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FG7142 Causes Opposite Changes in [^{3}H]GABA Release From Nigrocollicular Regions

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PERIS, J. AND J. D. SCOTT. *FG7142 causes opposite changes in [^{3}H]GABA release from nigrocollicular regions.* PHARMACOL BIOCHEM BEHAV **44**(2) 333–338, 1993.—The activity of GABAergic neurons projecting from the striatum to the substantia nigra (SN) and from the SN to the superior colliculus (SC) may be involved in regulating seizure sensitivity such that striatonigral transmission is decreased and nigrocollicular transmission is increased in proconvulsant states. To test whether these changes occur in FG7142-treated rats, GABA transmission was assessed by measuring [^{3}H]GABA release from superfused slices of the SN and SC and measuring [^{35}S]TBPS binding to $GABA_A$ receptors throughout the brain. Nine daily injections of FG7142 (30 mg/kg IP) greatly increased myoclonic seizures in about one half of the animals. These animals exhibited a decrease in stimulated [^{3}H]GABA release from the SN and an increase in both basal and stimulated release from the SC. Animals that were less sensitive to FG7142 treatment also had increased collicular release but not decreased nigral release. [^{35}S]TBPS binding was unchanged by FG7142 treatment. Thus, decreased nigral GABA release may contribute to decreased striatonigral transmission after seizure occurrence whereas increased collicular GABA release may contribute to increased nigrocollicular transmission preceding multiple-seizure occurrence.

Seizures GABA FG7142 Substantia nigra Superior colliculus

REPEATED administration of the β-carboline FG7142 increases seizure susceptibility (5,15) but the exact mechanism of action for this sensitization is unknown. FG7142 is an inverse agonist at the benzodiazepine binding site on the $GABA_A$ receptor ionophore, thereby decreasing $GABA_A$ receptor binding and $GABA_A$-coupled Cl^- conductance (13,29,30). Repeated injection of high doses of FG7142 increases seizure sensitivity over long-term periods. A decrease in the number and functional activity of $GABA_A$ receptors in cortex and cerebellum (5,14) resulting in loss of GABA-mediated inhibition throughout the brain has been suggested as a mechanism for the increase in seizure occurrence caused by repeated FG7142.

Recently, changes in GABA activity in both the striatonigral and nigrocollicular pathways have been associated with increases in seizure sensitivity (9,18,23). Specifically, decreased GABAergic transmission in the striatonigral pathway increases seizure sensitivity (8) whereas increased activity of GABAergic neurons originating in the substantia nigra (SN) and terminating in the superior colliculus (SC) occurs after a variety of proconvulsants (1,7,9,11,27). Because these two inhibitory pathways are connected in series (Fig. 1), an increase in inhibitory striatal output would decrease nigral activity and inhibitory GABA output, thereby decreasing nigrocollicular GABA activity (4,12). As substantiated by the literature (8,9,23,28), these changes in transmission are associated with a decrease in seizures (Fig. 1A) whereas decreased striatonigral and increased nigrocollicular output increases seizures (Fig. 1B). The effects of repeated FG7142 injections on the GABAergic activity of the striatonigral and nigrocollicular pathways have not yet been measured.

The occurrence of specific neurochemical changes during periods of increased seizure sensitivity that would decrease striatonigral and/or increase nigrocollicular GABA transmission has been substantiated in a number of different proconvulsant models. Ethanol withdrawal decreases GABA release from slices of the SN and increases GABA release from slices of the SC (23), bicuculline kindling increases GAD activity in the SC (10), and seizure-sensitive animals exhibit increased $GABA_A$ receptor number in the striatum and SC but decreased number in the SN (17,23). Thus, it appears as if different proconvulsant states may result in different neurochemical changes in GABA neurons that all ultimately decrease striatonigral and/or increase nigrocollicular transmission. It is also certainly possible that these changes do not occur in all proconvulsant models (6,30).

