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Behavioral sensitization and long-term neurochemical alterations associated with the fungicide triadimefon

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Abstract

Triadimefon (TDF), a widely used triazole fungicide, blocks reuptake of the neurotransmitter dopamine (DA), similarly to cocaine. Preliminary studies show that intermittent intraperitoneal injections of TDF increase ambulatory and vertical activity across repeated injections [Neurotoxicology (in press)] leading to the hypothesis tested here, that exposure to TDF may influence the development and expression of behavioral sensitization, a model of psychostimulant-induced psychosis. Exposure of adult male C57BL/6 mice to 75 mg/kg ip TDF (TDF75) twice a week for 7 weeks increased vertical activity at each injection. Following a 2-week withdrawal period, a TDF challenge to test for expression of behavioral sensitization revealed further increases in vertical activity levels relative to all other conditions. TDF induction/expression of behavioral sensitization was associated with long-term, perhaps permanent modulation of dopaminergic function that included increases in striatal dihydroxyphenylacetic acid (DOPAC) and DA turnover, increases in medial prefrontal cortex (mPFC) dopamine transporter (DAT) binding, as well as decreases in DA D1 and increases in DA D2 and DAT receptor binding that appeared to target the nucleus accumbens shell (NAs) subregion. Thus, TDF exposure may serve as an environmental risk factor for DA system dysfunctions.

Keywords: Triadimefon; Behavioral sensitization; Neurotoxicity; Pesticides

1. Introduction

Triadimefon [TDF; 1-(4-chlorophenoxy)-3,4-dimethyl-1-(1*H*-1,2,4-triazol-l-yl)-2-butanone; trade name Bayleton] is a triazole fungicide commonly applied to cereal and fruit crops (Walker et al., 1990) and commercially used on golf courses and home lawns and gardens. In fact, TDF is the active ingredient in many commercially available fungicides (see http://www.scorecard.org). While information regarding human exposure to TDF is not readily available, TDF exposure has been associated with contact dermatitis (Winter and Kurtz, 1985), and residues of TDF and of its primary metabolite triadimenol are reported in FDA bread-basket surveys (see http://www.cfsan.fda.gov).

Like psychostimulants, TDF increases locomotor and stereotypic activity (Crofton et al., 1988; Moser and MacPhail, 1989; Walker et al., 1990), as well as overall response rates of schedule-controlled operant behavior (Allen and MacPhail, 1991; Moser and MacPhail, 1989). Several lines of evidence point to changes in CNS dopaminergic function as the primary mediator of TDF-induced behavioral effects. By binding to the dopamine transporter (DAT), TDF effectively increases dopamine (DA) levels in the synapse, which is thought to give rise to its acute stimulant-like effects (Ikaiddi et al., 1997; Walker and Mailman, 1996). Indeed, the hyperactive effect of TDF is attenuated by reserpine (Crofton et al., 1989), and by D1 and D2 DA receptor antagonists (MacPhail et al., 1993). Acute administration of 100–200 mg/kg TDF leads to changes in DA metabolism in both the nigros-

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triatal and mesolimbic DA pathways (Ikaiddi et al., 1997; Walker et al., 1990). Overall, it appears that TDF acts as an indirect DA agonist, with a mechanism similar to that of cocaine.

A recent study in this laboratory revealed that 50 mg/kg TDF (TDF50) administered to mice twice weekly over a 12-week period acutely increased vertical and ambulatory activity, with a lack of recovery 24 h postdosing (Reeves et al., in press). Based on these observations and the associated neurochemical changes, it was hypothesized that TDF could lead to behavioral sensitization, an animal model of psychostimulant-induced sensitization observed in human drug abuse (Segal and Mandell, 1974) and of drug craving and relapse after withdrawal (Ohmori et al., 1995; Post et al., 1981). Behavioral sensitization has two temporally distinct phases, induction and expression. Induction is a progressive enhancement of locomotor activity following repeated dosing with a pharmacological challenge, while expression is an augmented locomotor activity following challenge with the agent after a withdrawal period (Kalivas et al., 1993). Behavioral sensitization to psychostimulants is most often represented in terms of ambulatory or vertical activity, but is also observed with stereotypical (Karler et al., 1996; Wallace et al., 2001) and vertical activity (Chin et al., 2002; Kalivas and Duffy, 1987; Tzschentke and Schmidt, 1998) as well.

Behavioral sensitization involves changes in dopaminergic transmission, particularly in the mesolimbic, but also in the nigrostriatal DA system. One of the most consistent findings is that following sensitization to cocaine or amphetamine, there is an augmentation in extracellular DA levels in the nucleus accumbens (NA) and striatum in response to challenge with the sensitizing drug (Kalivas and Duffy, 1993; Wolf et al., 1993). Other adaptations include a transient increase in the basal activity, or supersensitivity, of DA neurons in the ventral tegmental area (VTA), and a subsensitivity of VTA DA D2 autoreceptors (Henry et al., 1998). More enduring cellular changes include supersensitivity of NA DA D1 receptors, an effect that persists for up to 1 month following withdrawal from cocaine (Henry and White, 1995). For cocaine, the region that appears most critical for mediating the induction of behavioral sensitization is the VTA, based mainly on intra-VTA microinjection experiments (Cador et al., 1999; Cornish et al., 2001; Vezina, 1993). A primary anatomical substrate for the expression of sensitization is the NA, based on findings such as the augmented release of DA and glutamate in the NA following challenge with cocaine or amphetamine (Pierce et al., 1996; Pierce and Kalivas, 1995). In addition, the medial prefrontal cortex (mPFC), specifically the dorsal subregions of the mPFC (Pierce et al., 1998), as well as amygdala (Wolf et al., 1995) and striatum (Mayfield et al., 1992) have also been implicated as anatomical substrates for expression of both amphetamine and cocaine sensitization.

The current study tested the hypothesis that intermittent injections of TDF would lead to the induction of behavioral sensitization in adult male C57BL/6 mice, as well as expression of behavioral sensitization following challenge with the same dose of TDF after a 2-week withdrawal period. Mice were sacrificed 1 month after challenge to measure changes in neurotransmitter levels, and of DA D1-like, DA D2-like and DAT receptor binding in the nucleus accumbens core (NAc) and shell (NAs) subregions, striatum and mPFC, anatomical areas known to be involved in the neuroadaptations of behavioral sensitization. These endpoints were included to determine whether such TDF exposures might result in any long-term or residual changes in associated neural circuitry.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice (6–8 weeks old) from Taconic Farms (New York) were housed one to a cage with food and water available ad libitum in a room maintained under constant temperature and humidity conditions with a 12:12 light–dark cycle. They were habituated to the vivarium for 3 weeks 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.

