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Enhanced Benzodiazepine Responsiveness in Rats With Increased Cholinergic Function

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PEPE, S., D. H. OVERSTREET AND A. D. CROCKER. *Enhanced benzodiazepine responsiveness in rats with increased cholinergic function.* PHARMACOL BIOCHEM BEHAV 31(1) 15–19, 1988.—The effects of diazepam and muscimol on locomotor activity were examined in Flinders Sensitive Line (FSL) rats, derived by selective breeding methods from randomly bred Sprague-Dawley (RB) rats for increased behavioural and physiological sensitivity to the anticholinesterase, diisopropylfluorophosphate (DFP). Previous reports of increased behavioural sensitivity to oxotremorine, associated with increased striatal and hippocampal muscarinic receptor concentrations, were confirmed in FSL rats compared to RB rats. The FSL rats were more sensitive to the locomotor suppressant effects of diazepam and muscimol compared to RB. Binding experiments with [^{3}H]-diazepam showed that FSL rats had an increased benzodiazepine receptor concentration in the striatum and hippocampus compared to Flinders Resistant Line rats (FRL). FRL rats did not differ significantly from RB in diazepam-induced changes in locomotor activity or the concentration of benzodiazepine receptors. No significant differences in the affinity of benzodiazepine receptors was detected between the three rat lines in the brain regions investigated. Thus FSL rats showed an increased behavioural sensitivity to both diazepam and muscimol which was associated with a greater concentration of benzodiazepine receptors in the striatum and hippocampus compared to RB and FRL rats.

Flinders Line Rats Muscarinic receptors Diazepam receptors Striatum Hippocampus Diisopropylfluorophosphate

THE Flinders Sensitive Line (FSL) and Flinders Resistant Line (FRL) rats, derived from randomly bred Sprague-Dawley rats (RB) by selective breeding methods, differ in their behavioural and physiological sensitivity to the anticholinesterase, diisopropylfluorophosphate (DFP) (12). FSL rats are not only more sensitive to the effects of DFP, but also to the effects of direct and indirect cholinergic agonists, compared to FRL and RB rats. However FRL rats did not differ in sensitivity from RB rats (11). The increased sensitivity to anticholinesterases and cholinergic agonists in FSL rats has been shown to be unrelated to changes in the enzyme acetylcholinesterase (12). More recently it was shown that this increased behavioural sensitivity to muscarinic agonists was associated with an increased concentration of muscarinic acetylcholine receptors (mAchR) in the striatum and hippocampus of the FSL compared to FRL and RB rats. However, experiments failed to show any changes in acetylcholine synthesis or turnover in these regions (13).

The greatest concentration of acetylcholine in the brain has been shown to be present in striatal interneurons (1) which form synapses with gamma-amino-butyric acid (GABA) interneurons and GABA striatal output neurons (2). We predicted that since FSL rats had an increased concentration of striatal mAchR, some of which are located on GABA neuron cell bodies, their striatal GABA system functioning may also be altered. In addition, since considerable evidence exists that benzodiazepines facilitate GABA binding [16] by binding to a benzodiazepine receptor which is part of a macromolecular membrane complex containing the $GABA_A$ receptor [10], we proposed that FSL rats may also show differences in their responsiveness to benzodiazepines which may be associated with benzodiazepine receptor changes.

Thus the present study investigated the possibility that genetically selected differences to the cholinergic system of FSL rats may also be associated with modifications to the

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GABA/benzodiazepine system. This hypothesis was tested using both behavioural and neurochemical assessments of benzodiazepine receptor changes in the rat lines.

METHOD

Animals

Male rats (250–400 g) from the 29th and 30th generations of the Flinders lines and RB rats were caged in groups of 10, containing equal numbers of FSL and FRL or RB rats, and were maintained at a constant temperature (22°C), relative humidity (35%) and 24 hour continuous light to reduce circadian variations in acetylcholine [3]. Food and water was freely available to all animals. All animal handling occurred between 0730 and 1200 hours.

Drugs

Muscimol was synthesized by Dr. Bruno Kasum (Chemical Research, School of Physical Sciences, The Flinders University of S.A.), using a short 3 step 30% yield sequence method [5]. Muscimol, oxotremorine solution (Aldrich), atropine methylnitrate (Sigma) and diazepam solution (David Bull) were diluted in 0.9% saline and injected subcutaneously in a volume of 1.0 ml/kg.