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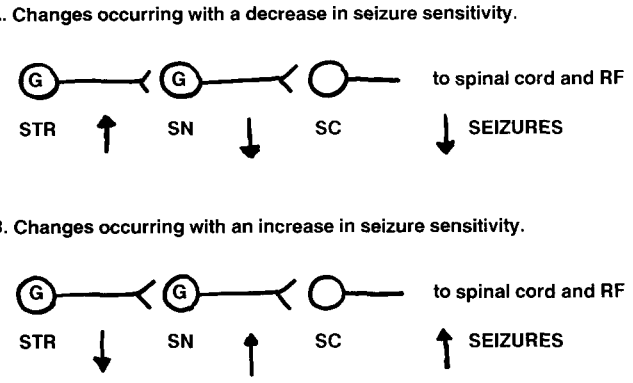

FIG. 1. Schematic diagram of the series of GABAergic neurons (G) originating in the striatum (STR) and terminating in the substantianigral (SN), originating in the SN and terminating in the superior colliculus (SC) and originating in the SC and terminating in the spinal cord and reticular formation (RF). Arrows indicate the relative neuronal activity associated with different states of seizure sensitivity.

We compared the effects of repeated FG7142 injection on indices of GABAergic transmission in rats that exhibited a high sensitivity to drug treatment, rats that were relatively insensitive to FG7142 treatment, and vehicle-injected control rats. To determine changes in presynaptic GABAergic transmission after FG7142 injections, [^{3}H]GABA release was measured from superfused slices of the SN and SC. Changes in binding parameters of postsynaptic $GABA_A$ receptors were assessed from [^{35}S]TBPS binding to brain sections using quantitative autoradiography (QAR). This ligand binds to the convulsant site, which is closely linked to the Cl channel of the $GABA_A$ receptor and has been used as an indicator of $GABA_A$ receptor number (25). Because it is possible that increases in nigrocollicular activity rely solely upon decreased GABAergic transmission in striatonigral pathways, terminal regions of both areas were studied in vitro to assess changes in one area independent of the proceeding pathway.

METHOD

Animals

Male Sprague-Dawley rats (85–175 g at the start of injections) from University of Florida Farms (Gainesville, FL) were used for these studies. Rats were housed under a 12 L : 12 D cycle and allowed rat chow and water ad lib.

Drug Treatment

Rats were injected with FG7142 IP at a dose of 30 mg/kg every day for 9 consecutive days (Group F). FG7142 was suspended in distilled water (30 mg/5 ml) containing one drop of Tween-80/5 ml. Control rats received a corresponding volume of water containing Tween-80 (Group V). Two additional groups of rats received only one injection of either 30 mg/kg FG7142 (Group F1) or vehicle (Group V1). Rats were observed for 90 min after each daily injection and the frequency and latency of each of the following behaviors was recorded: myoclonus, forelimb clonus, and loss of righting. The total number of seizures after nine injections was used to further divide Group F animals into two groups: Group F_{HI} consisted of animals exhibiting greater than 50 myoclonic seizures and/or a clonic seizure; Group F_{LO} consisted of rats exhibiting less than a total of 50 myoclonic seizures and never experiencing a clonic episode. Rats were killed by decapitation with a guillotine 4–7 days after the last injection; half the rats in each treatment group were used for release studies and half for binding studies.

[^{3}H]GABA Release From Superfused Rat Slices

Release experiments were performed as described previously (23). The SN and SC were dissected from each half of a rat brain, sliced with a Brinkman tissue chopper (Brinkman Instruments, Westbury, NY) (0.4 mm thick) and incubated for 30 min in Krebs' solution (35°C, pH = 7.4) saturated with 95% O_2/5% CO_2. The composition of the Krebs' buffer was (in mM): NaCl, 118; glucose, 11.1; $NaHCO_3$, 25; KCl, 4.7; NaH_2PO_4, 1.0; $MgCl_2$, 1.2; EDTA, 0.004; aminooxyacetic acid, 0.1. After replacing with fresh medium containing 32 nM [^{3}H]GABA (4-amino-*n*-2,3-^{3}*H*-butyric acid, 80 Ci/mmol; New England Nuclear, Newton, MA), the incubation was continued for 30 min. Rinsed, prelabeled slices were then placed into separate glass chambers maintained at 33°C and perfused with oxygenated Krebs' buffer at a rate of 1 ml/min. The superfusate was collected for 95 min at 5-min intervals beginning 60 min after the start of superfusion. After 20 min of collection, slices were exposed to electrical field stimulation (30 Hz, 8–30 mA, 2-ms duration) for 120 s. After superfusion, slices were solubilized and the radioactivity in both tissue and superfusate samples was determined by liquid scintillation counting.

