# Growth and Reshaping of Axons in the Establishment of Visual Callosal Connections

Abstract. *The visual cortical areas in the two hemispheres are interconnected by axons running through the corpus callosum. In adult cats, these axons originate from, and terminate in, tangentially restricted portions of each area. In young kittens, however, callosal axons originate from the entire extent of each area, although they apparently enter the gray matter only in the restricted regions where they will also be found in adults. In kittens, but not in adults, callosal axons also reach other regions, but there they appear to be confined to the lowest part of layer VI. During the first two postnatal months, the callosal efferent zones become progressively restricted to their adult locations. During this process, many neurons eliminate the axons (or axon collaterals) that they had formerly sent through the corpus callosum and form permanent connections ipsilaterally.*

In eutherian mammals, neurons whose axons travel through the corpus callosum (callosal neurons) provide the main connection between the cerebral hemispheres. In adults, this connection originates from, and terminates in, tangentially restricted regions of the neocortex. In immature animals, however, callosal neurons have a widespread tangential distribution but disappear from large portions of the neocortex during development (*1*, *2*). The questions addressed here are whether terminating callosal axons undergo a similar reduction in their tangential distribution and whether neuronal death or elimination of callosal axons is responsible for the disappearance of callosal neurons. These questions were approached by injecting the visual areas 17 and 18 of kittens at ages up to 9 days (Tables 1 and 2) with anterograde or retrograde (or both) axonal tracers: radioactive leucine and proline, horseradish peroxidase (HRP), granular blue, or fast blue. The latter two substances, which are fluorescent, have been used with other fluorescent tracers to double-label individual neurons that project through bifurcating axons to different targets (*3*). These substances yield long-lasting neuronal labeling (*3*). I exploited this property to monitor during postnatal development the fate of juvenile callosal neurons. I labeled callosal neurons during the first postnatal week and then studied their distribution after survival times as long as 51 days. In one kitten, some of these neurons were relabeled by the later reinjection of areas 17 and 18 with a second fluorescent dye (nuclear yellow) (*3*, *4*).

In anesthetized kittens (*5*) the injections (Table 1) were delivered through glass micropipettes (tip diameter, ~ 50 μm) connected to a pneumatic pump (amino acids alone) or through a Hamilton microsyringe (needle diameter 0.5 mm). After various survival times, all animals were reanesthetized with heavy doses (50 to 60 mg per kilogram of body weight, injected intraperitoneally) of Nembutal and intracardially perfused with fixatives (Table 1). The sections containing HRP were reacted with tetramethylbenzidine (TMB) (*6*) or with diaminobenzidine (DAB) alone or combined with cobalt chloride (*7*) (Table 1).

Those containing radioactive amino acids were coated with Ilford K5 or Kodak NTB2 nuclear emulsion, stored in the dark at 4°C, and developed after 5 to 12 weeks. Those containing fluorescent dyes were simply mounted and observed without coverslips through a microscope (Zeiss) whose stage movements could be directly fed into a computer, thus allowing the distribution of labeled neurons to be charted. Selected sections of some of the brains were counterstained with neutral red (HRP and fluorescent material) or toluidine blue (autoradiographic material).

As in our previously studied kittens (*1*), in the injected hemispheres, the labels spread through most of the visual areas 17, 18, and part of area 19. Labeled neurons or terminating axons were found in most of the visual relay nucleus of the thalamus (dorsal nucleus of the lateral geniculate body) ipsilateral to the injections; this suggests that the labels were available for uptake in most of areas 17, 18, or both, to which the nucleus projects in a topographic manner. At the concentrations and amounts used here the granular-blue injections provoked lesions; these, however, did not interfere with the uptake and transport of the dye, although they may have caused retrograde degeneration of some callosal neurons.

