Synaptic Properties of Serotonergic Growth Cones in Developing Rat Brain

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In order to gain insight into the events that take place when serotonergic growth cones are remodeled into synapses, we tested the hypothesis that neurotransmitter-related properties of presynaptic terminals are already present in these growth cones before synaptogenesis begins. The ontogeny of markers for the specific reuptake of 5-HT and for 5-HT-storing synaptic vesicles was studied in isolated growth cone (IGC) fractions from developing rat brain. High-affinity 3H-imipramine binding (a marker for the plasma membrane 5-HT transporter) was significantly enriched in IGC fractions prepared before the beginning of cortical synaptogenesis [embryonic day 15 (E15) and E20]. Radioautography with 3H-imipramine or 3H-paroxetine (another marker for the transporter) confirmed that the 5-HT transporter is present in the cerebral cortex when it contains serotonergic growth cones, but not serotonergic synapses. Specific uptake of 3H-5-HT was found in IGC fractions as early as E15; this uptake was inhibited by fluoxetine. Electron microscopic radioautography demonstrated directly that growth cones were the structures in IGC fractions that took up 3H-5-HT. The synaptic vesicle protein synaptophysin and a 45 kDa protein found specifically in serotonergic synaptic vesicles, serotonin-binding protein (SBP), were each enriched in IGC fractions from E15 to postnatal day 5; SBP immunoreactivity increased ~10-fold between E15 and E20. Endogenous 5-HT was detected in IGC fractions at E15 and increased in amount as development proceeded. The ratio of 5-HT to 5-hydroxyindole acetic acid suggested that 5-HT within growth cones is protected from catabolism by monoamine oxidase. Reserpine-induced depletion of 5-HT, a marker for the vesicular carrier of 5-HT, was apparent in IGC fractions at E20, but not at E15. These data suggest that properties that characterize the presynaptic components of mature serotonergic synapses develop in growth cones before synapses are formed. The early development of these properties may permit neurotransmission to be established rapidly during synaptogenesis or, alternatively, enable 5-HT to play a role in ontogeny.

[Key words: growth cones, growth and development, rat brain, 5-HT uptake, synaptic vesicles, 5-HT, GAP43, serotonin-binding protein, synaptophysin]

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The functional properties of the vertebrate nervous system are critically dependent on the connections between neurons that are established during development. Axonal growth cones are the structures that are responsible for enabling axons to find their targets. In order to do so, growth cones must exhibit motility, have a guidance mechanism, and recognize target structures (Landis, 1983; Lockerbie, 1987; Bray and Hollenbeck, 1988; Devoto, 1990). Following synaptogenesis, properties associated with neurotransmission become critical. In order to function synaptically, monoaminergic axon terminals must contain synaptic vesicles, which enable the neurotransmitter to be stored, protected from catabolism by monoamine oxidase (MAO), and secreted. The terminals must also exhibit a reuptake mechanism to inactivate released transmitter, which is associated with the presence of a specific transporter molecule in the plasma membrane (Blakely et al., 1991; Hoffman et al., 1991). After contact with the prospective postsynaptic structure has been made, therefore, growth cones must be either replaced or remodeled to give rise to the presynaptic components of synapses. A major difference between these two possibilities is that a replacement hypothesis predicts a relative absence of synaptic mechanisms in growth cones, while the remodeling hypothesis predicts their presence. Replacement and remodeling are not necessarily mutually exclusive. Synaptogenesis might involve adding some new structures to axon terminals, while others are retained. Previous studies have demonstrated that growth cones may contain neurotransmitters and their biosynthetic enzymes (McLaughlin et al., 1976; Gotow and Soteio, 1980; Westenbroek et al., 1988). Release of transmitters prior to the development of discernible synaptic contacts has also been observed (Hume et al., 1983; Young and Poo, 1983; Sun and Poo, 1987). What has not yet been established is whether or not the plasma membrane of growth cones is like that of mature presynaptic nerve terminals or whether growth cones contain synaptic vesicles. In order to study the degree to which growth cones share the neurotransmitter-related properties of presynaptic axon terminals, we analyzed the ontogeny of markers for the specific reuptake of 5-HT and for 5-HT-storing synaptic vesicles in isolated growth cone (IGC) fractions from developing rat brain. Serotonergic neurons were selected for this investigation, because although they are among the earliest neurons to develop in the mammalian brain, they do not form synapses until relatively late (Lauder and Bloom, 1974; Lauder et al., 1982; Lidov and Molliver, 1982a,b; Wallace and Lauder, 1983; Fujimiy et al., 1986). The delay in serotonergic synaptogenesis provides a window of opportunity during which the growth cones of serotonergic neurons can be investigated in the absence of synapses.