The amount of tritium released in each fraction was expressed as a percentage of total tritium present in each slice at the time of the sample collection. The evoked tritium release occurring after stimulation was calculated from the sum of all fractions greater than spontaneous release occurring within 10 min before stimulus onset. Under these conditions, 93–96% of the tritium remaining in basal and stimulated samples from the SN and in homogenized slices of the SN or SC exists as [^{3}H]GABA (23). Slightly less of the tritium in basal and stimulated samples from the SC elutes as [^{3}H]GABA (86 and 89%, respectively), indicating that about 10% of the tritium released from these fractions is probably ^{3}H metabolites rather than [^{3}H]GABA (23). Each release assay contained at least two slices each from both the SN and SC of a single rat.

QAR of $GABA_A$ Receptor Binding

Specific binding of [^{35}S]TBPS was used to quantitate the convulsant binding site on the $GABA_A$ receptors of differently treated rats as described previously (25). Brains were frozen in powdered dry ice, stored at −80°C, and then sectioned sagittally (30 μm) at −20°C to induce the SN, SC and striatum (22). Duplicate sections were thaw mounted onto gelatin-coated slides and stored at −80°C. Indirect saturation analysis of receptors using unlabeled TBPS was used to determine differences in the affinity (K_d) or number (B_{max}) of binding sites for [^{35}S]TBPS (60 Ci/mmol, New England Nuclear). Slide-mounted sections were thawed, dried at room temperature, and then transferred to slide mailers containing tissue buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM D-glucose, 1 mM $CaCl_2$, and 10 mM HEPES adjusted to pH 7.5 with Tris base, 25°C) for 20 min to remove endogenous GABA. Sections were then incubated for 90 min in buffer (25°C) containing 10 nM [^{35}S]TBPS alone or with either 100 μM picrotoxin to define nonspecific binding or one of eight

concentrations (20–1,000 nM) of unlabeled TBPS (Research Biochemicals, Inc., Natick, MA). Sections were then rinsed for 5 min in ice-cold buffer, dipped in ice-cold distilled water, and dried on a hot plate at 50°C. The sections were apposed along with plastic ^{14}C standards (American Radiolabeled Chemicals) to X-ray film (Amersham Corp., Arlington Heights, IL) for 5 days at 25°C. There is a direct one-to-one relationship between the values of binding calculated using these standards and standards containing ^{35}S (19).

The illuminated image of each autoradiogram was collected by a Nikon camera and digitized using the Turnkey Imaging System (Imaging Research, Inc.). A Michaelis-Menton function was fit to the values generated from the standards and used to transform the digitized gray values collected from the samples into concentrations (fmoles/mm^2) of radioligand bound. The images were digitized, areas of interest were defined, and the binding in these areas was determined for each section. Data were then subjected to Scatchard analyses.

Data Analysis

All neurochemical measures were subjected to one-way analysis of variance (ANOVA) for between-groups factors followed by Neuman-Keuls posthoc analyses when necessary. Behavioral data were subjected to two-way ANOVA using one between-groups factor and one within-subjects factor.