In the uninjected hemispheres, the distributions of HRP-labeled callosal neurons visualized with TMB are similar to those observed at comparable ages with the less sensitive DAB technique (*1*). The main finding is the complete absence in the first postnatal week of the wide acallosal regions that in adult cats occupy parts of each visual area, in particular the medial part of area 17 and the lateral part of area 18 (Fig. 1B). In kittens, callosal neurons have a higher packing

Table 1. Labeling of callosal neurons with HRP and radioactive amino acids. Tracers were dissolved in sterile saline. Age is counted from day of birth (day 0). Animals were perfused with saline followed by paraformaldehyde (3 percent) and sucrose (2 percent) solutions in 0.1*M* phosphate buffer (*p*H 7.3 to 7.6) (except for D29 and D30, which were perfused with 10 percent Formalin). Brains were stored in a 20 percent sucrose solution at 4°C. Formalin was neutralized. Nonidet P-40 is a substance that has been reported to increase HRP uptake, transport, or both (*15*).

| Animal code | Tracer | Injections (No.) × volume (μl) | Age (days) | | Histological procedures |
|---|---|---|---|---|---|
| | | | At injection | At death | |
| D18 | HRP 33 percent, [$^3$H]leucine-proline, 100 μCi/μl | 3 × 0.2 | 3 | 4 | Frozen sections, DAB, autoradiography |
| D29 | [$^3$H]leucine-proline, 400 μCi/μl | 2 × 0.5 | 5 | 6 | Paraffin sections, autoradiography |
| D30 | [$^3$H]leucine-proline, 400 μCi/μl | 2 × 0.5 | 8 | 9 | Paraffin sections, autoradiography |
| D34 | HRP 50 percent, [$^3$H]leucine-proline, 200 μCi/μl | 3 × 0.7 | 4 | 5 | Frozen sections, DAB-Co, TMB, autoradiography |
| D35 | HRP 50 percent | 3 × 0.5 | 8 | 9 | Frozen sections, DAB-Co, TMB |
| D36 | HRP 50 percent, [$^3$H]leucine-proline, 125 μCi/μl Nonidet 5 percent | 2 × 0.5 | 2 | 3 | Frozen sections, TMB, autoradiography |
| D37 | HRP 50 percent | 2 × 0.4 | 9 | 10 | Frozen sections, TMB |
| D38 | HRP 50 percent, Nonidet 6 percent | 2 × 0.4 | 2 | 3 | Frozen sections, DAB-Co, TMB |
| D39 | HRP 50 percent, Nonidet 5 percent | 3 × 0.4 | 6 | 7 | Frozen sections, DAB-Co, TMB |


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density than in adult cats, although, as in adults, the neurons lie principally in layers III, IV, and VI.

The diffuse tangential distribution of callosal neurons contrasts with the selective growth of callosal axons into the restricted regions that receive (and send) callosal connections in adults. Indeed by postnatal day 3, some HRP-labeled axons have penetrated the dorso-occipital cortex but only near the boundary between areas 17 and 18, in part of area 19, and in part of the suprasylvian areas (compare with Fig. 1B for the distribution of axons on day 5). In particular, near the border between areas 17 and 18, terminating fibers can be seen on days 3 and 4; they are especially densely packed in layer VI, but some can be followed as far as layers III, II, and I. By day 5 (Fig. 1C), as in adults, these fibers can be seen in all layers although they remain more densely packed in layer VI. During the following days, the distribution of terminating axons does not change significantly, although their density increases. The autoradiographic material confirms that, by the end of the first postnatal week, the callosal afferents are restricted to the gray matter near the 17/18 border and span its entire thickness (Fig. 1A).

In the HRP material, the callosal axons directed to the restricted cortical regions mentioned above, seem to travel in three separate bundles that acquire their individuality near the fundus of the splenial sulcus. Corresponding bundles of callosal axons exist in the adult cat, although they are somewhat displaced from their original trajectories by the development of the sulci. In adults, individual axons in these bundles can be resolved, whereas in the kitten, the bundles appear as condensations of a dust-like precipitate. Furthermore, it is characteristic for the kitten that a similar but lighter HRP precipitate almost fills the white matter and the deepest part of layer VI of the lateral and postlateral gyri (Fig. 1, B and D). This diffuse HRP precipitate does not correspond to retrogradely filled axons of layer III and IV callosal neurons, since it is almost completely absent in lower layer IV, in V, and in upper layer VI (Fig. 1D).