The precocious development of serotonergic neurons has led
to proposals that 5-HT plays a role in the developmental process itself (Lauder, 1991). 5-HT has been demonstrated to be able to act as a growth/differentiation factor (Mattson, 1988, Hannon et al., 1989; Lipton and Kater, 1989; Lauder, 1991). Whether or not 5-HT functions in the development of the nervous system, the presence of 5-HT in the fetal brain before synaptogenesis takes place is compatible with the idea that 5-HT is located in growth cones. If so, then growth cones might be expected to share properties of presynaptic terminals. Synaptic vesicles in growth cones, for example, could accumulate 5-HT and thus protect the amine from catabolism by MAO, which is present in the fetal rat brain (Levitt et al., 1985; Liu et al., 1987). Storage in synaptic vesicles and a reuptake mechanism might also enable 5-HT to play a role in developmental signaling. Finally, the occurrence of transmitter-related properties in growth cones would speed the process by which growth cones are converted into synapses. Extensive synthesis of new protein would not be required if critical elements of the transmitter apparatus were preassembled prior to the initiation of neurotransmission.

Previous studies of the development of 5-HT-related markers have concentrated on whole-brain homogenates (Loizou, 1972; Loizou and Salt, 1970; Hamon and Bourgoin, 1982; Liu et al., 1987; Herregodts et al., 1990) or synaptosomes (Tissari, 1975). None have been focused on the extent to which serotonergic mechanisms are present in growth cones. We have studied the presence of the plasma membrane transporter, which is responsible for the uptake of 5-HT, and 5-HT-storing synaptic vesicles in isolated growth cone fractions as a function of age. Markers for the 5-HT transporter included the specific binding of 3H-imipramine (Marcusson et al., 1986, 1989; Graham et al., 1990) and 3H-paroxetine (Habert et al., 1985; DeSouza and Kuyatt, 1987; Hrdina et al., 1990), as well as the ability to take up 3H-5-HT. Synaptic vesicle markers included synaptophysin, a protein found in the membrane of nearly all synaptic vesicles (Jahn et al., 1985; Wiedemann and Franke, 1985; Navone et al., 1986; Floor and Feist, 1989; Sudhof and Jahn, 1991) and a form (~45 kDa) of serotonin-binding protein (SBP), which is found only in the synaptic vesicles of serotonergic neurons and the secretory vesicles of other 5-HT-storing cells that are derived from neuroectoderm (Jonakait et al., 1979; Tamir and Gershon, 1979; Gershon et al., 1983; Gershon and Tamir, 1984, 1985; Barasch et al., 1987; Kirchgessner et al., 1988; Tamir et al., 1990). Finally, we investigated, as a function of age, the ability of reserpine, which blocks the transport of monoamines into synaptic vesicles (Carlsson, 1965; Giachetti et al., 1974; Shore and Giachetti, 1978; Maron et al., 1979; Angelides, 1980), to deplete 5-HT. Our data are compatible with the idea that components of the transmitter machinery are present in growth cones prior to synaptogenesis.

Materials and Methods

Animals. Timed-pregnant Sprague-Dawley female rats were used. The gestational age of fetal rats was counted from the day on which a vaginal plug was found (E0). Postnatal animals were kept with their mothers and were weaned at postnatal day 21 (P21). Pregnant dams and postnatal rats were anesthetized with methylxylutane or pentobarbital (Pitman-Moore Inc., Washington Crossing, NJ) and killed by decapitation.

