Septal deafferentation increases hippocampal adrenergic receptors: Correlation with sympathetic axon sprouting

(septo–hippocampal system/radioligand binding)

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ABSTRACT   Denervation of the hippocampal formation in adult rats through lesion of the medial septum and diagonal band or by transection of the fimbria/fornix elicits an increase in the number of putative α-adrenergic receptor binding sites labeled by the antagonist ligand [³H]WB4101 [2-(2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4-benzodioxane]. This increase in [³H]WB4101 binding is observable at 6 days postlesion, preceding the ingrowth of sympathetic axons into the partially denervated regions of the hippocampus. The receptor up-regulation is specific for lesions of the septal (primarily cholinergic) innervation of the hippocampus. Damage to noradrenergic, dopaminergic, or serotonergic afferents as well as kainate injections in the lateral septum had no effect on [³H]WB4101 binding levels. In vivo muscarinic/cholinergic-receptor blockade does not mimic the effects of the lesion on receptor binding levels or upon axonal sprouting of the sympathetic neurons. Although [³H]WB4101 binding consistently increased after septal deafferentation, there was no clear-cut effect upon the other adrenergic ligands, including [³H]prazosin, [³H]-yohimbine, P-[³H]aminoclonidine, or [³H]dihydroalprenolol. These observations can be interpreted as demonstrating a unique and selective adrenergic receptor increase after a nonadrenergic denervation but accompanying the ingrowth of anomalous adrenergic fibers. We suggest several possible relationships between the new binding sites and the ingrowing axons.

The ability of neurotransmitter receptors to regulate in response to the level of their activation has been documented in several monoaminergic and cholinergic systems (1). Paradoxically, in the hippocampus, lesion of the septal afferents, which are primarily cholinergic, does not alter the binding of either [³H]quinuclidinyl benzilate (QNB) or α-[³H]bungarotoxin, putative muscarinic and nicotinic receptor-antagonist ligands (refs. 2, 3, and 4, but see ref. 5, which reports an increase in [³H]-QNB binding, restricted to the dorsal hippocampus, after septal destruction). Furthermore, damage to the locus coeruleus or the noradrenergic dorsal bundle fails to change the binding capacity of [³H]WB4101 in the hippocampus (6–8). Because the hippocampus is renowned for its structural and functional plasticity, these exceptions to the widely acknowledged concept of receptor adaptation are perplexing. Lesion of the septal afferents to the hippocampus not only removes its major cholinergic input but also elicits an ingrowth of sympathetic axons into the partially denervated regions of the hippocampus (9–11). The functional consequences of this anomalous innervation are not yet known; however, the terminals form synaptic junctions and demonstrate a capacity for high-affinity uptake and storage of norepinephrine (12, 13). The present study was designed to examine the relationship of this anomalous afferent input to the adrenergic receptor population in the hippocampus

and to determine if a functionally appropriate receptor population would arise or, conversely, if a compensatory decrease in adrenergic receptors would be observed, as reported in the hyperinnervated cerebellum (14). We find that one specific population of adrenergic binding sites increase in number after septal damage, that this increase precedes the arrival of the anomalous sympathetic axons, and that the increase in adrenergic receptor sites is related to selective damage of nonadrenergic afferents to the hippocampus.

METHODS

Surgeries. Adult female Sprague–Dawley rats (Simonsen Laboratories, Gilroy, CA; Charles River Breeding Laboratories) were used for all the studies described. Transection of the fimbria/fornix unilaterally ($n = 34$) or bilaterally ($n = 15$) was done by stereotaxically lowering a blade 6.0 mm ventral from the dura at 1.0 mm posterior to bregma. The blade was moved 5.0 mm laterally from the midline. Thirty rats were administered radiofrequency lesions of the medial septal and diagonal band nuclei by using the following stereotaxic coordinates: (*i*) vertical limb of the diagonal band (incisor bar at −1.0 mm; AP, +0.8; ML, +1.5; and DV, −8.1 from dura); (*ii*) horizontal limb of the diagonal band (incisor bar, −1.0; AP, +2.0; ML, +1.5; and DV, −8.9); (*iii*) medial septal nucleus (incisor bar at 0.0; AP, 1.2; ML, +0.6; and DV, −5.0 and −7.0 with the electrode angled 10° toward the midline). The electrode (1 mm) was heated to 60°C for 10 sec at each position.