Table 2. Labeling of callosal neurons with fluorescent tracers (10 percent solutions in sterile distilled water). Each animal received three injections of 0.5 μl each. Animals were perfused with 10 percent neutralized Formalin in saline. Brains were stored in 20 percent sucrose in 0.1*M* cacodylate buffer (*p*H 7.3 to 7.6). Frozen tissue was sliced 30 μm thick.

| Animal code | Age (days) | |
|---|---|---|
| | At injection | At death |
| | *Fast blue* | |
| DL6 | 3 | 9 |
| DL7* | 3 | 30 |
| DL8 | 3 | 31 |
| DL13 | 8 | 52 |
| | *Granular blue* | |
| DL9 | 5 | 46 |
| DL10 | 5 | 11 |
| DL11 | 5 | 54 |
| DL12 | 8 | 59 |

*Reinjected on day 27 with 2 percent nuclear yellow in sterile distilled water (three injections of 0.5 μl each) (*16*).

Kittens injected with granular blue or fast blue during the first postnatal week show, at 9 and 11 days, respectively, a widespread tangential distribution of retrogradely labeled callosal neurons (blue cytoplasm with silver or orange granules [compare with (*3*)] similar to that described for the HRP material (Fig. 2, DL6). An almost identical widespread distribution of labeled neurons is still found in kittens injected about the same time and killed near the end of the first or second postnatal month (Fig. 2, DL13).

After 1 month's survival, the neurons are more intensely labeled by the fluorescent dyes than after shorter times and are often closely surrounded by labeled satellite glia [see (*3*) for similar effects of long survival] but not by other labeled neurons. This suggests that no significant transneuronal diffusion of the dyes has occurred, in agreement with the absence of neuronal labeling in the superior colliculus ipsilateral to the injection (where anterogradely labeled terminating fibers as well as glia can be seen) or in the dorsal lateral geniculate nucleus contralateral to the injections.

Near the end of the second postnatal month, the intensity of retrograde labeling has further increased in many neurons within regions that give rise to callosal fibers in the adult, whereas it has decreased elsewhere. Although many of the neurons labeled near the 17/18

