*Pharmacology Biochemistry & Behavior*, Vol. 38, pp. 659–663. © Pergamon Press plc, 1991. Printed in the U.S.A. 0091-3057/91 $3.00 + .00

# Chronic Flurazepam Differentially Regulates a Behavioral Effect of GABA Agonists

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RAMSEY, V. A., E. I. TIETZ AND H. C. ROSENBERG. *Chronic flurazepam differentially regulates a behavioral effect of GABA agonists*. PHARMACOL BIOCHEM BEHAV **38**(3) 659–663, 1991.—Subsensitivity to γ-aminobutyric acid (GABA) agonists was sought in rats treated 1 or 4 weeks with flurazepam (FZP). Sensitivity to GABA and 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP) was assessed by measuring contralateral rotation following unilateral microinjection of drug into the substantia nigra pars reticulata (SNpr). Immediately and 48 h after chronic treatment GABA, 200 μg or THIP, 60 ng was infused into SNpr. Immediately, but not 48 h after 1 week of FZP treatment, GABA subsensitivity was shown by a significantly reduced total number of contralateral turns and peak rotation rate. There was no change in the response to THIP after 1 week FZP treatment. Following 4 week FZP treatment, no subsensitivity to GABA or THIP was evident. Previous results showed subsensitivity to muscimol after 4, but not 1 week of FZP treatment. Since muscimol and THIP are not subject to uptake, there may be increased uptake of GABA after 1 week of FZP treatment, though it may not persist during continued treatment. Differential regulation of GABA agonist effects in SNpr may be related to their acting at differing $GABA_A$ receptor subpopulations, and variable responses of these subpopulations to chronic BZ treatment.

GABA Substantia nigra pars reticulata Flurazepam THIP Rotation Subsensitivity

CHRONIC treatment with benzodiazepines (BZs) is associated with the development of functional tolerance, i.e., reduced sensitivity of the nervous system to the actions of these drugs (26, 27, 30, 32). The mechanisms that are the basis for tolerance may include alterations in all components of the $GABA_A$ receptor. BZs bind to a site on the $GABA_A$ receptor and act as positive modulators of GABA action, increasing GABA-mediated chloride ($Cl^-$) channel function [cf. (9)]. Thus tolerance could result from changes in function of the GABA recognition site, the BZ recognition site, the $Cl^-$ channel (picrotoxinin-sensitive site), or the coupling between them.

Several changes in the BZ/$GABA_A$ receptor have been measured after chronic BZ treatment. Reduced numbers (downregulation) of BZ binding sites (21, 26, 31) and low-affinity $GABA_A$ receptor sites (7) have been found after some chronic BZ treatments. However, downregulation of the site on the $Cl^-$ ionophore, measured by [$^{35}$S]TBPS binding, was not found (10,21). Reduced functional coupling of the BZ site to the $GABA_A$ receptor/$Cl^-$ channel was suggested by the decreased ability of GABA to stimulate BZ binding after chronic BZ treatment (20,29). Decreased functional coupling of the BZ receptor to the $GABA_A$ receptor is also suggested by the reduced ability of BZs to enhance GABA-stimulated $Cl^-$ flux in the absence of any decrease in the ability of GABA to stimulate $Cl^-$ flux (22,37). A decreased capacity of GABA or muscimol to stimulate $Cl^-$ flux was found after other chronic BZ treatments (18,21), showing that reduced effectiveness of GABA agonists can also be a response to chronic BZ treatment.

The changes in the GABA receptor complex that result from chronic BZ treatment, and the associated decreases in the actions of BZ and GABA agonists, show regional variation. For example, chronic diazepam treatment that resulted in subsensitivity to microiontophoretically applied GABA in dorsal raphé (8) did not change the effectiveness of GABA in substantia nigra pars reticulata (SNpr) (34). Another indication of regional localization of changes in the GABA receptor complex is the nonuniformity of BZ receptor downregulation (21,25) and GABA/BZ receptor uncoupling (29). Downregulation of BZ binding sites was found to be greatest in SNpr (31), suggesting that this site might be a useful one for studying tolerance.