Locomotor Activity

Rats were injected with oxotremorine (0.15 and 0.3 μmoles/kg), 15 minutes prior to locomotor activity measurement; 15 minutes preceding oxotremorine injection, animals received atropine methylnitrate (2 mg/kg) to minimize peripheral effects of oxotremorine. Diazepam (2.25, 2.7, 3.15, 3.6, 4.05, 4.5, 5.4, 6.3, 7.2 μmoles/kg) and muscimol (4.4, 8.8, 21.9, 26.3, 30.7, 35.1 μmoles/kg) were injected 15 and 25 minutes respectively before locomotor activity measurement. Activity assessment took place under dim red light in perspex chambers (60×30×30 cm) which had 10×10 cm grid markings on the floors. Each rat was placed in the centre of a chamber and after a 1 minute habituation period the number of lines crossed per minute was recorded. Locomotor activity was expressed as the mean percentage of the activity level after vehicle treatment (baseline) for each of the rat lines.

Measurement of Muscarinic and Benzodiazepine Receptors

Animals were sacrificed by cervical dislocation and decapitated. Brains were rapidly removed and the frontal cortex, striatum and hippocampus from each were dissected over ice. These brain regions were then homogenized (30 seconds, setting 3) with a Polytron (Kinematica) in 10 volumes of Tris-HCl buffer (0.05 M, pH 7.4). The homogenates were then stored at −80°C and used in receptor binding assays within 7–14 days.

For muscarinic receptor binding, tissue homogenates were thawed and diluted in Na-K Phosphate buffer (0.05 M, pH 7.4) such that 250 μl aliquots contained 0.5 mg wet weight of tissue. Each aliquot was added to a tube containing varying concentrations (0.05–1.0 nM) of [^{3}H]-quinuclidinylbenzilate ([^{3}H]-QNB, 35 μCi/mmol, Amersham) and Na-K phosphate buffer in a total volume of 2.5 ml. Tubes were vortexed and incubated at 37°C for 15 minutes. Nonspecific binding was determined in the presence of 1.0 μM atropine.

[^{3}H]-Diazepam (1.0 nM; 85 μCi/mmol, Amersham) was incubated with 100 μl of homogenate (0.5 mg wet weight of tissue/tube) in Tris-HCl buffer (0.05 M, pH 7.4), in a total volume of 0.5 ml and displaced by varying concentrations of unlabelled diazepam (10^{-11} M–10^{-3} M). Nonspecific binding was defined using 1.0 μM of unlabelled diazepam. The assay tubes were incubated for 60 minutes in ice on a shaking platform.

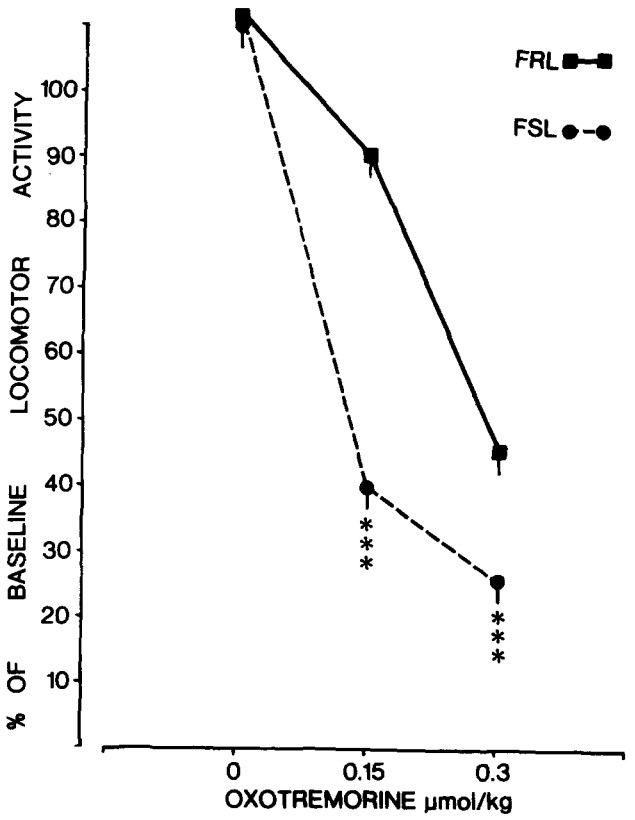

FIG. 1. The effect of oxotremorine on locomotor activity 15 minutes after subcutaneous administration to FRL and FSL rats. Each point (N=8) is the mean percentage (±S.E.M.) of the activity level after vehicle treatment (baseline) for each of the two rat lines. ***$p<0.001$ significantly different from FRL (Student's t-test).