2.2. Chemicals

All solvents for high-performance liquid chromatography with electrochemical detection (HPLC-EC) were obtained from Sigma (St. Louis, MO). TDF was obtained from Chem Service, West Chester, PA. All other chemicals were at least analytical grade and purchased from Sigma unless otherwise noted.

2.3. Experimental design and TDF administration

Prior to the initiation of TDF treatment, mice were habituated to intraperitoneal injections and to the locomotor activity chambers. At 9–11 weeks of age, mice ($N=60$ total) were injected intraperitoneally with saline (0.2 ml) and immediately placed in locomotor chambers on three consecutive days. The next day, mice ($n=30$ per group) were injected with either saline or 75 mg/kg TDF (TDF75) dissolved in corn oil. Studies in our laboratory have found no significant difference in locomotor activity levels between mice repeatedly treated with saline in comparison to corn oil (unpublished observations). Injection volumes, based on body weight, ranged from 0.22 to 0.30 ml. The intraperitoneal LD_{50} value for TDF in mice is reported to be 1000 mg/kg (RTEC, 1983). TDF was administered in this study twice a week for 7 weeks for a total of 14 injections. Locomotor activity was evaluated after alternating injections, both immediately after injection (Session 1) and 24

h later (Session 2). Thirteen days after the last TDF administration, saline was administered and locomotor activity measured to assess potential conditioning to the injections per se.

The next day, i.e., 14 days after the last TDF injection, mice were split into four groups ($n = 15$ per group): saline-pretreated mice challenged with saline (control–saline), saline-pretreated mice challenged with TDF75 (control-TDF75), TDF75-pretreated mice challenged with saline (TDF75–saline) and TDF75-pretreated mice challenged with TDF75 (TDF75–TDF75), and locomotor activity tested to measure expression of behavioral sensitization. One month later, mice were injected intraperitoneally with saline on two consecutive days, each followed by assessment of locomotor activity levels. Mice were sacrificed immediately after the second locomotor testing session to collect striatum (combined dorsal and ventral) and frontal cortex for neurotransmitter analyses to determine any long-term effects on levels of DA/metabolites from the treatments.

Separate groups of mice ($n = 8$ per group) were subjected to the same TDF treatment regimen without evaluation of locomotor activity and concurrently sacrificed (i.e., 1 month after the 2-week TDF challenge) for autoradiographical analyses to evaluate potential residual changes in binding to DA receptors and the DA transporter in striatum, NA and mPFC from these treatments.

2.4. Locomotor activity

Automated chambers (Opto-Varimex Minor, Columbus Instruments International, 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 were located 57 mm above the horizontal photobeams. Photobeam breaks were recorded each minute for 75 min for horizontal, vertical, and ambulatory movements, with activity counts totaled in 5-min blocks across the session. However, since TDF-induced hyperactivity was much more robust in terms of vertical activity, changes in ambulatory and horizontal activity were not included in the results.

2.5. Neurochemical analysis

Following cervical dislocation, striatum (dorsal and ventral striatum) and frontal cortex (FC) samples were dissected and placed in 0.5 ml 0.1 N perchloric acid. Tissues were sonicated and then centrifuged for 8 min at $1000 \times g$. 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 (5-HT) by HPLC-EC. The pellets were later digested in 1ml of 0.5 N NaOH for measurements of protein concentration using Bio-Rad assay reagents. Methods for HPLC-EC analysis are described in detail elsewhere (Thiruchelvam et al., 2000). The final concentration of neurotransmitter was expressed in terms of nanograms per milligrams of protein. DA turnover was separately expressed as the ratio of DOPAC/DA and as HVA/DA.

2.6. Autoradiography

Measurements of DA D1-like and D2-like receptors and DAT density were made in dorsal striatum, NAc and NAs, and ventromedial prefrontal cortex (vmPFC) and dorsomedial prefrontal cortex (dmPFC) using autoradiography. Brains were harvested following decapitation, and rapidly frozen in isopentane at $-35°$ for 15 s, then put on dry ice and kept at -80 °C until further analysis. Brains were warmed to -20 °C and coronal sections cut on a cryostat, thaw-mounted onto gelatin-coated slides, allowed to dehydrate and stored at -20 °C until used in assays.

For receptor assays, slides were warmed to room temperature then incubated with tritiated ligand using [^{3}H]-SCH-23390 for DA D1-like, [^{3}H]-YM09151-2 for DA D2-like and [^{3}H]-GBR12935 for the DAT (NEN Life Science Products, Boston, MA) at room temperature (except for DAT done at 0°) for varying periods (until equilibrium: 150 min for DA D1-like, 240 min for DA D2-like and 30 h for the DAT). All incubations were done at a concentration of tritiated ligand equal to the K_d of that ligand for the specific receptor in the rat striatum. After incubation, the slides were washed for varying times (10 min for DA D1-like, 60 min for DA D2-like and 2 h for DAT) in cold buffer, then dried using a stream of cool air.

For autoradiographical analysis, dried slides were placed in X-ray cassettes with [^{14}C]-labeled plastic standards previously calibrated with tritiated brain paste sections. The slides and standards were exposed to Amersham Hyperfilm for varying time periods (7 days for DA D1-like, 14 days for DA D2-like and 10 days for DAT) and the film developed in Kodak D19 for 3 min then fixed with Kodak rapid fix for 3.5 min. The atlas of Franklin and Paxinos (1997) was used to assist in verification of structures. Binding density in dmPFC (defined as cingulate area 1 and dorsal prelimbic cortices), vmPFC (defined as ventral prelimbic and infralimbic cortices), NAc, NAs and striatum was determined using densitometric measurements described in detail elsewhere (Richfield et al., 1989). Delineation between NAc and NAs was not possible for DA D2-like binding because sections were too rostral and thus values are presented for NA as a whole.

2.7. Statistical analysis

Overall effects of treatment on locomotor activity were first analyzed using a 2-within 1-between repeated-measures analyses of variance (RMANOVA) with blocks (across a single session) and injection number as within-group factors

and treatment group as a between-groups factor. If main effect or interactions were confirmed, subsequent RMANOVAs were performed with treatment group as a between-groups factor and injection number as a within-groups factor. If significant main effects or interactions were confirmed, individual one-way ANOVAs or Fishers PSLD post hoc tests were used to further assess the nature of the effect. Evaluation of locomotor activity across blocks within a session was also analyzed using 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.