Rotational behavior is reliably produced following unilateral microinjection of BZs or GABA agonists into the SNpr (13, 19, 30, 33). In an earlier study, circling behavior was measured and compared in BZ-treated and control rats in order to measure tolerance to flurazepam (FZP) and subsensitivity to the GABA agonist, muscimol, in SNpr (30). The time-course observed for the development and reversal of FZP tolerance did not match that for subsensitivity to muscimol, suggesting differential regulation of the responsiveness to GABA agonists and BZ agonists in SNpr. Muscimol has actions in SNpr that are not shared by other GABA agonists (2,3), and may not bind to the same receptor pool as GABA (4). Additionally, subsensitivity to muscimol-induced rotation has been reported in SNpr (30), whereas no subsensitivity to neuronal suppression by GABA, when microiontophoresed into

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SNpr, was found following prolonged BZ treatment (34). Therefore, this study was performed to examine the rotational behavior elicited by GABA and another GABA agonist, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP), in SNpr following chronic FZP treatment.

## METHOD

### *Treatment*

Male Sprague-Dawley rats were treated by providing, as the sole source of drinking water, FZP in a dilute saccharin solution for 1 week (initial weight 250–275 g) or 4 weeks (initial weight 200–225 g), according to a previously described treatment method (26,30) known to cause BZ receptor downregulation in SNpr (31) and tolerance to many BZ actions including locomotor impairment (26), anticonvulsant activity (27), drug-induced rotational behavior in SNpr (30), suppression of SNpr neuronal firing rate (32), and enhancement of GABA-gated $Cl^-$ flux (37). Rats weighed approximately 300 g at the time of testing. Briefly, rats were given 0.02% saccharin water for a 2-day adjustment period, then offered FZP in saccharin water for 1 or 4 weeks. The target dose for days 1–3 of the 1 week FZP treatment and for days 1–7 of the 4 week treatment was 100 mg/kg/day. The target dose for days 4–7 of the one week treatment and days 8–28 of the 4 week treatment was 150 mg/kg/day. Doses were calculated daily from water intake and body weights. The average doses of FZP consumed by rats injected with GABA and THIP were not significantly different from each other (Student's $t$-test, $p>0.05$) after either 1 week ($111.5 \pm 3.2$ vs. $121.6 \pm 10.2$ mg/kg/day) or 4 week chronic treatment ($122.7 \pm 3.4$ vs. $111.5 \pm 3.7$ mg/kg/day). Control rats received saccharin water for the duration of treatment, and were handled identically to the FZP-treated rats.

### *Stereotaxic Surgery*

No later than 7 days before testing, rats were deeply anesthetized with sodium pentobarbital (65 mg/kg, IP), and given atropine sulphate (0.5 mg/kg, IP). Rats were placed in a stereotaxic frame with head level. Two 26-gauge stainless steel cannulae measuring 9 mm in length with close-fitting obturators were implanted bilaterally 2 mm dorsal to SNpr. Coordinates from lambda were: 3.1 mm anterior, 2.0 mm lateral, and 6.0 mm ventral to skull surface (23). The guides were fixed in place with dental acrylic and an anchoring screw.

### *Testing*

A stainless steel bowl, 13 cm high, with a flat base of 15.5 cm diameter and maximal diameter of 40.6 cm, served as a rotation chamber. Just before drug infusion, baseline rotational behavior was recorded for 5 min. If a rat completed a net total of more than 5 complete 360° turns to either side, that rat was not included in the study. Chronic treatment did not significantly increase this rejection rate in any test group ($\chi^2 \geq 0.14$). Each rat received 2 infusions, one into each SNpr. The first side of injection (0 h after FZP treatment) was randomly assigned. The other side was injected 48 h later. Not all rats included in 48-h groups had an injection histologically verified to be in SNpr at 0 h. Likewise, rats included in the 0-h groups may not be included in the corresponding 48-h groups.