To control for the effect of interrupting monoaminergic afferents, five rats received injections of 6.0 µg of 6-hydroxydopamine HBr (Regis) into the fimbria. Similar doses have been shown to produce >90% depletion of norepinephrine in the hippocampus when injected into the dorsal noradrenergic bundle (8). A total volume of 0.2 µl of 0.9% NaCl/0.2% ascorbic acid was injected at the following stereotaxic coordinates: AP, +10; ML, 0.2; and DV, −5.5 with a needle angled at 10° to the midline. Selective lesions of the lateral septum, a target for hippocampal efferent fibers (15), were accomplished by injecting 0.2 µg of kainic acid (Sigma) in 0.2 µl 0.9% NaCl 1.0 mm lateral of the medial septal coordinates. The ascending serotonergic fibers were destroyed by radiofrequency lesion at the following coordinates: incisor bar, 0.0; AP, 1.0 mm; ML, +0.8 mm; and D.V., −6.0 with the electrode angled 10° toward the midline, (60°C for 5 sec). This procedure resulted in a 40 ± 5% depletion of serotonin in the hippocampus over sham-operated controls. The radiofrequency lesion of the septum and diagonal band produced an identical serotonin depletion in the hippocampus.

Abbreviations: WB4101, 2-(2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4-benzodioxane; QNB, quinuclidinyl benzilate.
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Serotonin was assayed by the spectrofluorometric method of Curzon and Green (16). Sham-operated rats ($n = 60$) were matched to the above groups, operated the same day, and sacrificed at the same time; all assays were run in parallel.

In Vivo **Drug Treatment.** The potent muscarinic/cholinergic antagonist, scopolamine hydrochloride (10 mg/kg per day) was administered subcutaneously to 15 rats by means of Alzet osmotic minipumps. Control rats were implanted with a size-matched silastic pellet containing saline. The implants were removed and replaced after 2 wk for a total treatment of 28 days. The stability of scopolamine in the pump at 37°C was checked once every week by competition of [^{3}H]QNB binding with the eluate.

Histology. After survival times ranging from 6 to 33 days, rats were decapitated, and the brains were placed rapidly in chilled saline. The forebrain region anterior to the hippocampus was reserved in 10% Formalin and sectioned coronally at 40 μm on a freezing microtome. Alternate sections were stained for acetylcholinesterase according to Koelle (17) or with cresyl violet for lesion verification. The extent of each lesion was confirmed prior to receptor assay both by lesion reconstruction and the extent of cholinesterase depletion in the fimbria. The medial septal and diagonal band ablation left a small group of cells in the anterior diagonal band intact, but cholinesterase was depleted in the fimbria. The 6-hydroxydopamine injections produced no obvious cell loss in the septum or hippocampus. Cell losses after kainic acid injection were confined to areas adjacent to the needle tract in the dorsal aspect of the lateral septal nucleus.