The incubation reactions were terminated by the addition of ice cold phosphate buffer (for muscarinic receptor assay) or Tris buffer, pH 7.7 (for benzodiazepine receptor assay) and rapid vacuum filtration through buffer presoaked GF/B glass fibre filters (Whatman). The filters were then washed with 3×2.5 ml buffer, transferred to scintillation vials and 4 ml of scintillant (4 g 2,5-diphenyloxazole, 750 ml toluene, 350 ml triton X100) was added. Radioactivity was allowed to elute from the filters for 3 hours and then counted by liquid scintillation spectrometry on a Beckman LS3801 liquid scintillation counter.

An iterative, extended least squares algorithmic nonlinear curve fitting programme, MK MODEL Version 3.03 (4), was used to analyze the binding data to estimate the concentration (B_{max}) and affinity (K_d) of muscarinic and benzodiazepine receptors.

Protein concentrations of each of the tissue samples were measured by the Lowry assay method (6), using bovine serum albumin as the standard. Values for B_{max} were expressed as pmol/g protein.

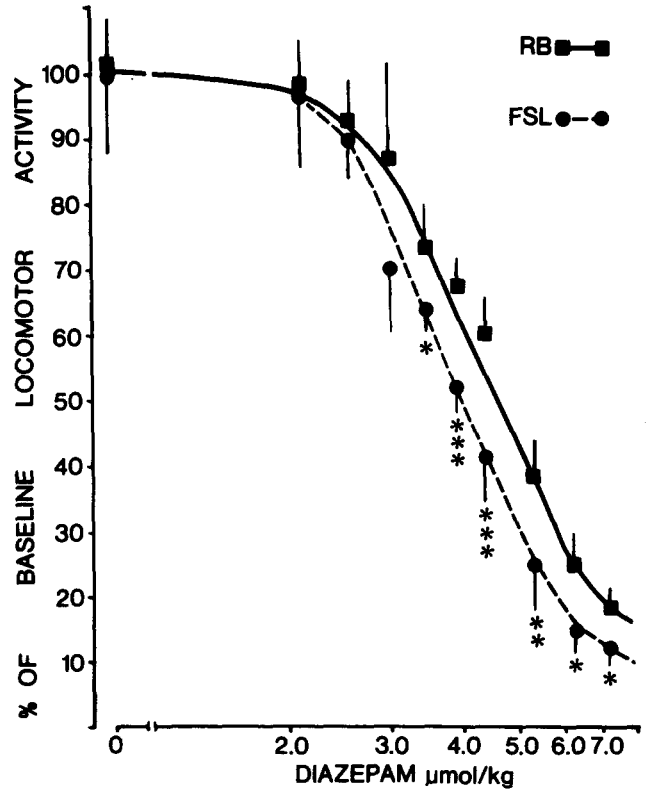

FIG. 2. The dose-dependent decrease on locomotor activity by diazepam 15 minutes after subcutaneous injection to RB and FSL rats. Each point (N=5) is the mean percentage (±S.E.M.) of the activity level after vehicle treatment (baseline) for each of the two rat lines. *$p<0.05$, **$p<0.01$, ***$p<0.001$, significantly different from RB rats (Student's t-test).

