamatergic mechanisms was hypothesized based on known involvement of both systems in sensitization to cocaine (Li et al., 1997; White et al., 1995), a psychostimulant that shares the ability with TDF to block the DA transporter (Walker and Mailman, 1996). Results show that pretreatment with the DA D1-like antagonist SCH 23390, the NMDA antagonist CPP, and the AMPA antagonist NBQX all blocked the development of TDF vertical, but not ambulatory,
sensitization. In contrast, pretreatment with the DA D2 receptor antagonist remoxipride failed to block the development of either vertical or ambulatory sensitization. The ability of antagonists to attenuate vertical TDF sensitization did not appear to be due to residual effects of antagonist treatment alone on spontaneous activity, since activity levels of antagonist+veh–veh and antagonist+TDF–veh groups were not significantly lower than those of the veh+veh–veh group (Figs 1B, 5B, and 7B; 2-week veh challenge). Also, no evidence of cross-sensitization in terms of vertical activity between these antagonists and TDF was observed (Figs 1B, 5B, and 7B; cf. antagonist+TDF–TDF groups vs. veh+veh–TDF groups at 2-week challenge). Compounds that blocked TDF-associated vertical sensitization also blocked the associated increase in striatal extracellular DA turnover. Since only single doses of these antagonists were tested here, doses that were based on pilot studies confirming their moderate behavioral activity, it is possible that results could differ at either higher or lower doses.

It is important to note that the criteria we used to determine if antagonist pretreatment successfully blocked the development of TDF sensitization was whether locomotor activity levels in response to TDF challenge after 2-week withdrawal in groups that received antagonist before each of the fourteen

FIG. 7. Effect of pretreatment with 10.0 mg/kg NBQX during the development phase on TDF behavioral sensitization. Total ambulatory (A) and vertical (B) locomotor activity counts measured immediately after injection as a percentage change from the veh+veh and veh+veh–veh groups. Each data point represents a group mean ± S.E. (n = 14–24 for dose 1-14 and 2-week veh, n = 7–12 for 2-week challenge), across 14 intermittent injections, challenge with veh 2 weeks after last of 14 injections, and after 2-week challenge with vehicle or TDF. For explanation of treatment groups, see Table 1. Following RMANOVA, subsequent Fischer post-hocs were used to compare treatment groups with the respective control value for each dose. *p ≤ 0.05 compared to veh+veh or veh+veh–veh groups, †p ≤ 0.05 compared to veh+TDF group, and #p ≤ 0.05 compared to veh+veh–TDF group.
intermittent TDF doses (ant+TDF–TDF) was significantly lower than levels produced by fourteen intermittent doses of TDF alone (veh+TDF–TDF). This comparison is the primary measure used to evaluate the ability of antagonists to alter development sensitization (Jackson and Sanger, 1988; Kalivas and Alesdatter, 1993; Li et al., 1999; White et al., 1998; Wolf and Jeziorski, 1993). While the ability of antagonist pretreatment to modify the acute hyperactive effects of TDF across the fourteen intermittent doses (ant+TDF vs. veh+TDF) was evaluated, it was not considered the ultimate assessment of altered development of TDF sensitization per se. Indeed, the ability of an antagonist to attenuate the behavioral effect of a psychostimulant across repeated doses is not always an indication of its ability to block behavioral sensitization when tested with a challenge (Li et al., 1997), and in our present study NBQX did not attenuate TDF-induced hyperactivity across the repeated doses, but finally blocked the sensitized response to TDF challenge.