Radioligand Binding. All radioligands were purchased from New England Nuclear at the highest specific activity available, excepting [^{3}H]etorphine, which was obtained from Amersham. Individual hippocampi were stored at −70°C prior to assay. Crude membrane preparations were prepared by homogenization (speed 7, Tekmar Tissuemizer) for 8 sec at 5 mg/ml in cold 50 mM Tris HCl (pH 8.2) and were centrifuged twice at 48,000 × g, with resuspension in fresh buffer. Duplicate or triplicate tubes contained 5 mg of tissue in 800 μl, 100 μl of "blank" drug to determine nonspecific binding or of vehicle, and 100 μl of radioligand. After incubation at 25 or 37°C to equilibrium, the tissue was filtered rapidly under reduced vacuum over Whatman GF/B glass fiber filters and washed three times with 5 ml of Tris buffer. Radioactivity trapped on the filters was assayed by liquid scintillation spectroscopy. For [^{3}H]yohimbine binding, final assay volume was 250 μl, tissue concentration was 10 mg/ml, and assay buffer contained 100 mM NaCl, 10 mM MgCl$_2$, and 1 mM EDTA. Radioligands and "blank" drugs used were: [^{3}H]WB4101 (0.005–10 nM) and norepinephrine (0.1 mM); [^{3}H]prazosin (0.02–1.5 nM) and phentolamine (10 μM), [^{3}H]yohimbine (2.5–60 nM) and phentolamine (10 μM); [^{3}H]etorphine (0.5 nM) and naloxone (1 μM); [^{3}H]QNB (0.1–3.2 nM) and atropine (1 μM); [^{3}H]dihydroalprenolol (1 nM) and propranolol (1 μM); and [^{3}H]p-amino-clonidine (1 nM) and norepinephrine (10 μM). Assays in which norepinephrine was utilized contained 0.01% ascorbic acid. Data was computer-analyzed with a weighted, nonlinear, least-squares program based on the Law of Mass Action (18). Models for one, two, or more sites were successively used, and the magnitude of the residual variance, unaccounted for by the model, was statistically compared between models to determine the "best fit." Thus, it is assumed that deviations of the binding curve from that expected for a bimolecular reaction are due to differential affinities of the ligand for separate (e.g., noncooperative) receptor binding sites. In the case of [^{3}H]WB4101 saturation binding experiments, the model involves the binding of the radioligand
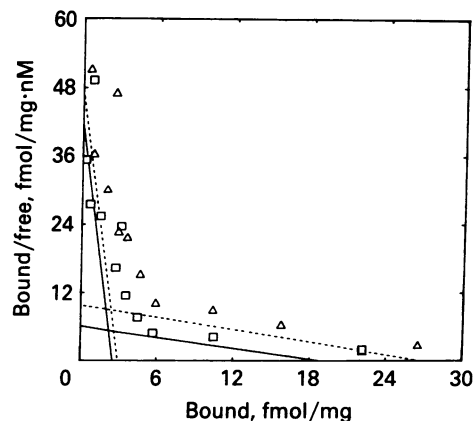

FIG. 1. Scatchard plot of [^{3}H]WB4101 binding in hippocampus 8 days after a sham lesion (□) or complete bilateral ablation of the medial septal and diagonal band nuclei (△). The hippocampi from four rats were pooled in each group. The straight lines represent the two individual binding components obtained by computer analysis. The data were analyzed by a nonlinear regression program (LIGAND) (16) that best fits the data to two sites. There was no increase in the B_{max} [expressed in fmol/mg (wet weight) of tissue] of the higher affinity site (K_{high}, 66 pM; control B_{max}, 2.51; lesion B_{max}, 3.0), whereas the second site showed a 47% increase in this representative experiment (K_{low}, 3.0 nM; control B_{max}, 17.8; lesion B_{max}, 26.3).

to two sites, which are apparently distinct, for which the ligand has different affinity.

RESULTS

[^{3}H]WB4101 Binding. [^{3}H]WB4101 binds reversibly and with high affinity to two sites in hippocampus that are saturable and have high affinities for α-adrenergic agonists and antagonists (19–21). In preliminary studies we have found a 39% increase over control binding of [^{3}H]WB4101 after either fimbrial transection or septal and diagonal band lesions (22). We investigated binding at three concentrations of ligand (0.25, 0.5 and 1.0 nM) because this was feasible with a single hippocampus. There was an approximately equivalent percentile increase in [^{3}H]WB4101 binding throughout this narrow range of concentration. In order to conduct detailed saturation studies, we pooled 4–6 hippocampi and again found an increase in specific [^{3}H]WB4101 binding after septal deafferentation. However, the increase in binding was restricted to higher ligand concentrations labeling the lower-affinity binding site ("nanomolar site"; Fig. 1). There was a 38 ± 5% increase in the number of these binding sites (B_{max}) with no change in affinity (K_d). This up-regu-
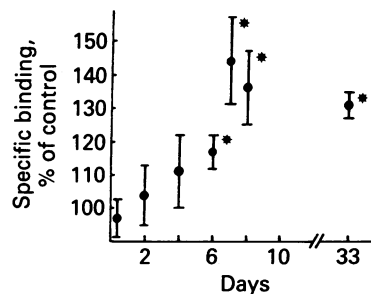