Changes in levels of neurotransmitters and receptor binding were analyzed using one-factor ANOVAs with treatment group as the between-groups factor with Fishers PSLD post hocs where appropriate. In all cases, P values of ≤ 0.05 were considered statistically significant.

3. Results

3.1. Body weight

There were no significant treatment-related effects on body weight, with all mice gaining an average of about 7–8 g over the experiment. Mean weight in grams as measured at the final challenge dose were: control–saline = 32.4; control–TDF = 31.1; TDF–saline = 31.3; and TDF–TDF = 31.0. Mean weight gain over the course of the experiment were: control–saline = 7.9; control–TDF = 8.1; TDF–saline = 7.7; and TDF–TDF = 8.0.

3.2. Locomotor activity

Fig. 1 shows group mean total vertical activity counts across the 14 intermittent injections. Vertical activity levels were higher for the TDF75 group than for control at each of the injection times. RMANOVA confirmed a significant main effect of treatment [$F(1,58) = 10.343$, P=.002] as well as an interaction of treatment by blocks by injection [$F(84,4872) = 1.808$, P=.0001]. Levels of ambulatory activity were similarly increased (data not shown) across injections compared to controls ($P < .05$ for each injection by one-way ANOVA). Historically, our laboratory has observed that TDF-induced increases in hyperactivity across injections and TDF-induced sensitization upon challenge is much more robust in vertical activity compared to ambulatory. Thus, only vertical data are included in the results.

Mice were injected 13 days posttermination of TDF treatment with saline to determine whether injections per se might be associated with increased spontaneous activity levels, i.e., whether there were conditioning effects. As can be seen in Fig. 1 (2-week saline), no significant difference between the TDF75-pretreated group and controls were evident following saline injection.

On the next day, each group (saline, TDF) was subdivided and half injected again with saline and the other

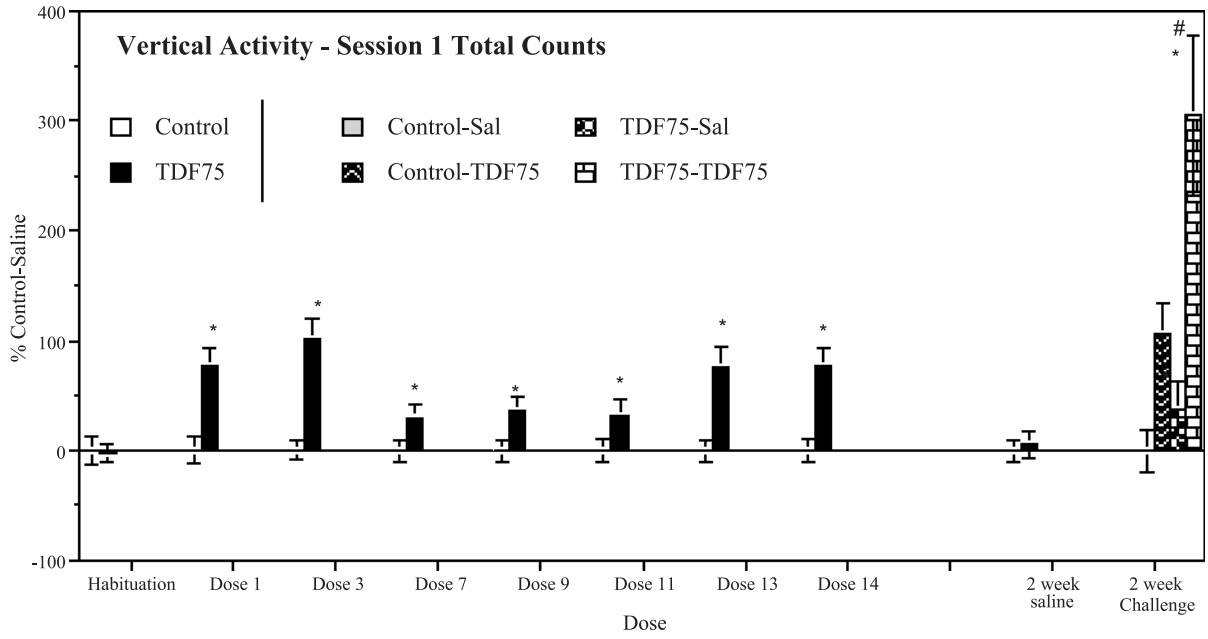

Fig. 1. Total vertical locomotor activity counts measured immediately after injection (Session 1) as percentage of control–saline group data. Each data point represents a group mean ± S.E. ($n = 30$ per group for Doses 1–14 and 2-week saline, $n = 15$ per group for 2-week challenge). Mean raw values for control vertical activity levels at habituation (third day of habituation) in cell mean = 598.8. Following RMANOVA, subsequent Fischer post hocs were used to compare treatment groups with the respective control value for each dose. Following challenge, Fischer post hocs revealed the TDF75–TDF75 group to be significantly higher than all other groups. * $P \leq .05$ compared to control–saline; #, compared to control–TDF75.

half with TDF75 to determine expression of behavioral sensitization following a withdrawal period (Fig. 1; 2-week challenge, control–saline, control–TDF75, TDF75–saline, TDF75–TDF75). In this assessment, mice pretreated with TDF75 and then challenged with TDF75 (TDF75–TDF75 group) displayed marked increases in vertical activity relative to all other groups, including control mice receiving their first acute administration of TDF75 (control–TDF75; P=.0002). This latter group exhibited increases in activity consistent with those associated with the first injection of TDF in the TDF-treated group. Ambulatory activity levels also exceeded those associated with challenge to saline-pretreated animals (data not shown).

Fig. 2 displays the vertical activity levels across successive blocks of 5 min over the course of the 75-min 2-week challenge session. Control–saline-treated mice showed the characteristic pattern of activity, with highest levels early in the session, then declined and stabilized. A similar pattern was seen for the TDF75–saline group, as expected. Control mice receiving their first acute administration of TDF75 (control–TDF75) exhibited vertical activity levels that never declined across the course of the session. Mice previously exposed to TDF75 and challenged following withdrawal (TDF75–TDF75) displayed activity levels that actually continued to increase across the session. RMANOVA confirmed a significant main effect of treatment [$F(3,56)=13.49$, $P<.0001$] as well as an interaction of treatment by blocks [$F(42,784)=7.784$, $P<.0001$].

Locomotor activity was also evaluated 24 h postdosing (Session 2) and outcomes for group mean total vertical activity counts are shown in Fig. 3. RMANOVA across the 14 initial injections revealed a significant main effect of treatment [$F(1,58)=5.379$, P=.0239] but no interaction of treatment by injection, consistent with uniform residual decreases in vertical activity of the TDF group relative to control across injections. This was confirmed in subsequent one-way ANOVAs.