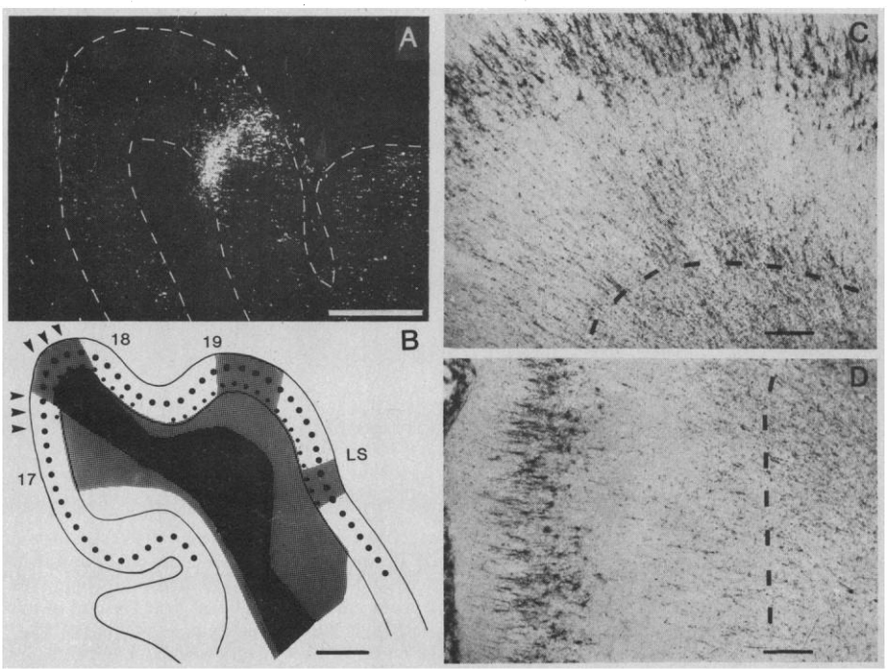


Fig. 1. (A) Dark-field photomicrograph of a coronal section through the occipital region in a 9-day-old kitten (D30). Terminating callosal axons labeled by contralaterally injected radioactive amino acids enter a restricted region of the gray matter corresponding to the boundary between visual areas 17 (medially) and 18 (laterally). Calibration bar, 1 mm. (B) Schematic drawing of a slightly more rostral section in a 5-day-old kitten (D34). Large and small dots schematize the distribution of HRP-labeled callosal neurons in layers III/IV and VI, respectively; the former are more numerous and occupy the entire tangential extent of the cortex. HRP-labeled callosal axons (stippled area) are grouped in a bundle (in black), three branches of which (only one is clearly distinguishable at this rostrocaudal level) enter (from left to right) the 17/18 border, part of area 19, and the suprasylvian region (*LS*). Elsewhere callosal axons do not extend beyond lower layer VI. Calibration bar, 1 mm. (C and D) Bright-field photomicrographs from the section shown in (B) showing, respectively, the 17/18 border and a neighboring portion of area 17 [as indicated by arrows in (B)]. Labeled axons have entered the cortex in the former, not in the latter, region. They are shown as far as layers III/IV (denoted by the presence of labeled neurons) in (C) but not in (D). The interrupted line corresponds to the lower boundary of layer VI. Calibration bars, 100 μm. The dorsal and medial surfaces of the brain are, respectively, up and toward the left.

boundary have acquired the typical large size of adult callosal neurons (*1*) those labeled in the medial bank of area 17 have not.

The persistence of labeled neurons in the medial part of area 17 and in the lateral part of area 18 at the end of the second postnatal month is remarkable since HRP injections made at the end of the third postnatal week do not label callosal neurons in the greater part of these regions (*1*).

This finding is due neither to failure in HRP uptake or transport in kittens older than 3 weeks nor to an interference with the normal reduction of callosal neurons after the early injections. The animal injected with fast blue on day 3 and reinjected with nuclear yellow on day 27 (Fig. 2, DL7) showed a distribution of neurons labeled by the second injection [yellow/green nucleus; compare with (*3*)] similar to that observed in kittens injected with HRP at a comparable age (*1*). Therefore, near the 17/18 border, in parts of area 19 and of the suprasylvian cortex, most neurons were double-labeled (blue cytoplasm and yellow nucleus); elsewhere, the neurons were labeled only by the early (blue) injection. However, intermingled with the double-labeled neurons, a few neurons were labeled only by the first or by the second injection. This finding cannot be interpreted unless axonal damage due to the first injection, fading of the early injected dye, and selectivity of some neurons for one of the two dyes can be ruled out (*8*).

These results suggest the following interpretations. (i) In adult animals, only some tangentially restricted portions of the visual cortex give rise to callosal axons, but the tangential location of juvenile cortical neurons apparently does not determine whether or not they will initially send axons through the corpus callosum, although it may determine whether these axons will form permanent connections in the contralateral hemisphere. (ii) Most terminating callosal axons acquire their restricted adult distribution earlier than the callosal neurons [compare with (*2, 9*)]. However, the diffuse anterograde labeling of lower layer VI in regions contralateral to the injections suggests that some callosal axons (or their collaterals) have a more widespread distribution. They may be allowed to penetrate the cortex more fully only under extraordinary morphogenetic conditions, such as those induced by strabismus (*10*) or by genetic abnormalities of the optic pathway (*11*). Under these conditions, callosal neurons also remain more widespread than they do normally (*11, 12*). Thus, the distributions of callosal neurons and terminals are interrelated in development, although, at least normally, the latter precedes and possibly conditions the former. (iii) Many of the neurons that can be labeled through the corpus callosum during the first postnatal week in parts of areas 17 and 18 that are acallosal in the adult are apparently still alive over more than 1 month after they can no longer be labeled in this way. Thus, a substantial number of juvenile callosal neurons eliminate their callosal axons (or axon collaterals) and presumably form permanent connections ipsilaterally. The possibility that, in addition, some juvenile callosal neurons die cannot be ruled out by the data.