FIG. 2. Time course of [^{3}H]WB4101 binding increase in hippocampal membranes after unilateral fimbria transection. Each point represents the mean ± SE of specific binding ($n = 5$ except day 7 when $n = 4$) in the deafferented hippocampi compared to the contralateral side. The concentration of ligand was 0.5 nM. *, $P < 0.05$, Student's t test).
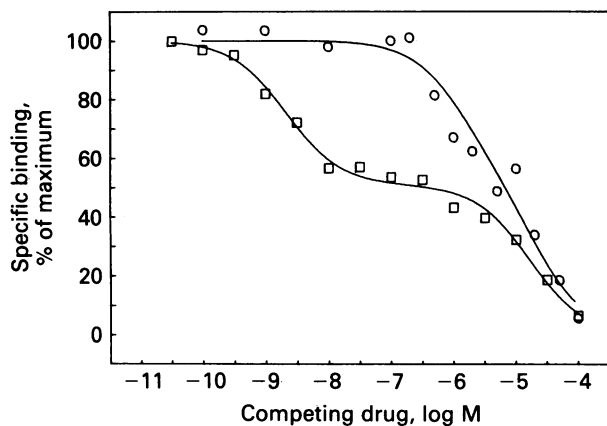
FIG. 3.   Prazosin (□) and yohimbine (○) competition with [³H]WB4101 (1.5 nM) in rat hippocampus. The open symbols represent the experimentally determined data points, while the solid lines are computer generated curves fitting the observed data points. The affinities of [³H]WB4101 for its two binding sites were set constant (K_{SH} = 50 pM) (K_H = 1.5 nM) for this analysis, whereas the affinities of prazosin and yohimbine and the proportion of the two binding sites were obtained by the computer analysis. Prazosin affinities were K_{SH} = 0.11 nM and K_H = 6.15 μM, labeling 28% and 72% of the sites, respectively. Yohimbine affinities for the two sites (K = 2.54 μM) were not significantly different from each other.

lation of [³H]WB4101 binding is observed as early as 6 days, is maximal by 8 days, and remains constant to 33 days, the latest time point investigated (Fig. 2).

Ligand Selectivity of Adrenergic Effects. Several investigators have suggested that [³H]WB4101 labels both α_1 and α_2 subtypes of adrenergic receptors in some tissues with apparent equal affinity (23, 24). Therefore, we characterized the ability of the α_1-specific antagonist, prazosin, and the α_2-specific antagonist, yohimbine, to compete with [³H]WB4101 binding sites in hippocampus. Fig. 3 shows that prazosin has high affinity for less than half of the specific [³H]WB4101 binding. Using the affinity constants for [³H]WB4101 obtained through saturation studies and analyzing the data with LIGAND (18), we found that prazosin has an affinity constant K_d of 0.10 nM for the high-affinity [³H]WB4101 site ("picomolar site") and 1–5 μM affinity for the nanomolar [³H]WB4101 site. This picomolar binding site represents 25–30% of the total number of [³H]WB4101 binding sites. This site is not altered after fimbrial transection or medial septal and diagonal band damage (Fig. 1). Furthermore, Fig. 3 shows that yohimbine has low (micromolar) affinity for both binding sites labeled by [³H]WB4101 in the hip-

pocampus. A detailed characterization of the nanomolar affinity [³H]WB4101 binding site will appear elsewhere. However, it is important to note that the adrenergic agonists epinephrine, norepinephrine, phenylephrine, and isoproterenol demonstrate the same potency at both [³H]WB4101 binding sites. Dihydroergocriptine is a potent antagonist of both sites, whereas prazosin is selective for the picomolar site. Thus, the up-regulation of [³H]WB4101 after cholinergic deafferentation represents an α-adrenergic binding site distinct from that labeled by [³H]prazosin or [³H]yohimbine. Indeed there is no change in the number or affinity of [³H]prazosin or [³H]yohimbine sites 8 days after fimbrial damage (Table 1). To further investigate the selectivity of this effect we tested p-[³H]aminoclonidine (an agonist ligand for α_2-adrenergic receptors) and [³H]dihydroalprenolol (an antagonist ligand for β-adrenergic receptors). The level of specific binding (see Methods) was unaltered 33 days after fimbrial transection (n = 5). There was no change in [³H]-etorphine binding, which labels opiate receptors. In addition, we confirmed earlier studies (refs. 2, 3, and 4, but see ref. 5) that report no change in [³H]QNB binding after electrolytic or knife lesions of the septal afferents.