Twenty-four hours after the 2-week challenge, only the TDF75–TDF75 sensitized group exhibited significant residual decreases in vertical activity, as compared to the control–saline group (P=.037).

One month following this challenge, all mice received saline for two consecutive days and locomotor activity was measured immediately postdosing. Fig. 4 shows results from the first and second day of locomotor activity assessment, with reductions in vertical activity of 25–45% seen in all groups previously receiving TDF, as compared to control–saline treated mice, but these effects were not statistically significant.

3.3. Neurotransmitter determinations

Figs. 5 and 6 display striatal (combined dorsal and ventral striatum) and frontal cortical levels, respectively, of DA and metabolites and DA turnover measured 1 month after the 2-week challenge. This time point was 4 weeks after the last TDF treatment for groups control–TDF75 and TDF75–TDF75 and 6 weeks after the last TDF treatment for the TDF75–saline group. Groups that exhibited sensitization following challenge with TDF75 (TDF75–TDF75) also showed statistically significant increases in striatal DOPAC levels (P=.018) and DA turnover (P=.023) that were 40–50% greater than control–saline

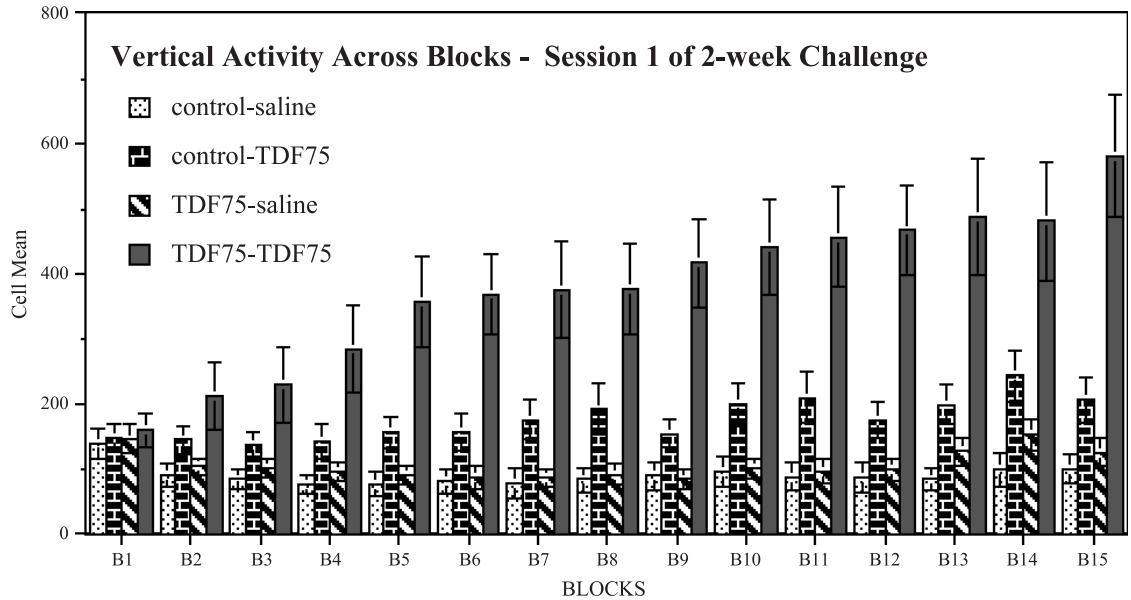

Fig. 2. Vertical activity counts measured immediately after 2-week challenge, in 5-min blocks across the 75-min session. Each data point represents a group mean ± S.E. for 15 mice per group. Mean value for control–saline group at B1 = 140.9. RMANOVA analysis revealed a significant main effect of treatment ($P<.001$) and subsequent Fischer post hocs revealed the TDF75–TDF75 group to be significantly higher than all other groups ($P \leq .05$).

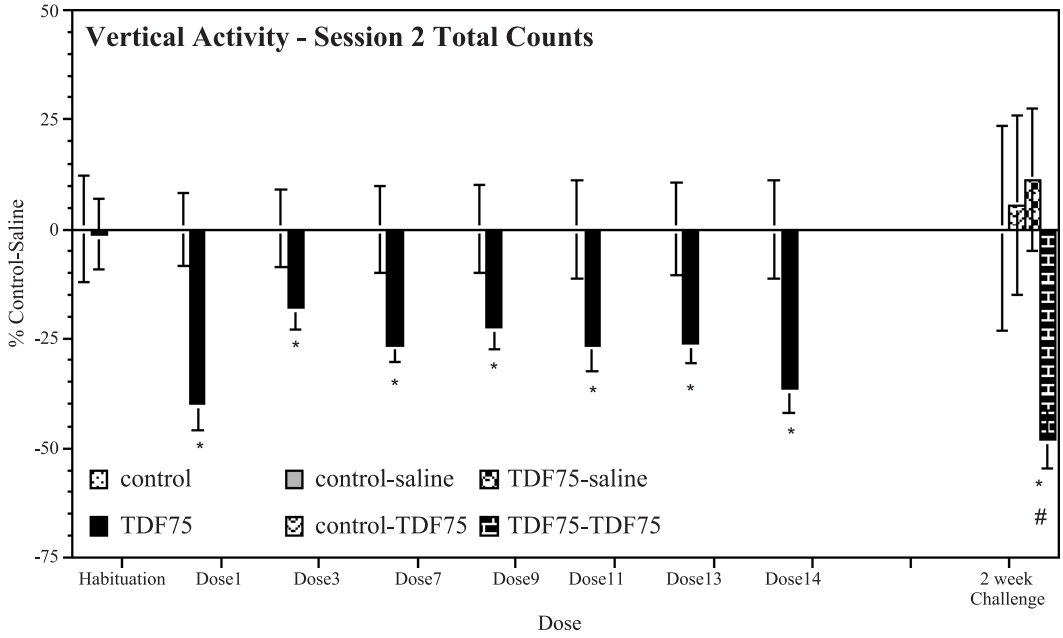

Fig. 3. Total vertical locomotor activity counts measured 24 h after injection (Session 2). Each data point represents a group mean ± S.E. ($n = 30$ per group for Doses 1–14; $n = 15$ per group for 2-week challenge Session 2 and 1 month saline). Raw data for control group vertical activity levels at habituation (third day of habituation) = 598.8 cell mean. Following RMANOVA, subsequent Fischer post hocs were used to compare treatment groups with the respective control value for each dose. * $P \leq .05$ compared to control–saline; #, compared to control–TDF75.

values. Mice that were pretreated with TDF75, but received saline at the 2-week challenge (TDF75–saline), showed no corresponding changes, nor did the control group that received its first injection of TDF at the 2-week challenge (control–TDF75).