In rats (*2*) and cats (*1*), most callosal axons are eliminated by the end of the second postnatal week and of the first postnatal month, respectively—that is, immediately before the onset of callosal myelination (*13*). If this correlation holds for humans, a similar reshaping of callosal connections ought to occur in children during the first four postnatal months; afterward myelination of the corpus callosum begins (*14*).

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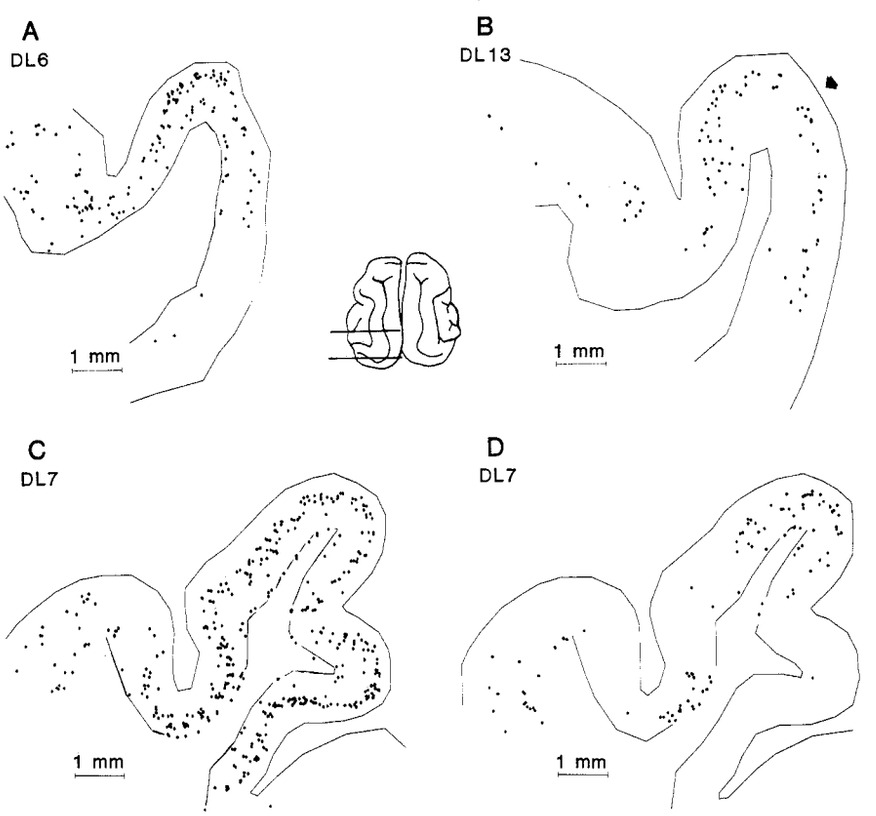


Fig. 2. Computer charts of the distribution of neurons labeled by fluorescent dyes. Coronal sections at corresponding levels from DL6 and DL13; more rostral in DL7 (inset). The dorsal and medial surfaces of the brain are, respectively, up and to the right. The arrow approximates the most medial part of area 17, where callosal neurons can still be labeled near the end of the second postnatal month. (A) Neurons labeled with fast blue on postnatal day (pd) 9 after injection on pd 3; (B) neurons labeled with fast blue on pd 52 after injection on pd 8; (C) neurons labeled with fast blue on pd 30 after injection on pd 3; and (D) neurons labeled with nuclear yellow on pd 30 after injection on pd 27.