Control Lesions of Monoamine and Serotonin Afferent and Hippocampal Efferents. Injections of 6-hydroxydopamine into the anterior aspect of the fimbria had no effect upon [³H]WB4101 binding. This finding is in agreement with the work of U'Prichard et al. (8) in which destruction of the dorsal noradrenergic bundle by 6-hydroxydopamine did not alter [³H]WB4101 binding in the hippocampus. The dose used in the present study was great enough to also destroy the dopaminergic fibers within the fimbria (see ref. 25 for example). Thus, it appears that the increase in adrenergic binding is not due to the removal of monoaminergic inputs. The removal of serotonergic inputs resulted in a 40.3 ± 5.3% decrease in hippocampal serotonin, somewhat less than would be expected with the fimbria transection (26) but identical to that found with septal damage (39.5 ± 7.5%). [³H]WB4101 binding was unaltered after every lesion that spared the septal cholinergic tract. Others have reported that a complete depletion of serotonin from hippocampus increases [³H]-WB4101 binding (27). It is not clear which binding site was involved in that study, but clearly the alteration in the nanomolar [³H]WB4101 binding site reported here is not caused by the serotonergic disruption. Kainate injections into the lateral septal nucleus had no effect on hippocampal [³H]WB4101 binding levels.

Chronic Muscarinic Receptor Blockade. In order to determine whether the observed increase in [³H]WB4101 binding could be induced by a loss of cholinergic tone through chronic blockade of cholinergic receptors, in the absence of cholinergic

Table 1.   Effects of cholinergic denervation and blockade on adrenergic receptor binding in rat hippocampus

	Bilateral septal or fimbrial lesion			Chronic scopolamine		
	Control	Experimental	n	Control	Experimental	n
[³H]WB4101 Specific binding,* % of control	100 ± 8	139 ± 9[†]	16	100 ± 5	104 ± 6	15
[³H]Prazosin B_{max}, pmol/g	4.5 ± 0.33	4.0 ± 0.48	9	4.2 ± 0.28	4.2 ± 0.36	15
K_d, nM	0.18 ± 0.03	0.13 ± 0.02		0.16 ± 0.01	0.16 ± 0.01	
[³H]Yohimbine B_{max}, pmol/g	15.4 ± 1.9	17.2 ± 2.7	6			
K_d, nM	24.1 ± 1.9	24.9 ± 4.4				

* Specific binding was compared in tissue run simultaneously with [³H]prazosin or [³H]yohimbine. The concentration of [³H]WB4101 was 1.0 nM.
[†] P < 0.025.

terminal degeneration, rats were continually administered scopolamine (a muscarinic/cholinergic receptor antagonist) by an implanted Alzet osmotic pump for 28 days. This treatment resulted in a significant increase in the B_{max} of [^{3}H]QNB binding to muscarinic/cholinergic receptors (70%) in the hippocampus. However, there was no increase in the binding of [^{3}H]WB4101 or [^{3}H]prazosin (Table 1). In additional rats treated for glyoxylic acid-induced fluorescence, sympathetic fibers did not enter the hippocampus after this treatment.

DISCUSSION

The major finding of this study is that removal of a nonadrenergic afferent to the rat hippocampus elicits an increase in a specific population of adrenergic receptor binding sites. The significance of such a finding may be interpreted in several ways. A compensatory relationship between cholinergic deafferentation and adrenergic receptor binding site number has not been shown previously and may reflect a molecular interaction between cholinergic and adrenergic receptors in the hippocampus. Because chronic exposure to scopolamine does not alter the adrenergic binding sites in the hippocampus, it is unlikely that these sites are linked to a muscarinic receptor. Alternatively a neuronal nicotinic receptor may be involved. Nicotinic regulation of norepinephrine levels in the hippocampus have been reported (28). There is considerable evidence for functional coregulation of cholinergic and adrenergic receptor binding sites in peripheral systems. Cholinergic agonists can alter the binding affinity of noradrenergic agonists to adrenergic receptors labeled by [^{3}H]WB4101 (29) and [^{3}H]dihydroalprenolol (30) in cardiac tissue. Parasympathetic denervation of salivary glands induces pharmacologic supersensitivity to noradrenaline and isoproterenol as well as to cholinergics such as methacholine (31–33). Such effects may be mediated (*i*) by alterations in the cell membrane that affect the availability of receptors not specifically associated with the pharmacological agent used or (*ii*) by competition between receptors for common membrane components of limited availability.