Despite trends in FC suggestive of increased levels of DA, DOPAC and DOPAC/DA turnover in the saline-pretreated group that received one injection of TDF75 upon challenge (control–TDF75), no statistically significant changes in levels of DA, metabolites or indices of DA turnover were confirmed, probably owing to the low levels of DA in this region and associated variability.

3.4. Autoradiographic determinations of DA receptor and transporter levels

Fig. 7A–C shows the results of autoradiographical measurements of striatal DA D1-like and DA D2-like

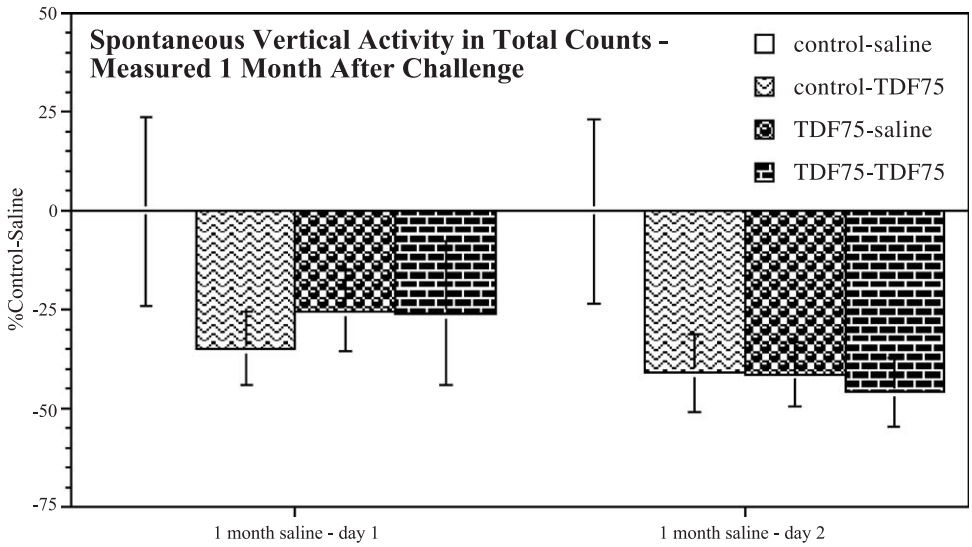

Fig. 4. Total spontaneous vertical locomotor activity counts 1 month after challenge. Mean value for control–saline group in cell mean = 2251.3 for saline Day 1, and 1816.4 for saline Day 2. All mice were injected with saline across two consecutive days. Each data point represents a group mean ± S.E. ($n = 15$ per group).

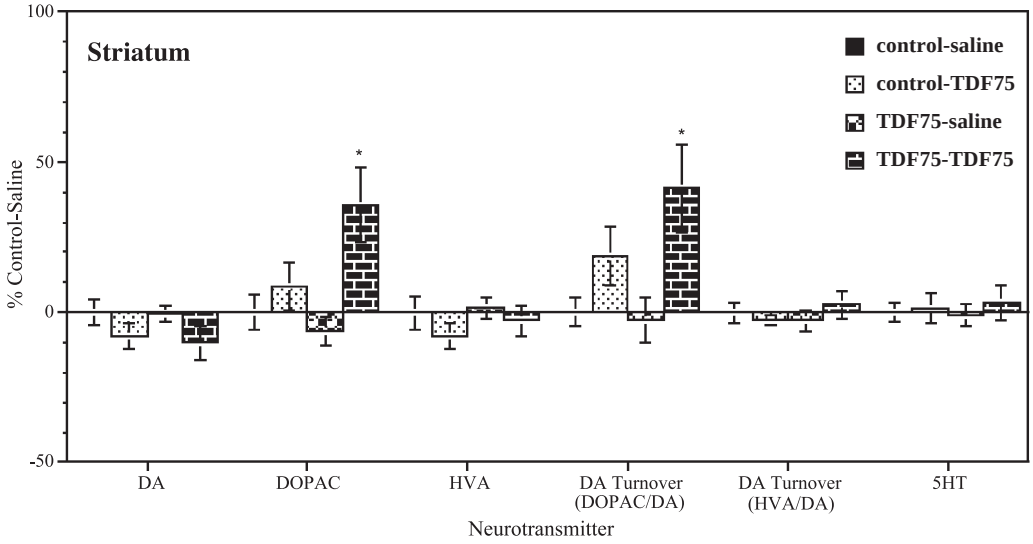

Fig. 5. Group mean ± S.E. levels of striatal dopamine (DA), DOPAC, HVA, intracellular DA turnover [DA turnover (DOPAC); DOPAC/DA], extracellular DA turnover [DA turnover (HVA); HVA/DA] and serotonin (5-HT) determined immediately after spontaneous locomotor activity 1 month following the 2-week challenge. (Time since last TDF injection is actually 4 weeks for the control–TDF75 and TDF75–TDF75 groups, and 6 weeks for the TDF75–saline group.) Mean values for control–saline group in nanograms per milligrams were: 198.78 for DA, 37.93 for DOPAC, 28.78 for HVA and 4.65 for 5-HT. One-factor ANOVAs were followed by Fischer post hoc tests. * $P \leq .05$ compared to control–saline. $n = 15$ per group.

receptor binding, and DAT binding, respectively, measured 1 month after the 2-week posttreatment challenge. Levels of binding are shown separately for dorsal striatum and NA for DA D2-like receptors, and dorsal striatum, NAc and NAs for DA D1-like receptors and the DAT. Delineation between NAc and NAs for DA D2-like binding was not possible because sections were too rostral.

Although general decreases in DA D1-like binding were seen across regions in groups that received TDF75 either acutely or for the 14 dosing regimen, statistically significant reductions, averaging between 10% and 20%, were seen only in the NAs (TDF75–saline vs. control–saline, P=.05; TDF75–TDF75 vs. control–saline, P=.0041).

For D2-like receptor binding, TDF changes were also confined to NA, and specifically in this case to the TDF75–TDF75 group, where elevations of almost 20% relative to control–saline group (P=.007) were observed.