Subfractionation of fetal and neonatal rat brains. Isolated growth cone (IGC) fractions were prepared by the procedure of Pfenninger et al. (1983). Briefly, brains from fetal or neonatal animals were gently homogenized at 4°C. The homogenates were centrifuged at 1660 × gmax (for 15 min) to obtain a low-speed supernatant (LSS). The LSS was layered onto a discontinuous sucrose gradient and centrifuged at 242,000 × gmax (for 40 min). The band at the load/0.75 M sucrose interface (the A fraction) was collected, diluted, and centrifuged at 39,000 × gmax. This pellet was then resuspended in a minimal volume of the buffer used for binding assays (120 mM NaCl, 5 mM KCl, and 50 mM Tris pH 7.4) containing aprotinin (Sigma Chemical Co., St. Louis, MO), and was kept frozen at -80°C until assayed. The IGC fraction collected from the load/0.75 M interface is contaminated by supernatant material. The IGC fraction was therefore diluted to a final concentration of 0.25 M sucrose (containing 1 mM MgCl2 and 1 mM Na3-hippurisulfonyl methyl-2-aminoethane-sulfonic acid, pH 7.3) and centrifuged (116,000 × gmax for 90 min) through a layer of 0.45 M sucrose. IGCs were collected above a cushion of Maxidens oil (Sigma Chemical Co.). 3H-5-HT was used to evaluate the degree to which IGC fractions were contaminated by soluble material. 3H-5-HT was added to the diluted IGC fraction immediately before it was subjected to centrifugation through 0.45 M sucrose. The amount of radioactivity present in the IGC fraction collected over Maxidens oil was then determined. Since the growth cones were exposed to 3H-5-HT in a medium that did not contain Na+, the temperature was 4°C, and membranes were impermeable to 5-HT, accumulation of 3H-5-HT within growth cones should be minimal. 3H-5-HT appearing in the material at the 0.45 M sucrose/Maxidens oil interface, therefore, provides an estimate of how much soluble material is trapped in the final IGC fraction. In the absence of 5-HT, the amount of 3H-5-HT in IGC fractions (in addition to added 3H-5-HT) was also measured. These measurements indicated that very little of the endogenous 5-HT found in IGC fractions could have been derived from supernatant contamination. In a typical experiment (E20), the concentration of endogenous 5-HT in the supernatant fraction was 11.8 ± 2.5 pmol/ml. On the basis of this value, and the volume of supernatant present in the fraction collected over Maxidens oil (estimated from the amount of 3H-5-HT recovered in this fraction), the supernatant was calculated to contribute 0.4 pmol of 5-HT to the IGC fraction. The purified IGC fractions, however, were observed to contain 10.4 ± 2.0 pmol of 5-HT per fraction. Supernatant contamination, therefore, is responsible for only about 4% of the 5-HT experimentally found in the purified IGC fraction.

Synaptosomes were prepared according to the method of Cohen et al. (1970) and kept at -80°C until used.

Preparation of fractions for electron microscopy. The fractions were processed for electron microscopy (EM) as described previously (Pfenninger et al., 1983). Briefly, aliquots of the various fractions were mixed with gradually increasing amounts of 1.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.3) to which 120 mM glucose and 0.4 M CaCl2 were added. The fixed material was pelleted, washed with arsenate buffer, stained en bloc with magnesium uranyl acetate, dehydrated, and embedded in Epon 812. Thin sections prepared from these pellets were doubly stained with uranyl acetate and lead citrate and examined with a JEOLCO 100S electron microscope.

Galactosyltransferase assay. In order to evaluate the relative contamination of the IGC fraction by membranes derived from the Golgi apparatus, activity in the IGC fraction of a Golgi marker, galactosyltransferase, was assayed and compared to that of the LSS fraction. Activity of the enzyme in the fractions was determined as previously described (Bretz and Staubli, 1977). Several concentrations of membranes were tested to make certain that the activity was measured within a linear range of enzyme activity. Blank values were determined in the absence of membranes.

Phospholipids. In order to determine the phospholipid content of fractions, lipids were extracted with chloroform/methanol (2:1) and the lower phase was used for measurement of phosphate. The protein concentration in the fractions was determined according to the method of Lowry et al. (1951). Proteins in the fractions were separated and their globular fibrillary acidic protein (GFAP) immunoactivity was analyzed on cellulose blots.

Assays of 3H-imipramine binding by rapid filtration. 3H-imipramine binding to brain fractions was measured by a modification of the method of Raisman et al. (1979). In order to construct saturation isotherms, the LSS was collected and centrifuged again (65,000 × gmax for 1 hr), and the pellet was resuspended in a minimal volume of the incubation buffer and diluted to a concentration of ~1.0 mg protein/ml. Twelve concentrations (0.3–10 nm) of 3H-imipramine (0.3–10 nm) were used. Nonspecific binding was estimated by using norzimelidine as a displacing agent (Marcusson et al., 1985). Specific binding was defined as total binding minus that found in the presence of 1 μM norzimelidine. The assay was initiated by adding 100 μg of membranes to each of the solutions containing 3H-imipramine (final volume, 0.3 ml/tube). Membranes were incubated for
60 min at 0–2°C. The reaction was terminated by adding 3 ml of ice-cold buffer and filtering the reaction mixture through Whatman GF/B filters that had been presoaked in incubation buffer containing 0.1% (v/v) polyethyleneimine to diminish nonspecific binding to the filters. A 24-

channel harp sampler (Brandel, Gaithersburg, MD) was used for the filtration and the filters were washed twice with 3 ml of ice-cold buffer. Radioactivity trapped by the filters was determined by liquid scintillation spectroscopy. For experiments in which the relative binding of 3H-imipramine was determined, one single concentration of 3H-imipramine, 6 nM (3 × 10^6 cpm), was employed.