Conversely, it should be considered that the appearance of these receptors might not reflect an interaction with cholinergic receptors *per se* but rather a direct relationship with the ingrowing noradrenergic fibers, which also result from the same manipulation. These lesions elicit both the α-adrenergic receptor binding site increase and the reinnervation of the hippocampus by sympathetic axon collaterals, originating in the superior cervical ganglion and usually found only on the cerebral vasculature. The sympathetic fibers begin to invade the hippocampal parenchyma 9–10 days after lesion and innervate the hippocampus maximally 21–30 days after lesion (34), whereas the α-adrenergic receptor binding sites demonstrate a 17% increase in number by as early as 6 days after lesion. Thus, it is unlikely that the new sites are presynaptically localized on the arriving noradrenergic terminals. However, it is possible that the new receptor binding sites are involved in eliciting or directing the sympathetic axon ingrowth. They may have, or may regulate, neurotrophic properties similar to that described by Bjorklund and Stenevi, who found a dramatic stimulation of ingrowth of both ganglion implants and axotomized central noradrenergic fibers into the hippocampus after septal and diagonal band lesions (35). Such a potent neurotrophic effect could involve a receptor mechanism. It is also possible that these adrenergic binding sites serve as targets and, therefore, determine the localization of sympathetic axons within the hippocampus. A functional relationship between extrajunctional receptors and sprouting also has been proposed at the neuromuscular junction (36–38). The present data correlate the new

appearance of receptors and collateral sprouting in the central nervous system of mammals.

In other systems (such as the dopaminergic projection to striatum and nucleus accumbens or the noradrenergic projection to cerebral cortex) denervation and receptor blockade both increase the binding capacity of the respective receptors (39–41). Thus, in these systems terminal degeneration does not appear necessary to elicit the receptor up-regulation. In the hippocampus, the effects of cholinergic denervation and muscarinic receptor blockade are incongruent with respect to adrenergic and muscarinic/cholinergic receptor binding. This could be explained by postulating that the septal afferents terminate at nicotinic/cholinergic sites, perhaps on interneurons (42). Therefore, it will be of interest to investigate the effect of nicotinic/cholinergic receptor blockade on [^{3}H]WB4101 binding in hippocampus.

The observation that the two high-affinity binding sites for [^{3}H]WB4101 regulate differentially after septal deafferentation also has important implications for the characterization of central adrenergic receptors. The multiphasic binding characteristics of [^{3}H]WB4101 may be due to negative cooperativity, differential affinity of the two isomers of commercial [^{3}H]WB4101 for a single receptor, or two affinity states of a single receptor protein. However these explanations are inconsistent with the present finding. In all cases one would not expect 70% of the binding sites labeled by [^{3}H]WB4101 to up-regulate after a manipulation that does not effect the other population of sites. Thus, we suggest that [^{3}H]WB4101 labels two distinct adrenergic binding sites in the rat central nervous system.

The nanomolar affinity binding site that demonstrates this cholinergic regulation cannot be considered an α_1- or α_2-adrenergic receptor binding site using present standards for adrenergic receptor classification. Whereas the pharmacological profile for agonists is consistent with α-adrenergic binding properties, the antagonists prazosin and yohimbine have only micromolar affinity at this binding site. On the other hand, the antagonists dihydroergocryptine, phentolamine, and WB4101 compete with nanomolar affinity. While it is possible that this investigation has identified a third α-adrenergic receptor subtype, extensive studies are necessary to demonstrate this. Nevertheless, it should again be considered that high affinity for prazosin does not unequivocally delineate α_1-adrenergic receptors (43, 44).

The behavioral implications of the receptor responses discussed here could be far-reaching. In the hippocampus, for instance, heterologous interaction of cholinergic and adrenergic receptors could account for the observed behavioral supersensitivity to cholinergic agents that follows destruction of the central noradrenergic innervation (45). In addition, we must now consider that many currently used psychoactive drugs may modify several neurotransmitter systems through such receptor interrelationships. And if, indeed, neurotransmitter receptors serve to regulate axonal sprouting and synaptogenesis during normal development or in response to injury, neuronal plasticity may be subject to pharmacological manipulations.

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