Small but significant increases in DAT binding in the NAs, but not in the NAc, were observed in all TDF-treated groups relative to control–saline group (P=.006 for con-

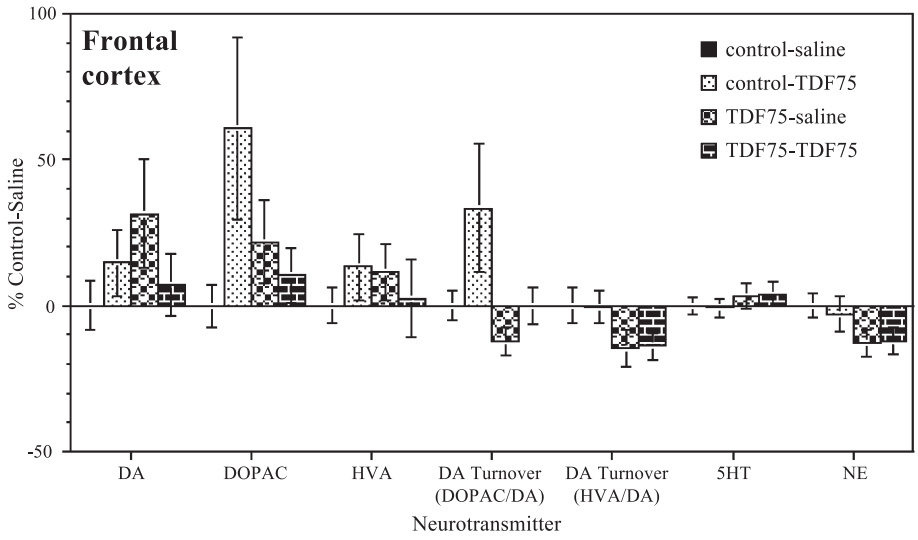

Fig. 6. Group mean ± S.E. levels of frontal cortical dopamine (DA), DOPAC, HVA, intracellular DA turnover [DA turnover (DOPAC); DOPAC/DA], extracellular DA turnover [DA turnover (HVA); HVA/DA], serotonin (5-HT) and norepinephrine (NE) determined immediately after spontaneous locomotor activity measured 1 month following the 2-week challenge. (Time since last TDF injection is actually 4 weeks for the control–TDF75 and TDF75–TDF75 groups, and 6 weeks for the TDF75–saline group.) Mean values for control–saline group in nanograms per milligrams were: 61.486 for NE, 7.17 for DA, 7.49 for DOPAC, 5.42 for HVA and 11.08 for 5-HT. One-factor ANOVAs were followed by Fischer post hoc tests. $n = 15$ per group.

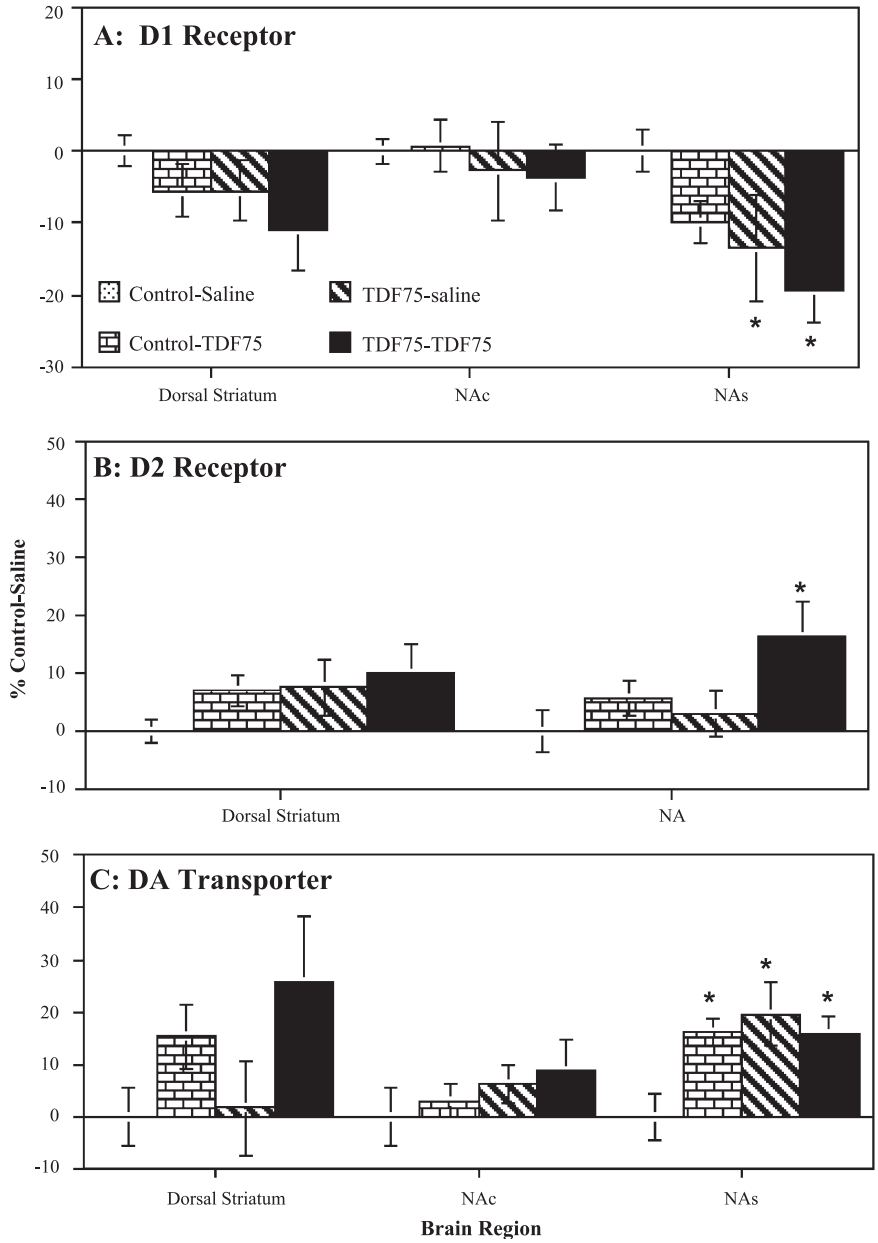

Fig. 7. Group mean ± S.E. levels of binding as percentage of control–saline group values in dorsal striatal (DS), nucleus accumbens core (NAc) and nucleus accumbens shell (NAs) for: DA D1-like receptors (A), DA D2-like receptors (B) and the DA transporter (C). Binding values were determined 1 month following the 2-week challenge. (Time since last TDF injection is actually 4 weeks for the control–TDF75 and TDF75–TDF75 groups, and 6 weeks for the TDF75–saline group.) Delineation between NAc and NAs for DA D2-like binding was not possible because sections were too rostral. Mean control values for control–saline groups in picomolars per milligrams were: (1) DA D1-like; 2.936 for DS, 2.808 for NAc and 2.44 for NAs, (2) DA D2-like; 0.126 for DS and 0.123 for NA, (3) DAT; 1.741 for DS, 1.572 for NAc and 1.319 for NAs. * $P \leq .05$ compared to control–saline. $n = 8$ per group.

trol–TDF75; P=.003 for TDF75–saline; P=.01 for TDF75–TDF75). Trends towards increased levels of the DA transporter in the TDF75–TDF75 group were evident in the dorsal striatum.