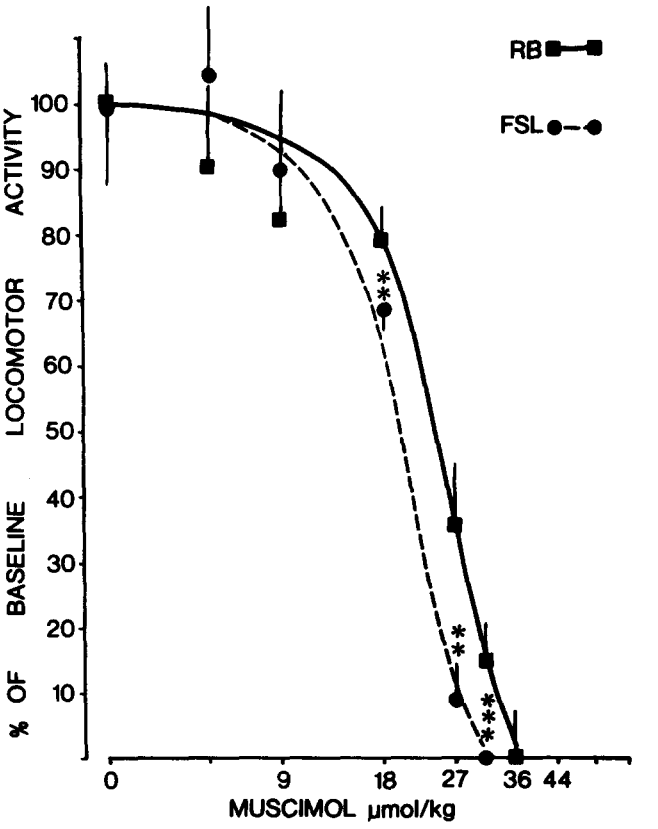

FIG. 3. The dose-dependent decrease on locomotor activity by muscimol 20 minutes after subcutaneous injection to RB and FSL rats. Each point (N=5) is the mean percentage (±S.E.M.) of the activity level after vehicle treatment (baseline) for each of the two rat lines. **$p<0.01$, ***$p<0.001$, significantly different from RB rats (Student's t-test).

Statistical Analyses

Locomotor activity differences between the two strains were analyzed using Student's t-tests for each dose of oxotremorine, diazepam and muscimol tests. Receptor binding data were analysed by two-way ANOVAs and further analysis of individual comparisons was carried out with Scheffe's multiple contrast tests.

RESULTS

In the behavioural experiments locomotor activity was expressed as the mean percentage of the activity level after vehicle treatment (baseline) for each of the rat lines because there was an initial baseline difference in activity of FSL compared to FRL and RB rats. Oxotremorine (0.15 and 0.3 µmoles/kg) dose-dependently decreased locomotor activity in FRL rats, but a significantly greater decrease in activity ($p<0.001$) occurred in FSL rats (Fig. 1).

Diazepam produced a dose dependent decrease in locomotor activity in RB rats (Fig. 2). This effect was significantly greater in FSL rats for doses of diazepam larger than 2.7 µmoles/kg ($p<0.05$). A similar dose-dependent decrease in the activity of RB rats was seen after the administration of muscimol (Fig. 3). Muscimol, a $GABA_A$ receptor agonist, produced significantly greater locomotor depression in FSL rats compared to RB rats between doses of 8.8 µmoles/kg and 30.7 µmoles/kg ($p<0.05$). Although sedation occurred in both FSL and RB rats at doses greater than 60 µmoles/kg of muscimol, activity was abolished at a lower dose (26.3 µmoles/kg) in the FSL than in the RB group (35.1 µmoles/kg).

Results of the [^{3}H]-QNB binding assays (Table 1) showed significant line differences, $F(2,99)=33.1$, $p<0.001$, with an increased concentration of mAchR in the striatum and hippocampus but not frontal cortex, of FSL compared to FRL ($p<0.01$) and RB ($p<0.01$) rats, confirming earlier findings (13).

Line differences were also observed in the [^{3}H]-diazepam binding assays, $F(2,135)=28.1$, $p<0.001$. An increased concentration of benzodiazepine receptors was detected in the striatum and hippocampus of FSL compared to FRL ($p<0.01$) and RB rats ($p<0.01$), however no differences in B_{max} were seen in the frontal cortex between the lines. There was no significant difference in B_{max} between FRL and RB rats, while the K_d did not differ significantly between the 3 groups (Table 2).