RESULTS

When rats were injected daily with FG7142, an increase in the frequency of myoclonic seizures was seen starting on day 2 and continuing across injection days (Fig. 2A). There was no incidence of seizures in any animal after one injection of either FG7142 or after one or more injections of vehicle. Animals in Group F were divided into two groups as described above, resulting in n = 10 and 14 for Groups F_{HI} and F_{LO}, respectively. As defined, Group F_{HI} exhibited more seizures than did Group F_{LO} [group effect, $F(1, 22) = 18.3$, $p < 0.001$], which was due to a greater increase in seizures over days compared to Group F_{LO} [group × days interaction, $F(8, 176) = 2.3$, $p < 0.05$]. Of the three animals in Group F_{HI} exhibiting clonic seizures, two experienced these episodes after the sixth injection while the other experienced one after both the fifth and sixth injections. When the latencies to the first seizure behavior each day were measured (Fig. 2B), there was a decrease in latency to seizures over days in both groups [days effect, $F(8, 176)$ 19, $p < 0.001$]. However, Group F_{HI} had an overall shorter latency compared to Group F_{LO} [group effect, $F(1, 22) = 4.3$, $p < 0.05$]. Although it appeared as if this difference was greater after the last four injections, the groups × days interaction was not significant. The further division of rats into groups for subsequent measurement of release and receptors resulted in an n = 6, 5, and 7 for Groups V, F_{HI}, and F_{LO}, respectively, for both the release and binding assays. At no time during the injection period did rats differ in body weight due to FG7142 treatment.

The effects of FG7142 injections on basal [^{3}H]GABA release from the SN and SC are shown in Fig. 3. When rats were killed after one injection of either vehicle or FG7142, there was no difference due to drug treatment in basal release from either the SN (Fig. 3A) or the SC (Fig. 3B). Repeated FG7142 treatment increased basal release from slices of the SC (Fig. 3B) regardless of whether rats exhibited pronounced behavioral effects, $F(1, 16) = 4.84$, $p < 0.05$, but did not affect basal release from slices of the SN (Fig. 3A). There was an increase in basal release from the SN caused by repeated injection of either vehicle or FG7142 (Fig. 3A).

When slices of the SN (Fig. 4A) or the SC (Fig. 4B) were exposed to electrical stimulation, there was no difference in evoked release between rats killed after one vehicle or FG7142 injection. After repeated drug treatment, evoked tritium release was increased from the SC (Fig. 4B) in both Groups F_{HI} and F_{LO} compared to Group V, $F(1, 16) = 4.1$, $p < 0.05$. On the other hand, electrically stimulated tritium release was decreased in the SN of Group F_{HI} compared to Groups F_{LO} and V, $F(2, 15) = 4.7$, $p < 0.05$, with no difference between

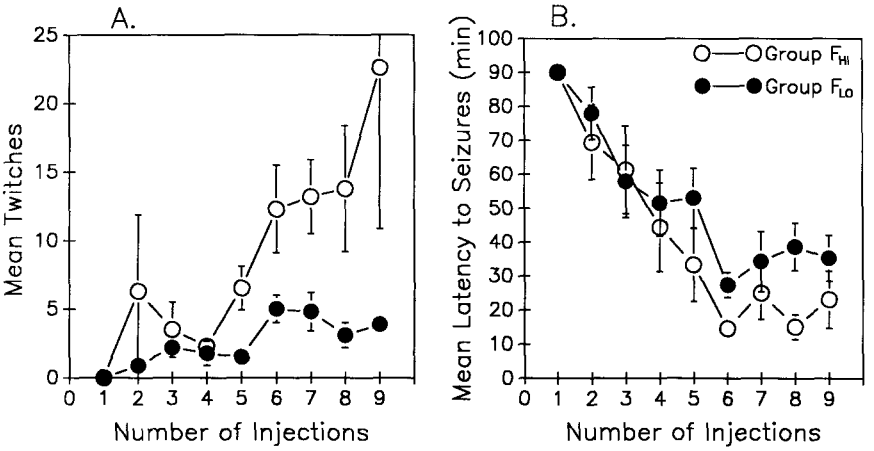

FIG. 2. A. Mean number of myoclonic seizures occurring after each daily injection of FG7142 (30 mg/kg, IP). B. Mean latency to the first seizure behavior occurring after daily FG7142 injection. In both figures, FG7142-treated animals were divided into Groups F_{HI} and F_{LO} according to the total number of myoclonic and clonic seizures experienced by each rat. n = 10 and 14 for Groups F_{HI} and F_{LO}, respectively. Vehicle-treated control animals (Group V) or animals receiving one injection of FG7142 (Group F1) or vehicle (Group V1) did not experience any seizures.