Autoradiographical measurements of DA D1-like receptor binding and DAT binding are shown for dmPFC and vmPFC in Fig. 8A and B, respectively. No significant treatment-related differences in DA D1-like receptor binding were found in either region of mPFC at this time point. Binding to PFC DA D2-like receptors was below detectible levels and thus not shown. Notable increases in DAT binding were detected in both the dmPFC and the vmPFC for both the control–TDF75 (P=.007 and P=.01, respectively) and the TDF75–TDF75 group (P=.001 and P=.002, respectively) relative to control–saline. These increases ranged from greater than 50% to approximately 80% of control–saline values. Both of these groups had received TDF75 at the time of the 2-week posttreatment challenge 1one month prior to these determinations. In contrast, the TDF75–saline group that had not received any TDF75

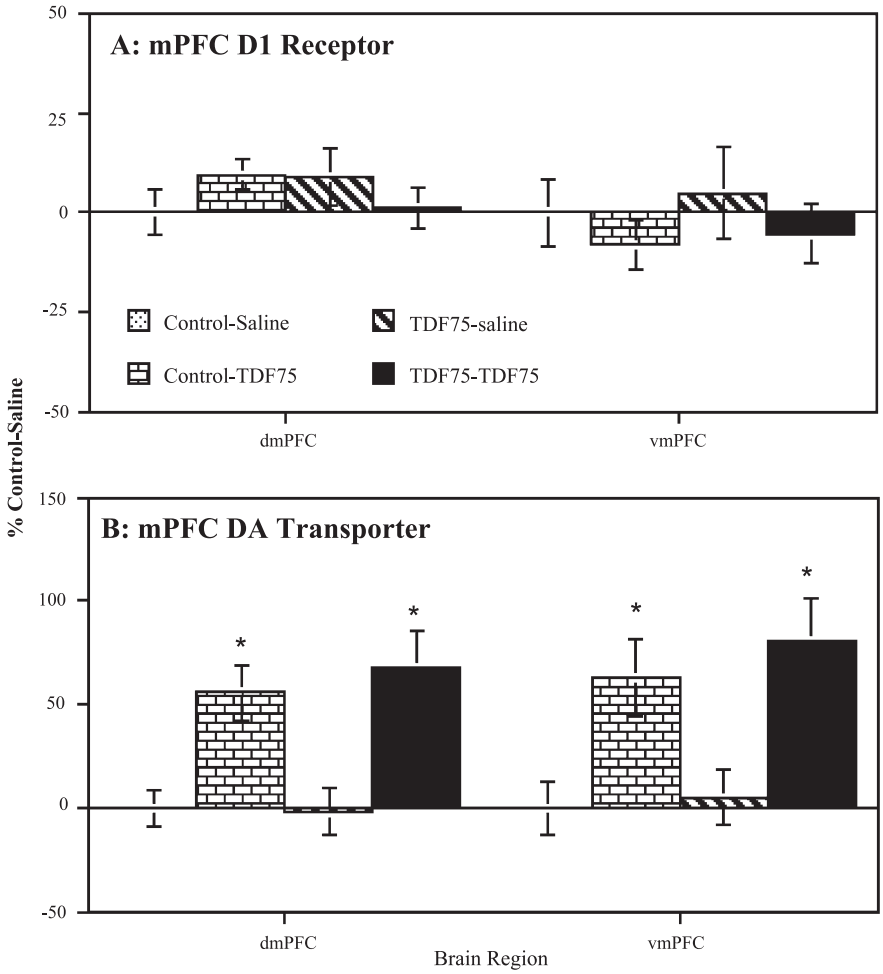

Fig. 8. Group mean ± S.E. levels of binding as percentage of control–saline group values in dmPFC and vmPFC for: DA D1-like receptors (A), and the DA transporter (B). Binding values were determined 1 month following the 2-week challenge. (Time since last TDF injection is actually 4 weeks for the control–TDF75 and TDF75–TDF75 groups, and 6 weeks for the TDF75–saline group.) Mean control–saline group values in picomolars per milligrams were: (1) DA D1-like; 0.283 for dmPFC and 0.219 for vmPFC, (2) DAT; 0.061 for dmPFC and 0.069 for vmPFC. * $P \leq .05$ compared to control–saline. $n = 8$ per group.

administration for 6 weeks prior to the determination of binding levels showed no corresponding increases.

4. Discussion

This study examined the hypothesis that repeated intermittent injections of TDF could lead to the development and/or expression of behavioral sensitization, with associated neurochemical effects on DA systems. In this study, TDF75 administration resulted in the induction and protracted expression of behavioral sensitization that was associated with long-term neurochemical changes in both the mesolimbic and nigrostriatal dopaminergic systems. These findings differ from the report of Hill et al. (2000) that failed to find expression of TDF sensitization in response to a TDF challenge administered 5 days after the last of 14 intermittent injections of 100 mg/kg TDF in rats. This difference may reflect the fact that Hill et al. employed a challenge dose of only 25 mg/kg for the TDF challenge, which by itself did not acutely increase activity. Other studies also indicate that 25 mg/kg TDF may be below the no-observed adverse effect level for hyperactivity (Moser and MacPhail, 1990; Reeves et al., in press). Hill et al. also employed a different animal model (rat) as well as a different posttreatment withdrawal period (5 days vs. 2 weeks) relative to the current study. Nevertheless, the present sensitized TDF response at the 2-week challenge produced increases in vertical activity of over 300%, an effect even more robust than what appears typical of cocaine sensitization (Henry and White, 1995). Furthermore, the longer withdrawal period used here (2 weeks) may be more relevant than the more typical 24- or 48-h challenge employed in many studies, which can be thought of as merely an extension of the development phase, particularly as there are a variety of transient neurochemical changes occurring during early withdrawal that may result in competing effects on behavior and confound the more permanent neuroadaptations associated with expression of long-term sensitization. It is also important to mention that the

dose of TDF used here was not associated with any changes in body weight and is only 7.5% of reported mouse intraperitoneal LD_{50} values (RTEC, 1983).