DISCUSSION

The results of this study confirm the previous findings of Overstreet and Russell (11) that FSL rats have an increased behavioural sensitivity to mAchR agonists, compared to

TABLE 1

CONCENTRATION (B_{max}) OF MUSCARINIC RECEPTORS AS DETERMINED BY [^{3}H]-QNB SATURATION ASSAY

Rats	N	Striatum	Hippocampus	Cortex
FSL	12	1621.4 ± 23*†	1317.4 ± 25*†	1468.2 ± 0.01
FRL	12	1352.8 ± 20	1145.0 ± 21	1445.4 ± 0.09
RB	12	1295.0 ± 31	1114.0 ± 34	1400.8 ± 0.05

*$p<0.01$, compared to FRL; †$p<0.01$, compared to RB (Scheffe's multiple contrast test). B_{max}=pmoles/g protein. All results expressed as means ± s.e.m. N=number of rats in each group. K_d ranged from 0.071 ± 0.01 to 0.16 ± 0.15 nM.

TABLE 2

CONCENTRATION (B_{max}) OF BENZODIAZEPINE RECEPTORS AS DETERMINED BY [^{3}H]-DIAZEPAM DISPLACEMENT ASSAY

Rats	N	Striatum	Hippocampus	Cortex
FSL	16	428.1 ± 16*†	910.2 ± 22.6*†	1101.5 ± 29
FRL	16	336.3 ± 26	740.2 ± 14.3	1066.2 ± 36
RB	16	370.6 ± 21	762.9 ± 25	1018.0 ± 21

*$p<0.001$, compared to FRL; †$p<0.001$, compared to RB (Scheffe's multiple contrast test). B_{max}=pmoles/g protein. All results expressed as means ± s.e.m. N=number of rats in each group. K_d ranged from 3.97 ± 0.3 to 5.4 ± 0.8 nM.

FRL rats and this is associated with an increased concentration of mAchR in the striatum and hippocampus (13).

It has been suggested (2), on the basis of histochemical studies (18), that striatal cholinergic interneurons do not synapse directly onto GABA striatonigral neurons, but on GABA interneurons shown to exist by McGeer and McGeer (9). These subsequently synapse with striatal efferents to the substantia nigra (2). Accordingly, we predicted that FSL rats, with genetically determined increased cholinergic function, would also have altered GABA/benzodiazepine function in the striatum. This was confirmed by our findings that FSL rats exhibited a greater decrease in locomotor activity in response to both the benzodiazepine agonist, diazepam and the $GABA_A$ agonist, muscimol (Figs. 2 and 3), compared to RB rats. Locomotor suppressant effects of muscimol have also been reported following its injection into the ventral striatum in rats [17] suggesting that the effects we observed may be due to changes in this area. This conclusion was supported by the demonstration that the increased behavioural sensitivity to diazepam was associated with an increased concentration of [^{3}H]-diazepam binding sites in the striatum of FSL compared to FRL rats (Table 2). An increased concentration was also found in the hippocampus but the functional significance of this is unknown.

Findings from studies of GABA/benzodiazepine function following chronic treatment with DFP, to change cholinergic function, are relevant to the present study. After chronic and acute treatment with DFP in rats there was a significant decrease in the number of mAchR (14,15), which was shown to be associated with an increase in $GABA_A$ receptor concentration in the striatum (15). Further, it was shown that DFP (10^{-4} M), in vitro, did not affect radioligand binding to muscarinic and GABA receptors. It was suggested that the increased concentration of GABA receptors may be a compensation for the anticholinesterase-induced cholinergic overactivity (15). Similarly, the increased cholinergic function in FSL rats may be compensated for by the observed changes in GABA/benzodiazepine function.

Other studies have investigated whether all the effects of DFP are due to its action in the cholinergic system or are the result of changes in GABA/benzodiazepine function. It has been shown that organophosphate induced convulsions can be completely blocked by low concentrations of benzodiazepines acting via GABAergic mechanisms, while atropine had no effect (8). In this same study it was demonstrated that aminooxyacetic acid, a GABA-transaminase inhibitor, also inhibited organophosphate-induced convulsions. Although there is also considerable evidence that chlorinated hydrocarbon insecticides such as dichlorodiphenyltrichloroethane and pyrethroids such as deltamethrin act directly at various binding sites on the GABA receptor ionophore complex (7), DFP does not act at this site (15). Overall, however, it would seem that changes in cholinergic function do affect GABA/benzodiazepine function.