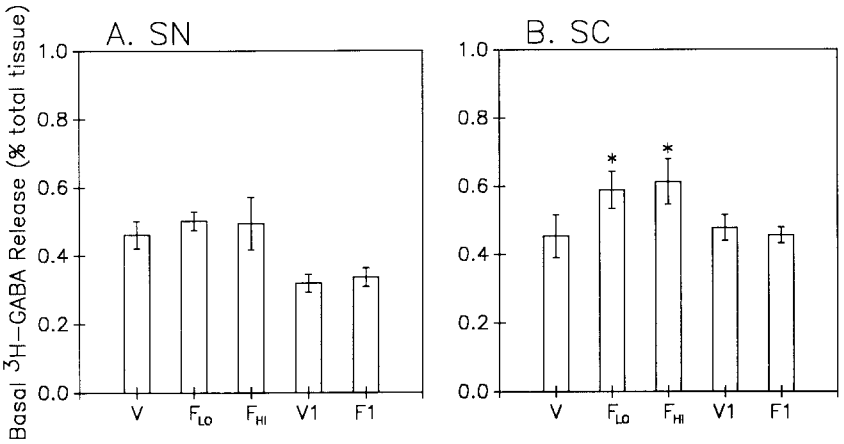

FIG. 3. Mean basal [^{3}H]GABA released from slices of the substantia nigra (SN) (A) and the superior colliculus (SC) (B) of vehicle-treated rats (V) and FG7142-injected rats that kindled (F_{HI}) or not (F_{LO}). Rats were divided into groups as described for Fig. 2. Also shown is basal release from rats receiving only one injection of FG7142 (Group F1) or vehicle (Group V1). In both graphs, tritium release was measured in a minimum of two slices from each rat. n = 6, 7, 5, 6, and 6 for Groups V, F_{HI}, F_{LO}, F1, and V1, respectively. *$p < 0.05$.

Groups V and F_{LO} (Fig. 4A). There was no difference between any of the groups in total tissue tritium measured in slices of the SN or SC. Total tissue tritium in the SN was equal to 440 ± 95, 437 ± 63, 461 ± 116, 348 ± 25, and 345 ± 19 Kdpms per slice and in the SC equal to 106 ± 43, 156 ± 30, 136 ± 64, 98 ± 12, and 111 ± 12 Kdpms per slice in Groups V, F_{LO}, F_{HI}, F1, and V1, respectively.

The effects of FG7142 on the number and affinity of [^{35}S]TBPS binding sites are listed in Table 1. The number of [^{35}S]TBPS receptors was greatest in inferior colliculus (IC), thalamus, superficial and deeper layers of the SC, and both the reticulata and compacta nuclei of the SN of all animals. There was no effect of treatment on B_{max} or K_d in any region. Although there was a trend for increased receptor number in striatum in Groups F_{HI} and F_{LO} vs. Group V, this difference was not significant, $F(2, 15) = 2.9, p = 0.7$.