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4. I propose to call this kind of experiment "sequential homothetic double labeling" to differentiate it from the double labeling obtained by simultaneous or sequential injections of different tracers in different structures (heterothetic labeling).
5. The kittens were medicated with atropine, anesthetized with Ketalar (40 mg per kilogram of body weight, injected intramuscularly) and with inhalation of Penthrane, while their electrocardiograms were continuously monitored. The association of these two anesthetics effectively and safely maintains analgesia in young kittens.
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16. While this manuscript was in preparation, kittens DL15 and DL18 received, respectively, three and two injections of 5 percent fast blue in areas 17 and 18 on postnatal day 6. These animals were reinjected in areas 17 and 18 with 2 percent nuclear yellow on postnatal day 50 (DL15, two injections, 0.5 μl each) and 43 (DL18, three injections, 0.5 μl each). The results were similar to those described for DL7.
17. Supported by the Swiss National Science Foundation grants 3.319-0.78 and 3.628-0.80. I am grateful to F. Amaudruz, P. G. H. Clarke, D. O. Frost, M. Gaillard, L. Garey, M. Gissler, C. Vaclavik, and H. Van der Loos for their help at different stages of this work. Fast blue and granular blue were kindly provided by O. Dann, Institut für Pharmazie und Lebensmittelchemie der Friedrich-Alexander-Universität, D-8520 Erlangen.

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# Two Distinct Central Serotonin Receptors with Different Physiological Functions

Abstract. *Two distinct serotonin (5-hydroxytryptamine) receptors designated serotonin 1 and serotonin 2 bind tritium-labeled serotonin and tritium-labeled spiroperidol, respectively. Drug potencies at serotonin 2 sites, but not at serotonin 1 sites, predict their effects on the "serotonin behavioral syndrome," indicating that serotonin 2 sites mediate these behaviors. The limited correlation of drug effects with regulation by guanine nucleotides suggests that serotonin 1 sites might be linked to adenylate cyclase. Drug specificities of serotonin-elicited synaptic inhibition and excitation may reflect serotonin 1 and serotonin 2 receptor interactions, respectively.*

Serotonin (5-hydroxytryptamine) elicits both synaptic inhibition and excitation in the brain (*1–4*) and plays a role in numerous behavioral systems. Increased concentrations of serotonin in the brain result in a behavioral hyperactivity syndrome with head twitching, resting tremor, and hypertonicity (*5, 6*). A serotonin-sensitive adenylate cyclase in brain homogenates may mediate some serotonin responses (*7–9*). Serotonin receptors in the brain can bind tritium-labeled lysergic acid diethylamide (LSD) (*10*), serotonin (*11*), and spiroperidol (*12*). Recently, we demonstrated that [$^3$H]serotonin and [$^3$H]spiroperidol bind to physically distinct populations of serotonin receptors in the brain, whereas [$^3$H]LSD binds to both of these sites with similar affinity (*13*). The receptors that bind serotonin (designated serotonin 1 receptors) are regulated by guanine nucleotides, whereas sites that bind spiroperidol (serotonin 2 receptors) are not influenced by nucleotides (*14*). By comparing drug affinities for serotonin binding sites with drug potencies in physiologic functions, we now provide evidence that serotonin 1 receptors might be related to the serotonin-sensitive adenylate cyclase, whereas the behavioral syndrome resulting from central serotonin stimulation is mediated by serotonin 2 receptors. Moreover, drug specificities of serotonin-elicited synaptic inhibition and excitation reflect serotonin 1 and serotonin 2 receptor interactions, respectively.