In conclusion, the results of the present study are in agreement with these findings, showing the changes to the GABA/benzodiazepine system were associated with the changes in the cholinergic system seen in FSL rats. We are currently investigating GABA receptor concentrations and the effect of GABA agonist and antagonist injections, in various brain regions of Flinders line and RB rats to confirm this change in the GABA/benzodiazepine system. It is yet to be determined whether FSL rats have altered concentrations of GABA/benzodiazepine receptors as a result of genetic selection, or because of compensatory changes in GABA metabolism as a consequence of their increased cholinergic activity. FSL rats are unique in having genetically determined increase in striatal mAchR concentration and thus provide an excellent model in which to investigate these interactions further.

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REFERENCES

1. Cheney, D.; LeFevre, H.; Racagni, G. Choline acetyltransferase activity and mass fragmentographic measurement of acetylcholine in specific nuclei and tracts of rat brain. Neuropharmacology 14:801–809; 1975.
2. Gale, K. Neurotransmitter interactions in the basal ganglia: GABAology versus DADAism. In: Hanin, I., ed. Dynamics of neurotransmitter function. New York: Raven Press; 1984:189–209.
3. Hanin, I.; Masarelli, R.; Costa, E. Acetylcholine concentrations in rat brain: diurnal oscillation. Science 170: 341–342; 1970.
4. Holford, N. MK MODEL; A modelling tool for microcomputers. Clin. Exp. Pharmacol. 12(Suppl.):179; 1984.
5. Jager, V. and Frey, M. A short synthesis of muscimol. Liebigs Ann. Chem. 1982:817–820; 1982.
6. Lowrey, O. H.; Rosebrough, H. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Pholin reagent. J. Biol. Chem. 193:265–275; 1951.
7. Lummis, S. C. R.; Chow; S. C.; Holan, G.; Johnston, G. A. R. Gammaaminobutyric receptor ionophore complexes: Differential effects of deltamethrin, dichlorodiphenyltrichloroethane and some novel insecticides in a rat brain membrane preparation. J. Neurochem. 48:689–694; 1987.
8. Lundy, R. M.; Magor, G. F.; Shaw, R. K. GABA metabolism in different areas of the rat brain at the onset of soman induced convulsions. Arch. Int. Pharmacodyn. Ther. 234:64–73; 1978.
9. McGeer, P. L.; McGeer, E. G. Evidence for glutamic acid decarboxylase-containing interneurons in the neostriatum. Brain Res. 91:331–335; 1975.
10. Olsen, R. Drug interactions at the GABA receptor ionophore complex. Annu. Rev. Pharmacol. Toxicol. 22:245–277; 1982.
11. Overstreet, D. H.; Russell, R. W. Selective breeding for sensitivity to DFP: Effects of cholinergic agonists and antagonists. Psychopharmacology (Berlin) 78:150–154; 1982.
12. Overstreet, D. H.; Russell, R. W.; Helps, S. C.; Messenger, M. Selective breeding for sensitivity to the anticholinesterase, DFP. Psychopharmacology (Berlin) 65:15–20; 1979.
13. Overstreet, D. H.; Russell, R. W.; Crocker, A. D.; Schiller, G. D. Selective breeding for differences in cholinergic function: Pre- and postsynaptic mechanisms involved in sensitivity to the anticholinesterase, DFP. Brain Res. 294:227–232; 1984.
14. Schiller, G. D. Reduced binding of [^{3}H]-QNB associated with chronically low acetylcholinesterase activity. Life Sci. 24:1149–1154; 1979.
15. Sivam, S.; Norris, J.; Lim, D.; Hoskins, B.; Ho, I. Effect of acute and chronic cholinesterase inhibition with DFP on muscarinic dopamine and GABA receptors of the rat striatum. J. Neurochem. 44:1414–1422; 1983.
16. Tallman, J.; Paul, S.; Skolnick, P.; Gallager, D. Receptors for the age of anxiety: Pharmacology of benzodiazepines. Science 207:274–280; 1980.
17. Turski, L.; Havemann, U.; Kuschinsky, K. GABAergic mechanisms in mediating muscular rigidity, catalepsy and postural asymmetry in rats: Differences between dorsal and ventral striatum. Brain Res. 322:49–57; 1984.
18. Woolf, N. J.; Butcher, L. L. Acetylcholinesterase-containing projection from the basal forebrain to the substantia nigra in the rat. Soc. Neurosci. Abstr. 264.8:850; 1981.