Radiolabeled homogenate of fetal hippocampus and 3H-paroxetine. The binding of 3H-imipramine to sections mounted on glass slides was assessed radioautographically (Grabowsky et al., 1983). Briefly, fetal rats (E20) were perfused intracardially with ice-cold 0.1% formaldehyde solution in isotonic phosphate-buffered saline (pH 7.4 at 25°C) for 15 min. Following this treatment, the tissues were exposed to 4–10 nM 3H-imipramine in 50 mM Tris-HCl buffer containing 120 mM NaCl and 5 mM KCl for 60 min at 4°C. Nonspecific binding was determined after washing slides with 0.1% Triton X-100 and 0.05% Tween-20. In order to ensure that the incubating solution also contained 1.0 mM norzimelidine. Dependence of 3H-imipramine binding on Na+ was investigated by omitting NaCl from the incubation buffer to which additional adjacent sections were exposed. Following incubation, slides were washed with a quick dip, followed by rinsing with ice-cold buffer solution for 10 and then 20 min. Sections were incubated with a 1:1 dilution of primary antibodies (anti-GAP43) overnight. Immunoreactivity was detected with affinity-purified biotinylated goat anti-rabbit secondary antibodies (diluted 1:400; 1 hr; Vector Laboratories). The nitrocellulose blots were then incubated at room temperature. Labelled sections were exposed to 3H-sensitive film for 12–16 weeks at 4°C. Radioautograms were then analyzed quantitatively by computer-assisted video microdensitometry (Imaging Research Corp., St. Catherines, Ontario, Canada).

Uptake of 3H-5-HT by IGC fractions. For measurement of 3H-5-HT uptake, IGC fractions were prepared as described above; however, since intact growth cones were needed, the last centrifugation step was omitted. The resulting supernatant was collected and dialyzed against a large volume of Krebs solution saturated with a mixture of 95% O2 and 5% CO2 for 1.5 hr at 4°C. The dialyzed IGC fraction (450 μl containing ∼1 mg protein) was added to a tube that contained 3H-5-HT (0.5 μM final concentration). The tubes were incubated in a shaker bath for 5–10 min at 37°C in an atmosphere of 95% O2 and 5% CO2. Uptake of 3H-5-HT in the presence of fluoxetine (25 μM) was used to estimate nonspecific binding. As a second control, nonspecific uptake was also measured by incubating the IGC fraction with 3H-5-HT at 4°C. Reactions were terminated by adding 3 ml of ice-cold buffer to each tube and immediately thereafter filtering the contents through Whatman GF/B glass fiber filters. Filters were washed twice with 3 ml of ice-cold buffer and counted by liquid scintillation spectroscopy.