Doses of GABA (200 μg) and THIP (60 ng) were chosen from preliminary trials to be approximately equieffective in producing contralateral rotation (data not shown), and equieffective to the doses of FZP and muscimol previously used to elicit rotation (30). GABA (Sigma Chemical Co.) and THIP were dissolved in 0.9% saline. In previous work, infusion of saline vehicle did not cause circling behavior (30). Drugs were infused into the SNpr through an 11 mm injector connected by polyethylene tubing to a 10 μl Hamilton syringe, driven by an infusion pump. The doses were infused in 0.5 μl for 2 min.

After drug infusion, the injector was kept in place for an additional minute to allow diffusion from the injector tip. The injector was then slowly removed, and replaced with an obturator. The number of complete 360° turns in either direction were then recorded in consecutive 2-min periods, until no net contralateral turns were recorded for 3 consecutive 2-min periods. For each injection site, the total number of turns, the duration of rotation, and the peak rotation rate (the greatest sum of total turns for any 5 consecutive 2-min periods) were calculated.

### *Histology*

Two days after the last injection, rats were deeply anesthetized with 65 mg/kg (IP) sodium pentobarbital and perfused intracardially with 0.9% saline followed by 10% formalin. SNpr injection sites were verified in cresyl fast violet-stained sections. As in the previous study (30), only those injections made into the SNpr between 6.0 and 6.5 mm posterior to bregma (23) were included in the data analysis.

### *Statistics*

For each FZP treatment (1 and 4 weeks), the effect of treatment was evaluated by planned comparisons for the 0-h and 48-h responses. As the responses were correlated variables (duration of turning, total turns and peak rotation rate), a one-way MANOVA (Wilk's lambda) was used. If a significant effect of chronic FZP treatment was found, additional univariate planned comparisons were made for each dependent variable, immediately and 48 h after treatment. The significance level was set to $p \leq 0.025$ by the Bonferroni method (14) to adjust for the number of comparisons made within each treatment.

## RESULTS

Time-action profiles of the rotation induced by GABA and THIP in control rats are shown in Fig. 1. While the peak effect of GABA in SNpr occurs immediately after infusion, the peak effect of THIP in SNpr is delayed 10 min. Muscimol (10 ng) infusion into the SNpr shows a delay of up to 30 min to peak effect (15,30). The peak turning rates produced by 200 μg GABA and by 60 ng THIP were similar to each other (Fig. 1), and similar to the peak turning rates produced by 10 ng muscimol and 50 μg FZP in the previous study (30).

There was a significant effect, $F(3,18) = 3.6$, $p = 0.03$, of 1 week FZP treatment on GABA-induced rotation immediately following chronic treatment. GABA infused into SNpr showed a significantly smaller effect in treated rats ($n = 9$) as compared to control rats ($n = 13$), as measured by total contralateral turns ($p = 0.01$, Fig. 2A) and by peak number of rotations per 10 min ($p = 0.004$, Fig. 2B). There was no significant difference ($p = 0.06$) between FZP-treated and control rats in duration of rotation at this time (treated, $84.2 \pm 8.2$ min; control, $112.0 \pm 10.3$ min). When GABA was infused 48 h after terminating 1 week of FZP treatment (Fig. 2), there were no significant differences, $F(3,12) = 0.93$, $p = 0.46$, between treated ($n = 8$) and control ($n = 8$) rats for any of the measures of rotational behavior (duration: treated, $123.0 \pm 14.0$ min; control, $116.5 \pm 16.7$ min). In contrast to the findings after 1 week of FZP treatment, immediately upon termi-