Acute TDF leads to increases in striatal DA levels in rats (Ikaiddi et al., 1997) approximately 4 h post-dosing, and we have observed similar increases in striatal DA after an acute dose of TDF75 to mice at earlier time points (unpublished observations). However, the more protracted TDF behavioral sensitization regimen as used here produces neurochemical changes that differ substantially from those associated with acute dosing. For example, the sensitized increase in striatal DA noted 2 h after TDF challenge following repeated TDF is not observed. This does not preclude the possibility that a sensitized increase in DA occurs before 2 h, but this may be compensated for by an immediate increase in DA turnover. Our findings suggest that TDF expression may result in an early increase in DA metabolism, especially in the extracellular compartment (HVA), as observed in the TDF-sensitized group at 8 h in the present study. Such hypotheses will require further experiments, perhaps using in vivo microdialysis to test TDF-induced fluctuations in DA metabolism at earlier time points.

Several studies in our laboratory show more prominent increases in TDF-induced vertical, as compared to ambulatory, activity. Here, however, similar effects of TDF on ambulatory and vertical activity were noted. Nonetheless, the antagonists tested here were successful in selectively blocking vertical activity. Additionally, these antagonists sometimes abolished the acute effect of TDF on vertical activity (rearing), while having no effect on horizontal activity (e.g., SCH, Fig. 1; cf. horizontal vs. vertical activity at doses 1 and 4). The reason for these discrepancies is not clear. Rearing may be considered a form of stereotypical activity. Given that stereotypical activity has been linked to nigrostriatal DA transmission, while locomotor (ambulatory) behavior is thought to be primarily mediated by the mesolimbic DA system (Swanson et al., 1997), it is possible that the NMDA, AMPA, and DA D1-like receptor populations necessary for the development of TDF vertical, but not ambulatory, sensitization are specifically located in the nigrostriatal, as opposed to the mesocorticolimbic, DA system. The fact that changes were found in striatal (Figs. 2, 4, 6, and 8), but not frontal cortical neurotransmitter levels (data not shown), 8 h after the 2-week TDF challenge further supports this conclusion.

The choices of antagonists for the present study were based largely on the similarities of cocaine and TDF (Crofton et al., 1988; Moser and MacPhail, 1989; Walker et al., 1990). Therefore, it is important to compare what is known about dopaminergic and glutamatergic involvement in the development of cocaine versus TDF behavioral sensitization.
DA D1 and D2 Receptors and the Comparative Development of Cocaine and TDF Behavioral Sensitization

Evidence for the involvement of DA transmission in the induction of cocaine sensitization remains inconsistent. For example, systemically administered or intra-VTA-microinjected DA D1 or D2 antagonists are sometimes ineffective in blocking the induction of cocaine sensitization (Pierce et al., 1996a), and repeated systemic administration of the DA D1 agonist SKF 38393 does not cross-sensitize with cocaine (Henry et al., 1998). In contrast, Pierce et al. (1996b) found that repeated intra-VTA injections of SKF 38393 produced cross-sensitization with cocaine when the challenge occurred after a 2-week withdrawal period. Repeated cocaine administration leads to a transient subsensitivity of VTA DA D2 autoreceptors (Henry et al., 1989), as well as an increased ability of presynaptic DA D1 receptors to increase extracellular glutamate (Pierce et al., 1996b). Thus, changes in DA neurotransmission may play a role in the development of cocaine sensitization, but other receptor types and neurotransmitter systems may also be required.

The present findings suggest that DA D1-like receptor activity is necessary for the induction of vertical sensitization to TDF, blocking the sensitized increase in locomotor activity and the increase in extracellular DA turnover associated with TDF sensitization (Fig. 2). Thus, TDF and cocaine sensitization are similar in that both involve DA in their acute locomotor effects and DA D1 receptors in the development of behavioral sensitization. A role for the DA D2-like receptors in TDF sensitization seems less pronounced, although it cannot be excluded. Indeed, as mentioned above, it is possible that a higher or lower dose of Rem may have been successful in blocking the development phase of TDF sensitization, especially given the inconsistent nature of remoxipride’s effect on TDF vertical activity across the development phase, and the fact that it completely abolished TDF-induced vertical activity at doses 4, 8, and 12 (Fig. 3).