EM radioautography of 3H-5-HT. The A fraction at P3 was incubated with 3H-5-HT in the presence or absence of fluoxetine as described above. At the end of the incubation period, the fractions were cooled on ice in order to stop the uptake process, and then mixed gradually with a 10-fold greater volume (to dilute the free 3H-5-HT) of 1.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.3) containing 120 mM glucose and 0.4 M CaCl2. Further processing was as described above for routine electron microscopy. The gold-colored sections were cut and prepared for radioautography with Ilford L-4 emulsion as described previously (Erde et al., 1985). After 3 weeks of exposure under carbon, the grids were developed with Kodak Microdrol-N (3 min) and fixed. Grain distribution on electron microscopic radioautographs was analyzed by the method of Williams (1969). Essentially, probability circles, equal in diameter (240 nm) to 1.5 times the half-distance (the distance from a radioactive line within which 50% of developed silver grains will lie; Salpeter et al., 1969), were placed around each silver grain and potential radioactive sources within the circles were scored. A regular grid of similar circles placed over the fraction and scored similarly served to evaluate the distribution that would be expected if the grains were randomly distributed in the fraction. Relative specific activity of the components was defined as the percentage of grains associated with the component, divided by the percentage of random circles (the effective area of the circle) (diluted 1:400; 1 hr; Vector Laboratories)
Figure 1. An electron micrograph of an IGC fraction prepared from postnatal rat brain (P9). A, The fraction consists primarily of membrane-bound cellular fragments (arrows) of variable size (0.3-1.5 μm). There are no rough microsomes. B-D, At higher magnification, the cellular fragments can be seen to contain variously shaped vesicles (B, D), vacuoles (C, D), and mitochondria (D). Some (C) contain dense-cored vesicles. These fragments are the structures identified as IGC. Note the absence of internal ribosomes and the paucity of bundles of microtubules and microfilaments. The single large vacuolar structure in D is probably an invagination of the plasma membrane (Lockerbie et al., 1991). Scale bars: A, 2 μm; B–D, 0.5 μm.
in sample buffer (120 mM KCl, 20.0 mM NaCl, 2.0 mM NaHCO₃, 2.0 mM MgCl₂, 5.0 mM HEPES pH 7.4, and 0.7% Triton X-100), spotted onto a nitrocellulose support, rinsed with water, air dried, and fixed for 15 min in a solution containing 10% acetic acid and 25% isopropanol. Following fixation, the nitrocellulose supports were washed and processed as described above for immunoblots. Antibodies to synaptophysin (diluted 1:500 in blocking solution containing 0.1% Triton X-100), were abundant. These fragments contained mitochondria, microfilaments, and irregularly shaped vesicles and vacuoles. As previously noted (Luccarbiec, 1990), internal membranes, which have been interpreted to be elements of smooth endoplasmic reticulum, were numerous. The cellular fragments lacked internal ribosomes (suggesting that they are derived from axons and not dendrites or glia) or assembled arrays of microtubules (suggesting that they are derived from terminals and not axonal shafts). Importantly, no evidence of myelin contamination was visible in electron micrographs of IGC fractions at P3 and almost no myelin was seen at P9.

The IGC fraction contains little GFAP: mature synaptosomes do not co-sediment with growth cones. The suggestion that the cellular fragments were not derived from glia was supported by immunoblots probed with antibodies to GFAP (data not illustrated). Almost no GFAP immunoreactivity was detected in the IGC fraction, but was present in small amounts in the LSS from which the IGC fraction was obtained. Although the accumulation of small vesicles in a few of the cell fragments caused them to resemble synaptosomes, adherent postsynaptic membranes, which characterize synaptosomes, were not observed; nevertheless, experiments were carried out to determine where mature synaptosomes would have sedimented if they had been present in the homogenates of developing brain. Synaptosomes were prepared from adult brain and labeled by incubation for 10 min with ³H-5-HT (0.5 μM). The ³H-3-HT-labeled synaptosomes were mixed with the LSS obtained from the brain of a rat at E20 and the IGC fraction was prepared in the usual manner. ³H-5-HT, which leaked from the synaptosomes, was recovered in the supernatant (~36% of the recovered ³H-5-HT), while most of the remaining ³H-5-HT (~58%) sedimented in the “C” band, which is collected at the interface between 1.0 and 2.55 M sucrose. Very little ³H-5-HT (~6%) was found in the remaining IGC and “B” fractions. Mature synaptosomes would thus not be likely to sediment with growth cones in the IGC fractions, even if they were to be present in the developing material subjected to fractionation. Instead, mature synaptosomes would probably be found in the heavier and very heterogeneous “C” fraction.