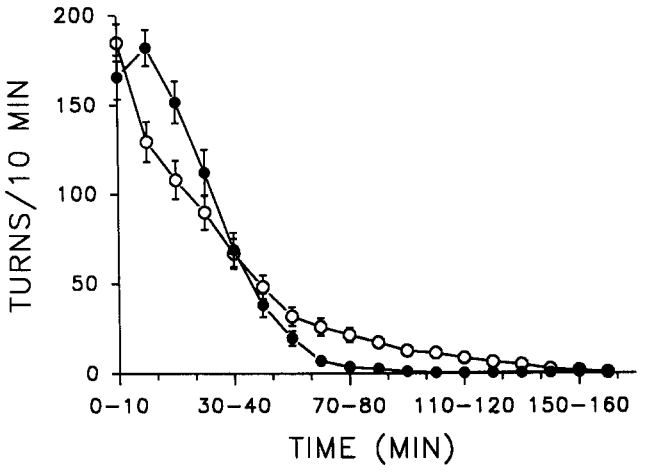


FIG. 1. Time-action profile of rotational behavior elicited by unilateral intranigral GABA (200 μg) or THIP (60 ng) infusion in control rats. Data were expressed as mean number of turns per 10-min block. (Time 0 = end of drug infusion.) Open circles, GABA (n = 33); closed circles, THIP (n = 21).

nation of 4 weeks of FZP treatment (Fig. 2), there was no significant effect, $F(3,11) = 0.53$, $p = 0.68$, of intranigral GABA in FZP-treated rats (n = 7) as compared to 4 week control rats (n = 8) (duration: treated, 132.0 ± 22.2 min; control, 108.5 ± 16.2 min). Nor were there significant differences, $F(3,12) = 0.50$, $p = 0.69$,

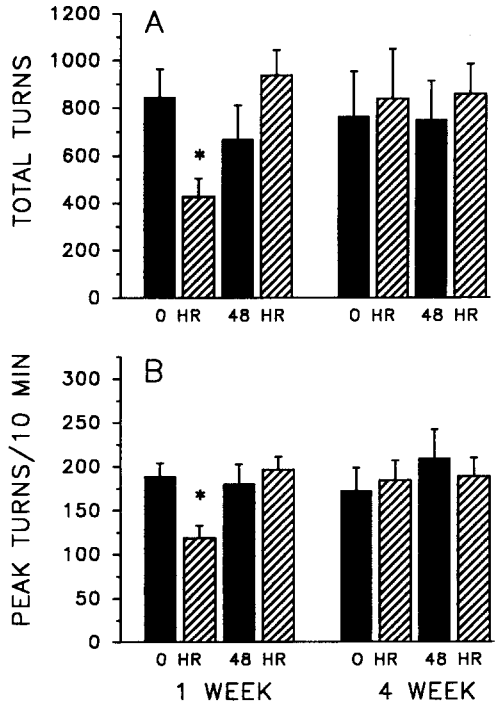


FIG. 2. (A) Total contralateral turns, and (B) peak turns/10 min elicited by unilateral intranigral infusion of 200 μg GABA, 0 or 48 h following 1 week or 4 weeks of FZP treatment. Filled bars, controls; striped bars, FZP-treated. *$p \leq 0.01$.