Behavioral hyperactivity follows central serotonin stimulation with drugs such as 5-hydroxytryptophan, tryptophan plus a monoamine oxidase inhibitor, D-LSD, and quipazine (*5, 6*). The syndrome includes resting tremor, hindlimb abduction, splayed hindlimbs, snake tail, side-to-side head weaving, and head twitching. The head twitch is an easily monitored and reliable measure of the presence of the syndrome. Accordingly, we evaluated the potencies of a wide range of drugs in inhibiting hydroxytryptophan-elicited head twitches in mice (Fig. 1, A and B). A number of drugs—including classical serotonin antagonists such as cyproheptadine and metergoline, neuroleptics such as spiroperidol and pipamperone, and antidepressants (*15*) such as mianserin and amitriptyline—are capable of preventing head twitches induced by hydroxytryptophan. Although amitriptyline and mianserin are potent inhibitors of the induced head twitches, other antidepressants, such as desipramine and iprindole, are much weaker; thus, serotonin blockade is not likely to account for the therapeutic efficacy of these latter drugs. The butyrophenone neuroleptic spiroperidol is the most potent inhibitor of hydroxytryptophan-induced head twitches; a 50 percent inhibitory dose ($ID_{50}$) of 0.18 μmole per kilogram of body weight also inhibited apomorphine-induced stereotypy (*16*), a dopamine-linked behavior. Pipamperone ($ID_{50}$ = 1.73 μmole/kg) and chlorpromazine ($ID_{50}$ = 2.41 μmole/kg), on the other hand, are more than 100 and 8 times, respectively, more potent inhibitors of the serotonin than of the dopamine behavioral syndrome. Conversely, haloperidol ($ID_{50}$ = 4.26 μmole/kg) is approximately 10 times more potent in blocking the dopamine than the serotonin syndrome. Thus, while neuroleptic drugs are potent antagonists of the hydroxytryptophan syndrome, these effects are not mediated through the dopamine system.

Drug affinities for serotonin 1 receptors labeled by [$^3$H]serotonin do not correlate with head twitch blockade (Fig. 1A). By contrast, drug potencies in blocking induced head twitches closely correlate with affinities for serotonin 2 receptors labeled by [$^3$H]spiroperidol ($r$ = .98, $P$ < .001). Since in vivo blockade of head twitches correlates with in vitro receptor affinity, the drugs tested presumably differ little in their ability to reach target sites in the brain.

Several lines of evidence suggest that

Table 1. Comparison of drug potencies at serotonin receptors with physiological actions, means ± standard errors of three to six experiments, each performed in triplicate. The influences of drugs on microiontophoretic serotonin effects are from the literature (*1–4, 23–25*).

| Serotonin-related drugs | Affinity for serotonin 1 receptors: $K_i$ versus [$^3$H]-serotonin (n*M*) | Affinity for serotonin 2 receptors: $K_i$ versus [$^3$H]-spiroperidol (n*M*) | Effects on serotonin synapses | |
|---|---|---|---|---|
| | | | Inhibition | Excitation |
| Serotonin | 2.7 ± 0.55 | 2700 ± 400 | Agonist (*1–4, 23–25*) | Agonist (*1–4*) |
| D-LSD | 9.8 ± 1.0 | 8.9 ± 1.7 | Agonist (*3*) | Antagonist (*1–3*) |
| Lisuride | 6.2 ± 1.1 | 11 ± 3.6 | Agonist (*25*) | |
| Metergoline | 9.9 ± 2.1 | 2.1 ± 0.67 | Antagonist (*24*) | Antagonist (*4*) |
| Cyproheptadine | 1100 ± 120 | 2.4 ± 0.21 | No effect (*4*) | Antagonist (*4*) |
| Bromo-LSD | 100 ± 15 | 2.5 ± 0.41 | No effect (*1*) | Antagonist (*1*) |
| Methysergide | 150 ± 17 | 3.1 ± 0.91 | No effect (*1, 2, 4*) | Antagonist (*1, 2, 4*) |
| Methiothepin | 300 ± 27 | 4.1 ± 0.67 | No effect (*4*) | Antagonist (*4*) |
| Cinanserin | 1800 ± 540 | 15 ± 3.1 | No effect (*1, 4*) | Antagonist (*1, 4*) |


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