DISCUSSION

Repeated administration of the benzodiazepine inverse agonist FG7142 resulted in a large increase in seizure occurrence in about 50% of animals after nine daily injections. The percentage of animals responding to FG7142 in our study was roughly similar to that found by others (5,16) even though different doses and schedules of administration were used. Unlike some of these previous reports, we observed relatively

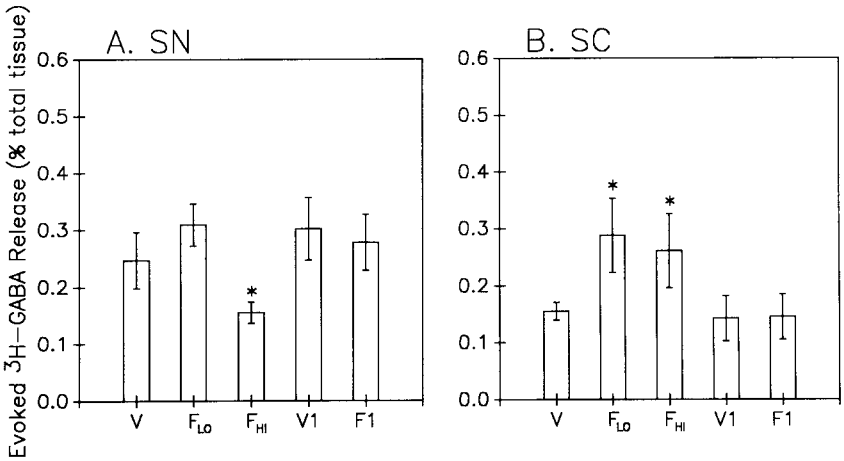

FIG. 4. Mean stimulated [^{3}H]GABA released from slices of the substantianigral (SN) (A) and the superior colliculus (SC) (B) in Groups V, F_{HI}, F_{LO}, V1, and F1. Rats were divided into groups as described for Fig. 2. Release was evoked by 120-s exposure to 30-Hz electrical pulses. In both graphs, tritium release was measured in a minimum of two slices from each rat. n = 6, 7, 5, 6, and 6 for Groups V, F_{HI}, F_{LO}, F1, and V1, respectively. *$p < 0.05$.

TABLE 1

NUMBER AND AFFINITY OF [^{35}S]TBPS BINDING SITES ON THE $GABA_A$ RECEPTORS OF RATS TREATED WITH FG7142

Region	B_{max}(pmol/mg protein)			K_d(nM) (all groups)
	Group F_{HI}	Group F_{LO}	Group V	
SN reticulata	6.0 ± 0.8	5.6 ± 0.9	5.9 ± 0.9	279 ± 13
SN compacta	5.9 ± 0.9	5.7 ± 0.9	5.2 ± 0.9	385 ± 44
SC (superficial)	5.4 ± 0.9	3.6 ± 0.9	5.2 ± 0.9	472 ± 17
SC (deep)	5.4 ± 0.6	6.0 ± 0.9	6.3 ± 0.8	325 ± 24
IC	5.7 ± 0.5	5.6 ± 0.9	5.9 ± 0.7	368 ± 23
Cerebellum	4.8 ± 0.3	5.1 ± 0.8	5.1 ± 0.8	557 ± 53
Cortex	5.0 ± 0.3	5.2 ± 0.4	4.6 ± 0.5	482 ± 18
Hippocampus	5.1 ± 0.5	5.4 ± 0.6	4.7 ± 0.8	547 ± 38
NDB	5.2 ± 0.9	5.6 ± 0.6	5.4 ± 0.8	363 ± 40
Thalamus	5.5 ± 0.7	5.6 ± 0.8	5.3 ± 0.5	358 ± 14
Striatum	5.2 ± 0.6	5.5 ± 0.9	4.2 ± 0.4	540 ± 53

Rats were divided into groups as described for Fig. 2. There was no significant difference between B_{max} or K_d values between groups. There was a significant effect of region on both B_{max}, $F(10, 150) = 1.93$, $p < 0.05$, and K_d, $F(10, 150) = 11.6$, $p < 0.001$. $n = 6$, 7, and 5 for Groups V, F_{HI}, and F_{LO}, respectively. NDB, nucleus of the diagonal band; IC, inferior colliculus.

few clonic episodes (3 of 24 rats) and these did not occur reliably even after later injections. The dosages used in the two previous studies were similar to that used at present; however, the dosing schedules differed in all three studies. Interestingly, the greatest number of clonic seizures was seen when drug was administered only twice weekly rather than daily. There is evidence that continuous administration of the same dose of FG7142 via osmotic minipumps is not proconvulsive (26). Thus, a widely intermittent schedule of administration may be an important determinant of the severity of the kindling effect of FG7142 and may explain the mildness of the seizures observed in the present study.