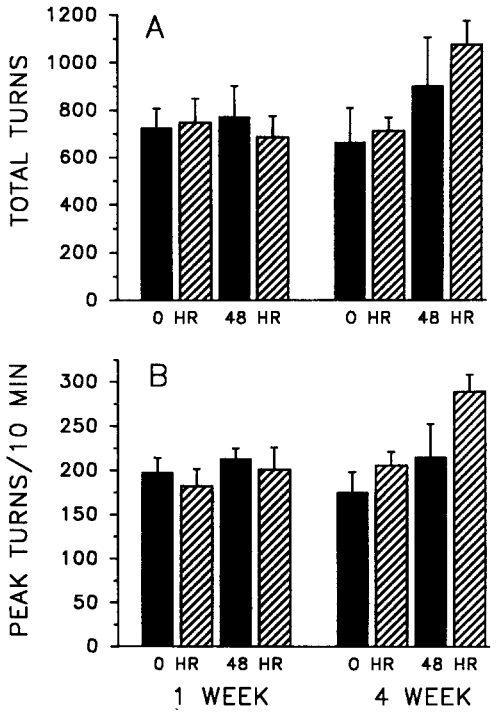


FIG. 3. (A) Total contralateral turns, and (B) peak turns/10 min elicited by unilateral intranigral infusion of 60 ng THIP, 0 or 48 h following 1 week or 4 weeks of FZP treatment. Filled bars, controls; striped bars, FZP-treated.

between treated (n = 12) and 4 week controls (n = 4) for any of the measures of rotational behavior 48 h after ending 4 week FZP treatment. Treated rats turned for 110.5 ± 10.3 min; control rats circled for 102.0 ± 19.1 min.

There was no significant effect of 1 week FZP treatment on THIP-induced rotation either 0 h [n = 6; $F(3,8) = 0.93$, $p = 0.47$] or 48 h [n = 5; $F(3,6) = 1.32$, $p = 0.35$] after ending treatment (Fig. 3), when compared to control rats (0 h, n = 6; 48 h, n = 5). One-week FZP-treated rats rotated for 78.7 ± 7.3 min (0 h) and 64.8 ± 3.1 min (48 h) after THIP microinjection. Control rats turned 68.7 ± 1.4 and 77.6 ± 5.4 min, respectively. When rats were tested with intranigral THIP immediately after 4 weeks of FZP treatment, there were no significant differences, $F(3,8) = 1.03$, $p = 0.43$, between treated rats (n = 6) and control rats (n = 6) for any parameter of rotation measured (Fig. 3). The duration of turning elicited by THIP at this time-point was 69.0 ± 6.1 min in treated rats and 91.7 ± 19.1 min in control rats. Likewise, there were no significant differences, $F(3,6) = 1.97$, $p = 0.22$, between treated (n = 6) and control (n = 4) rats for any measure of rotation elicited by THIP 48 h after 4 week FZP treatment (duration: treated, 79.3 ± 5.8 min; control 87.0 ± 9.5 min).

## DISCUSSION

When rats were tested after only 1 week of FZP treatment, there was subsensitivity to the action of intranigral GABA to induce rotational behavior. However, rats were no longer subsensitive to GABA after 4 weeks of FZP treatment. In a previous study in which muscimol-induced rotational behavior was studied (30), there was no effect of 1 week FZP treatment on muscimol-induced rotation, but subsensitivity to muscimol was evident af-

ter 4 week FZP treatment. These different results with GABA and muscimol suggest that more than one process determines the sensitivity of SNpr to different GABA agonists during chronic BZ treatment.

In contrast to the results with GABA, 1 week FZP treatment did not alter the ability of THIP to cause rotational behavior. Since THIP is not a substrate for the GABA uptake process (16), one possible explanation for the transient decrease in apparent sensitivity to GABA is that GABA uptake from the synaptic cleft may be increased after 1 week of FZP treatment. Previous results, which showed no change in the response to muscimol following only 1 week of FZP treatment (30), conform to the increased GABA uptake hypothesis since muscimol, like THIP, is not a substrate for the GABA uptake process (12). After 4 weeks of FZP treatment subsensitivity to GABA was no longer evident, in agreement with the previously reported finding that subsensitivity to GABA, microiontophoresed into SNpr, did not develop following prolonged (3 weeks) diazepam treatment (34). Extending treatment duration to 4 weeks may cause additional changes in the GABA system that mask the effects of increased GABA uptake. For example, $K^+$-evoked hippocampal [$^{14}$C]GABA release was reported to be increased by 3 weeks of daily diazepam injection (11). Alternatively, increased GABA uptake during chronic FZP treatment may be a transient phenomenon.