The neuroadaptations that occur during the induction of locomotor sensitization are a prerequisite for the expression phase (Druhan and Wilent, 1999; Li et al., 1997). It is interesting to note that a dampening of the stimulant response occurred between Doses 7–11 across the induction phase of TDF sensitization, with a return to a more heightened response at the last two injection (Fig. 1), a phenomenon we have previously seen (Reeves et al., in press). This is inconsistent with the more standard psychostimulant sensitization that manifests as a progressive augmentation in behavioral response across repeated injections (Kalivas et al., 1993). However, while the induction phase of TDF sensitization may be somewhat atypical, (except see Burger and Martin-Iverson, 1994; Capper-Loup et al., 2002), it is apparent that repeated exposure to TDF still resulted in neuroadaptations necessary for a robust expression of sensitization.

While acute administration of TDF75 increased activity, significant decreases were found in the TDF75 group 24 h postdosing, with similar but nonsignificant reductions in the sensitized TDF75–TDF75 group 24 h after the 2-week challenge (Fig. 3), confirming previous findings in our laboratory. Notably, such decreases were not found after TDF75 challenge in saline-pretreated rats (control–TDF75) where one might expect similarities to the TDF group effects during the induction phase. The reason for this discrepancy is not clear but may be related to the different behavioral histories of the TDF75 and control–TDF75 groups. In fact, time course experiments have shown that acute TDF75 administration in behaviorally naive mice leads to a fundamentally different profile of dopaminergic changes than that produced by TDF75 challenge in mice pretreated with intermittent injections of saline (control–TDF75, unpublished observations). Thus, these findings suggest that behavioral history could modulate TDF-induced sensitization.

In accord with the possibility of long-term changes in neural circuitry, significant increases in striatal DOPAC and intracellular turnover of DA were observed 1 month after the 2-week TDF challenge, changes that were specific to the sensitized TDF75–TDF75 group (Fig. 5) and not likely to reflect any TDF still present. Such increases in DA turnover could merely reflect the increase in DOPAC since no changes were seen in DA levels. However, these results could reflect a concurrent increase in DA synthesis that may compensate for increased DA metabolism, possibly through an upregulation of tyrosine hydroxylase or monoamine oxidase activity. Indeed, increases in striatal DA turnover are evident immediately after expression of methamphetamine sensitization (Nishikawa et al., 1983). While the relationship of these changes to TDF-induced behavioral sensitization is not yet clear, increases in DA turnover are associated with oxidative stress due to an increase in hydrogen peroxide formation (Spina and Cohen, 1989) and thus may be indicative of long-term or even permanent dopaminergic neurotoxicity. Thus, TDF induction and expression of behavioral sensitization are associated with protracted, perhaps permanent neurochemical changes.

Autoradiographic data carried out separately for dorsal striatum and core and shell subregions of NA suggest that striatal changes derive primarily from the shell subregion of the NA, where consistent decreases in D1 receptor binding and increases in D2 receptor and DAT binding were found (Fig. 7), again confirming what appear to be long-term effects. Sustained effects were also noted in DA transporter function in mPFC as well (Fig. 8). Although no significant changes in frontal cortical neurotransmitter levels were found (Fig. 6), residual increases in DOPAC, especially in the control–TDF75 group, while not statistically significant, were noteworthy and may be indicative of long-term alterations to frontal cortical dopaminergic neurotransmission after only a single injection of TDF.

The absence of decreases in striatal DA levels 1 month after TDF challenge (Fig. 5) might be considered surprising, given the decreases in activity in all TDF treatment groups at that time point. However, no sustained changes in basal DA release are found following long-term withdrawal from chronic amphetamine (Crippens et al., 1993) or cocaine (Meil et al., 1995). Moreover, decreases in NAs DA D1-like receptor binding (Fig. 7A) may be related to the observed decreases in locomotor activity, given that NAs DA D1-like receptor activity plays a critical role in spontaneous locomotion and rearing (Hoffman and Beninger, 1985; Meyer and Shults, 1993). In rats, DA D1-like receptor density decreases in NA immediately after cessation of chronic intermittent cocaine and recover after about 2 weeks of withdrawal (Farfel et al., 1992; Kleven et al., 1990). The current study suggests that chronic exposure to TDF may lead to more protracted effects on NAs DA D1-like binding than does cocaine.

It may be noted that many of the long-term neurochemical changes are observed only in the two groups that received a TDF challenge at the 2-week challenge and not in the group repeatedly administered TDF only (TDF–saline). Therefore, one could interpret that a TDF challenge "unmasks" potential neurotoxicity of repeated TDF exposure. However, it should be noted that decreases in striatal DA D1 receptor binding was observed in the TDF–saline group, which may reflect long-term (6 weeks) alterations in DA function due to repeated TDF treatment.

Expression of sensitization to TDF was also associated with long-term increases in DA D2-like and DAT binding (Figs. 7 and 8) that were statistically significant only in NAs and mPFC (although similar trends were noted in dorsal striatum and NAc). In contrast, downregulation in NA DAT binding (Pilotte et al., 1996; Sharpe et al., 1991), and changes in DA D2 density after withdrawal from cocaine sensitization are typically transient (Peris et al., 1990; Shilling et al., 1997).

Collectively, the current neurochemical and autoradiographical results are consistent with the possibility of TDF-induced long-term compensatory effects due to initial overactivity of dopaminergic neurotransmission, whereby overstimulation may lead to a kinetic or dynamic down-regulation of postsynaptic DA D1 receptors and excess synaptic DA may engender an increase in DA clearance (increases in DAT), or a decrease in terminal DA output (increases in presynaptic D2 autoreceptors). Likewise, it has been postulated that increased activation of DA D1 receptors brought about by repeated cocaine signals a reduction in DA D1 receptor production (Nestler et al., 1993); this may also be the case after repeated TDF treatment.

These findings demonstrate that TDF sensitization may share both similarities with and differences from psychostimulant drugs of abuse such as cocaine in the mechanisms whereby it produces induction and expression of behavioral sensitization. The high potential for abuse of TDF has been suggested elsewhere (Perkins et al., 1991; Walker and Mailman, 1996). Indeed, the data presented here call for further attention to this concern. Its ability to engender both induction and long-term (2 weeks) expression of behavioral sensitization with enduring changes in DA neurotransmission and receptor levels, effects that are observed even after a single injection of the fungicide, all suggest that TDF exposure may serve as an important environmental risk factor that could act in conjunction with other risk factors to detrimentally impact DA and other neurotransmitter systems and associated behavioral processes. These findings suggest the need for extended research on the mechanisms of TDF-induced sensitization and for its ability to cause long-term and/or permanent adverse effects on the brain, thereby its potential to serve as an environmental risk factor for DA-associated behavioral and psychiatric disorders.

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