The IGC fraction is enriched in membranes but myelin and Golgi contamination is minimal. An identifying feature of growth cones is their extensive system of internal membranes; therefore, the phospholipid content of the fractions was measured and used as a membrane marker (Fig. 2). The IGC fraction was found to contain significantly more phospholipid per unit of protein than did the LSS, the “C” fraction, or synaptosomes from adult brain. Since the phospholipid-to-protein ratio of myelin fragments would also be high, the potential contamination of the IGC fraction by myelin was assayed as a function of age (although almost no myelin was visible in electron micrographs as late as P9). Immunoblots were prepared and probed with antibodies to myelin basic protein (MBP). Little or no MBP immunoreactivity was detected in the IGC fractions between E15 and P3 (not illustrated); however, at P27 this fraction contained a great deal of MBP immunoreactivity. The high lipid-to-protein ratio found in the IGC fraction on P3 and earlier therefore cannot be ascribed to myelin. The absence of MBP immunoreactivity also implies that the IGC fractions contain little or no membrane derived from oligodendrocytes. Another potential source of membranous material that might sediment like growth cones is the Golgi apparatus. A significant proportion of the membranous material visible in electron micrographs of the IGC fraction (~19% of the volume of the fraction) could not be morphologically identified, because the particles either were cut tangentially or lacked sufficient ultrastructural detail; therefore, the extent of contamination by Golgi-derived membranes was estimated biochemically. Galactosyltransferase ac-
tivity was assayed (at E20, P3, and P9) and served as a Golgi marker (Table 1). No enrichment of galactosyltransferase activity in the IGC fraction was observed at any of these ages. In fact, the relative specific activity of galactosyltransferase was significantly lower in the IGC than in either the LSS or the two heavier subfractions (B and C). It is concluded that the IGC fraction is unlikely to contain mature synaptosomes, has a high phospholipid-to-protein ratio, is impoverished in Golgi-derived elements, and is predominantly composed of cellular fragments, the structure of which is consistent with a derivation of these fragments from axonal growth cones. The absence of ribosomes, GFAP, and MBP immunoreactivities supports the idea that the cellular fragments in this fraction (between E15 and P3) are derived from axons, rather than from astrocytes or oligodendrocytes.

Table 1. Distribution of galactosyltransferase in developing brain fractions

| Fraction | Protein (% Rec) | GalTase activity (% Rec) | RSA* | Protein (% Rec) | GalTase activity (% Rec) | RSA* | Protein (% Rec) | GalTase activity (% Rec) | RSA* |
|----------|----------------|-------------------------|------|----------------|-------------------------|------|----------------|-------------------------|------|
| LSS      | 100            | 100                     | 1.00 | 100            | 100                     | 1.00 | 100            | 100                     | 1.00 |
| IGC      | 2.4            | 0.8                     | 0.45 ± 0.05 | 2.3 | 1.4 | 0.59 | 4.9 | 2.0 | 0.40 |
| B        | 8.5            | 8.0                     | 1.15 ± 0.1 | 8.9 | 24.2 | 2.23 | 12.0 | 11.8 | 0.98 |
| C        | 23.8           | 21.2                    | 1.48 ± 0.2 | 20 | 34.1 | 1.33 | 34.9 | 33.3 | 0.95 |
| Total    | 34.6           | 30.0                    | -     | 31.3 | 59.7 | -     | 51.8 | 47.1 | -    |

Data show relative specific activity (RSA) of galactosyltransferase (GalTase) in IGC, B, and C fractions. Protein and GalTase activity measured in each fraction are expressed as percentage of LSS (% Rec). 100% of the enzyme activity and the protein applied to the gradient (i.e., LSS) was recovered when the material remaining in the gradient after the fractions were collected was also assayed. RSA = GalTase % rec/protein % rec.

* RSA values for E20 are an average ± SE of five different experiments, whereas the other values in the table are derived from one experiment.

"H-imipramine binding sites in subcellular fractions of developing and adult brain are comparable

"H-imipramine binding is a marker for the plasma membrane 5-HT transporter in adult animals (Marcusson et al., 1986, 1989; Graham et al., 1989); however, in order to use this parameter as such a marker in developing animals, it is necessary to show that the "H-imipramine binding sites in the developing brain are comparable to those of adults. "H-imipramine binding was therefore characterized in the developing brain. Initial experiments were carried out with synaptosomes isolated from adult rat brains and with the LSS fractions from fetal brains (El 5 and E20) in order to determine optimal parameters for analysis of the binding of "H-imipramine. "H-imipramine binds only to neuronal membranes; therefore, the LSS fraction can be used, despite its heterogeneity, to characterize "H-imipramine binding to membranes from the developing brain. When fractions were incubated with "H-imipramine (6.0 nM) at 4°C for 1 hr, specific binding was found to increase linearly as a function of protein concentration in the range of 20–140 µg of protein for synaptosomes and 60–210 µg protein for the LSS fractions. Specific binding of "H-imipramine obtained under these conditions was approximately 43% of the total binding and represented 1–3% of the total cpm added to the incubation medium. All subsequent assays, therefore, were conducted at protein concentrations of 80–120 µg/tube.