There was a concomitant decrease in the amount of [^{3}H]GABA released from the SN occurring in animals exhibiting a large number of seizures. The result of this neurochemical change would be to decrease nigral GABA transmission, which would effectively decrease inhibition of cell bodies in the SN. These data are consistent with evidence from the literature that nigral output is increased after proconvulsant treatments (9,23). Striatal GABA receptors were not significantly increased in Group F_{HI} of the present study, unlike changes previously found in this region of seizure-sensitive animals (23). On the other hand, GABA release was increased in the SC of all animals treated with FG7142 regardless of the number of seizures observed in those animals. This neurochemical change is in agreement with the hypothesis that nigrocollicular output is increased after proconvulsant treatments via a change in release (10,23) and not via a change in postsynaptic receptors in the SC, unlike that found previously (25). It is interesting to note that changes in the SC after FG7142 treatment were also found in Group F_{LO} instead of only in Group F_{HI}. Animals in Group F_{LO} did not exhibit large increases in seizure sensitivity to FG7142 but may have been more sensitive to other proconvulsant treatments. If these animals were more sensitive to seizures even though they did not exhibit many seizures under the conditions tested, this would indicate that the increased transmission in the SC may precede actual seizure occurrence while decreases in transmission in the SN may only occur during or after seizure episodes. It is necessary to test each of the three groups for sensitivity to other proconvulsant agents to determine whether animals in Groups F_{LO} and F_{HI} are in fact more sensitive to seizures than animals in Group V.

Unlike previous reports, FG7142 kindling had no effect on the density of $GABA_A$ receptors in the cortex (14) or the cerebellum (5). One possible explanation for these differences may be that both of the previous studies reported a greater incidence of clonic seizures than did the present study. In addition, the former study (14) used mice rather than rats. Another possibility for the discrepancy is the method of measurement of $GABA_A$ receptor number. Because the latter study used [^{3}H]GABA to label GABA receptors (5), it is possible that differences in the cerebellum or cortex might have been found using radiolabeled GABA or muscimol rather than TBPS. Even though these sites are presumed to coexist on the $GABA_A$ receptor complex, it is possible that drug treatment could cause dissociation of these sites. In addition, there is now substantial evidence that the protein subunit composition of the $GABA_A$ receptor is not homogeneous across brain regions (2,21) and this heterogeneity can result in differences in both receptor binding (3,20,24) and receptor function (24) across the brain. Therefore, not only is it likely that TBPS binding sites, GABA binding sites, and benzodiazepine binding sites are not always colocalized but the effect of inverse agonists on these different binding sites may also vary regionally. Thus, to determine completely how postsynaptic GABA responsiveness is affected by FG7142 in the SN or SC further experiments utilizing a variety of ligands should be performed. In addition, changes in receptor number should be confirmed using functional measurements of the $GABA_A$ receptor/chloride ionophore.

In summary, repeated FG7142 treatment decreased [^{3}H]GABA release from the SN and increase release from the SC. Animals that were relatively insensitive to FG7142 treatment also showed increased release from the SC but not decreased nigral release. Thus, decreased GABA release may contribute to a decrease in striatonigral transmission but only after treatments that increase seizure occurrence. On the other

hand, increased GABA release may contribute to increased nigrocollicular transmission and this change precedes a large increase in seizure occurrence. It is still unclear how FG7142 kindling induces these specific neurochemical changes in GABA transmission in these pathways. An action at the benzodiazepine receptor may be involved because concurrent administration of the benzodiazepine antagonist Ro 15-1788 completely prevents kindling (5). If so, similar treatment with Ro 15-1788 should abolish the changes in nigral and collicular release seen after FG7142 exposure.

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