Although pretreatment with SCH and Rem did not produce cross-sensitization to TDF (Figs. 1 and 3), Figures 2 and 4 reveal that both also decreased DOPAC, as well as DA in the case of SCH, effects that were significantly greater than those observed after a single dose of TDF (SCH+veh–TDF and Rem–veh+TDF vs. veh+veh–TDF). Therefore, pretreatment with these antagonists appears to “prime” the nigrostriatal dopamine system, such that a subsequent challenge with a DA agonist results in more pronounced alterations in DA metabolism and function. Many individuals require treatment with DA antagonists for disorders such as schizophrenia and psychosis (Maguire, 2002). Indeed, exposure to TDF may represent a particular risk factor for unwanted dopaminergic side effects in these individuals.

SCH 23390 has been shown to act at 5HT2 (Bischoff et al., 1986) and DA D2 receptors (Plantje et al., 1984), posing the possibility that an action at these receptors could be responsible for inhibiting TDF sensitization. Also, Rem has weaker antagonistic efficacy for presynaptic dopamine autoreceptors compared to postsynaptic DA D2 receptor activity (Ogren et al., 1994). Electrophysiological studies point to a specific involvement of VTA DA autoreceptors in sensitization (Henry et al., 1989), whereas a critical role for postsynaptic DA D2 receptors has yet to be established. Therefore, it is possible that the inability of Rem at 0.3 mg/kg to block the induction of TDF behavioral sensitization may stem from its inability to act at DA autoreceptors.

NMDA and AMPA Receptors in the Comparative Development of Cocaine and TDF Sensitization

Changes in glutamatergic pharmacology have been strongly implicated in the induction of cocaine sensitization. In particular, NMDA and AMPA receptors have repeatedly been shown to play a role, because systemic pretreatment with noncompetitive (MK-801) or competitive (CPP or CGS-19755) NMDA antagonists (Li et al., 1999; Wolf and Jeziorski, 1993) or AMPA antagonists (Jackson et al., 1998; Li et al., 1997) block the induction of cocaine sensitization as well as the associated cellular correlates. However, repeated intra-VTA microinjections of NMDA did not lead to cross-sensitization to a systemic cocaine challenge after a 2-week withdrawal (Pierce et al., 1996b). Therefore, it is possible that NMDA receptor function may be necessary, but not sufficient, for the development of cocaine sensitization, at least in the VTA.

The present study reveals that both NMDA and AMPA receptors are necessary for the development of TDF vertical sensitization, but whether action at these receptors is sufficient to induce TDF sensitization remains to be determined. Indeed, it appears that CPP is the most efficacious of all antagonists in blocking the development of TDF behavioral sensitization. This may warrant further research into the role of glutamatergic mechanisms in the behavioral effects of TDF, an avenue that has not yet been the focus of experimental research.

The experimental model of behavioral sensitization has clinical importance, given that chronic use of psychostimulants in humans can cause a progressive augmentation in paranoia than culminates in psychosis (Ellinwood et al., 1973), with symptoms that are often indistinguishable from paranoid schizophrenia (Strakowski et al., 1996). Behavioral sensitization has also been hypothesized as a model of drug craving and relapse after withdrawal (Childress et al., 1988) and is considered to reflect neuronal plasticity. The fact that TDF leads to behavioral sensitization warrants concern over its ability to act as an environmental risk factor for behavioral and affective disorders linked to dysfunction of glutamatergic and dopaminergic systems. TDF is a commercially available fungicide. It is widely used on cereal and grain crops, as well as golf courses, and is even the main ingredient in many commonly used products for home lawns and gardens (see the Federal Register; http://...
ACKNOWLEDGMENTS

The authors would like to thank Dr. Miriam Virgolini and Christine Hammond for their helpful comments on the manuscript. This work was supported by Grants T32ES07026, ES01247, and ES10791 from the National Institute of Environmental Health Sciences.

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