When binding of "H-imipramine to membranes derived from developing and adult rat brains was measured as a function of the "H-imipramine concentration, saturable, high-affinity, specific binding was found (Fig. 3). Computer-assisted nonlinear least-squares analysis (LUNDON-L) of the saturation isotherms indicated that the data were best described by a model that assumes a single population of 3H-imipramine binding sites; moreover, a linear Scatchard plot was obtained and the Hill coefficient (0.97) was close to unity. The Bmax and Ks values computed at different ages are summarized in Table 2. Note that specific high-affinity "H-imipramine binding sites could already be detected at E15. At this time the Ks for "H-imipramine binding was not significantly different from that determined for adult synaptosomes. The Ks, furthermore, was not found to change significantly during ontogeny. At E15, the Bmax for "H-imipramine binding was about 18% of that of adult synaptosomes. The Bmax increased at later ages and by P15 was about 60% of that of the adult synaptosomal value. Since only a single high-affinity "H-imipramine binding site was found with a Ks that did not change significantly during ontogeny, it is concluded that "H-imipramine binding sites in the developing brain are comparable to those in the brains of adults.

"H-imipramine binding sites are enriched in IGC fractions early in development

The binding of "H-imipramine (6 nM) was measured in LSS and IGC fractions derived from developing rat brain as a function of developmental age (Table 3). On E15, "H-imipramine binding in the IGC fractions was enriched 3.7-fold (p < 0.05) over that found in the LSS fractions from which the IGC fractions were derived. This enrichment persisted on E20 and was 2.4-fold greater in the IGC than in the LSS fractions (p < 0.05). After E20 the IGC fraction no longer exhibited significant enrichment in "H-imipramine binding sites. In fact, the binding of "H-imipramine in the IGC fraction declined sharply postnatally and by P27 was only 25% of that found on E20 (p < 0.01). As a control, the binding of "H-imipramine was assayed in the heavier “C” fractions, obtained from the same material from which the IGC fractions were isolated, and in which mature synaptosomes would be found had they been present (Table 3). At none of the ages tested was "H-imipramine binding significantly greater in the “C” band than in the LSS.

The developing rat cortex contains "H-imipramine and "H-paroxetine binding sites, when serotonergic growth cones are present prior to synaptogenesis, at E20

Previous studies have established that serotonergic axons reach the cerebral cortex prior to, or on, E20; however, these axons do not form synapses at this time (Lidov and Molliver, 1982a).
Synaptogenesis occurs mainly during postnatal periods. Since the cerebral cortex at E20 contains serotoninergic growth cones, but not synapses, the presence of the 5-HT transporter in the cortex at this age would support the hypothesis that the transporter is present in growth cones. Radioautography was used to analyze the location of potential 5-HT uptake sites in prenatal rat brain at E20. Both 3H-imipramine (±1.0 μM norzimelidine) and 3H-paroxetine (±30 μM fluoxetine) were used as probes. Specific 3H-imipramine and 3H-paroxetine binding sites were heterogeneously distributed in the brain at day E20. Binding sites were most concentrated in the region of the median raphe, septal nuclei, and the hypothalamus, but were also clearly present in the hippocampus and cerebral cortex (cingulate, frontal, primary olfactory areas and the hippocampus) at this age (Figs. 4, 5). The binding of 3H-imipramine to cortical sites was found to be highly dependent on the presence of Na+ (Fig. 5). When NaCl was omitted from the incubating solution, binding was greatly reduced.

**IGC fractions specifically accumulate 3H-5-HT**

In order to confirm that the 5-HT transporter is present on the plasma membrane of serotoninergic growth cones, accumulation of 3H-5-HT was measured in IGC fractions prepared from rat brain at E15, E20, and P3. Inhibition of 3H-5-HT accumulation by fluoxetine, which blocks the uptake of 5-HT, but not that of norepinephrine, was used to define the specificity of 3H-5-HT uptake. The further addition of DMI (0.1 μM), a drug that specifically blocks the uptake of norepinephrine, produced no additional antagonism of the accumulation of 3H-5-HT. No specific uptake of 3H-5-HT was observed when IGC fractions were incubated at 0-4°C. In contrast, when the assay was conducted at 37°C, IGC fractions specifically accumulated 3H-5-HT for 5 min, after which equilibrium was reached. Specific uptake of 3H-5-HT was observed in IGC fractions as early as E15 and increased significantly between E15 and E20 (Fig. 6); however, no further increase was observed between E20 and P3. These data suggest that transmembrane transport of 3H-5-HT develops in IGC together with 3H-imipramine/3H-paroxetine binding sites.