After 4 week FZP treatment, there was subsensitivity to rotation induced by intranigral muscimol (30), but not GABA or THIP. The different responses to these GABA agonists may be based on their actions at different receptor pools. Action at different populations of receptors is suggested by the fact that intranigral muscimol elicits behaviors not caused by GABA (2,3). Moreover, muscimol has more binding sites than GABA in rat brain (4). This surplus of muscimol binding sites may relate to receptors that are responsible for the unique actions of muscimol (2,3), and which may also mediate a portion of muscimol-, but not GABA-mediated rotational behavior.

The idea of pools of $GABA_A$ receptors that are differentially sensitive to various agonists is also supported by the observation that [$^3$H]THIP labels fewer high affinity $GABA_A$ sites than [$^3$H]GABA (5). THIP and isoguvacine, another $GABA_A$ agonist, have been proposed to be partial agonists/antagonists [cf. (15)]. Additional evidence for differences in effects between $GABA_A$ agonists includes the fact that GABA and muscimol stimulated BZ binding with much greater potency (300–1000-fold) than THIP and isoguvacine when assays were performed at nonphysiological temperatures (15,36). However, when enhancement of BZ binding affinity by $GABA_A$ agonists was measured at 37°C, THIP and isoguvacine had efficacies similar to GABA and muscimol (36). On the other hand, whereas GABA, muscimol and isoguvacine have similar efficacies to stimulate $Cl^-$ influx into mouse brain vesicles, THIP produces a smaller maximal effect and inhibits muscimol-stimulated uptake (1). The reduced efficacy of THIP, as compared with other $GABA_A$ agonists, to stimulate $Cl^-$ uptake and the lack of regulation of THIP-induced rotation in chronic FZP-treated rats may be related to its interaction with only a subset of the $GABA_A$ receptors (15). In contrast, isoguvacine, which binds to a similar receptor population as GABA (15), shows a reduced potency to inhibit CA1-evoked field potentials in in vitro hippocampal slices 48 h after 1 week FZP treatment (Xie and Tietz, unpublished observations). Nonuniform effects of chronic BZ treatment on the different receptor populations could explain the differences observed among the regulation of $GABA_A$ agonist effects.

Iontophoretic application of GABA in SNpr has been used previously to measure neuronal GABA sensitivity (34). Subsensitivity to GABA was not observed at various time points between 1 day and 11 weeks of diazepam treatment when this measurement was used. In contrast, subsensitivity is reported here to GABA-induced rotational behavior following 1 week of FZP treatment. These conflicting reports may be due to the different methods used to measure subsensitivity (extracellular single unit recording vs. rotational behavior). It is possible that subsensitivity was not observed after iontophoretic application of GABA because of the different BZ used for chronic treatment in that study (diazepam vs. FZP in this study). Also, the reduced effectiveness of GABA in SNpr is transient, so that it may not have been present at the particular time points studied in diazepam-treated rats (34).

The molecular basis for a diversity in responses to GABA agonists may be related to the recently demonstrated existence of multiple subtypes for the $\alpha$ and $\beta$ subunits of the $GABA_A$ receptor (6). The binding of muscimol, GABA, or other $GABA_A$ receptor ligands may be determined by the particular subunit isoforms present. The expression of these $GABA_A$ receptor subunit isoforms is regionally specific (17,35). Different isoforms may mediate different responses, and may not respond identically to chronic BZ treatment. Other $GABA_A$ receptor subunits may also play a role. Muscimol preferentially labels high-affinity $GABA_A$ sites which are colocalized with the $\delta$ subunit of the GABA receptor (28). The distribution of the $\delta$ subunit is distinct from that of the $\gamma_2$ subunit, which is responsible for BZ modulation of GABA function (24). The differential regulation of SNpr sensitivity to GABA agonists might be based on alterations in the expression of $GABA_A$ receptor subunit isoforms during chronic BZ treatment.

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