**Growth cones are the structures in IGC fractions that are responsible for uptake of 3H-5-HT**

The structures responsible for the uptake of 3H-5-HT in the IGC fraction at P3 were identified by EM radioautography. Fractions were incubated with 3H-5-HT as above; however, they were fixed so as to retain intracellular, but not extracellular, 3H-5-HT (Gershon and Ross, 1966). As a control, the IGC fraction was incubated with 3H-5-HT in the presence of fluoxetine. The grain distribution on the resulting EM radioautographs (Fig. 7) was analyzed statistically by the method of Williams (1969). For this purpose, three components were considered: (1) growth cones, (2) unoccupied space, and (3) unoccupied space.
identified cell fragments (non-growth cones). A χ² analysis was used to compare the number of silver grains associated with each of these components with the number predicted for a random distribution. The number expected for a random distribution was based on the measured effective areas of each item. The actual distribution of silver grains was found to differ significantly (p < 0.0001) from random (Table 4). Only growth cones were associated with a significantly larger than random number of silver grains. The number of grains associated with space and structures that were not identifiable as growth cones was significantly less than that expected for a random distribution. The distribution of silver grains on EM radioautographs of sections of IGC fractions incubated with [³H]-5-HT in the presence of fluoxetine also differed significantly (p < 0.01) from that expected for a random distribution; however, in the presence of fluoxetine, the difference from random was due to a greater than expected abundance of silver grains associated with space, not growth cones. These data confirm that growth cones are the structures in the IGC fraction responsible for the specific (fluoxetine-sensitive) accumulation of [³H]-5-HT.

5-HT, but not 5-HIAA, is retained within the IGC fraction

Levels of 5-HT and 5-HIAA were measured in LSS and IGC fractions of developing pre- and postnatal rat brains. An extra step was added to the fractionation procedure in order to be certain that IGC fractions were not contaminated with supernatant material. This problem is important with regard to 5-HT because it is soluble. Breakage of 5-HT-containing cellular constituents would thus lead to the appearance of 5-HT in the supernatant fraction. Growth cones were therefore separated from supernatant material with a sucrose step gradient. The initial IGC fraction was diluted and loaded onto a two-step gradient consisting of a layer of 0.45 M sucrose buffer and a cushion of Maxidens oil. Growth cones could thus be collected relatively quickly and were washed free of soluble contaminants as they sedimented through the 0.45 M sucrose layer. EM examination of the fraction collected over the Maxidens oil (data not illustrated) revealed its morphology to be essentially that of the IGC fractions described previously. Experiments in which known amounts of [³H]-5-HT were added to IGC fractions (under conditions that prevented uptake of [³H]-5-HT) indicated that the purified IGC fraction was not significantly contaminated by material from the supernatant (see Materials and Methods).

5-HT and 5-HIAA were found to be present in both LSS (5-HT, 5.8 ± 0.3 pmol/mg; 5-HIAA, 6.0 ± 0.4 pmol/mg) and IGC (5-HT, 4.5 ± 0.2 pmol/mg; 5-HIAA, 2.4 ± 0.8 pmol/mg) fractions as early as E15 (Fig. 8A). The concentrations of 5-HT in each of these fractions increased as a function of age during later development through P9. The increase in the levels of 5-HT in the IGC fractions (2.8-fold) was greatest between E15 and E20. The 5-HT concentration in the IGC fraction at P9 was significantly higher as a function of protein than that of synaptosomes prepared from adult brain (p < 0.05). Although the levels of 5-HT in the IGC fraction did not differ significantly from that of the LSS fraction at any of the ages tested, the concentration of 5-HIAA was significantly lower in the IGC fraction than in the LSS fraction (p < 0.05) at all ages after E15 (Fig. 8B). As a result, the ratio of 5-HT to 5-HIAA was substantially higher in the IGC than in the LSS fraction (Fig. 8C). A similarly high ratio of 5-HT to 5-HIAA was found in synap-

| Table 3. Enrichment of [³H]-imipramine binding sites in IGC fractions |
|-------------------------|----------------|----------------|
| Age        | LSS            | IGC         | C band |
| E15        | 38.36 ± 5.9    | 140.97 ± 34.0 | 47.69 ± 10.5 | 3.7* | 1.24 |
| E20        | 95.48 ± 6.6    | 228.43 ± 37.2 | 78.81 ± 11.7 | 2.4* | 0.83 |
| P3         | 134.30 ± 9.5   | 115.92 ± 37.5 | 93.43 ± 5.9  | 0.86 | 0.70 |
| P7         | 133.12 ± 10.2  | 130.04 ± 29.7 | 84.54 ± 9.4  | 0.98 | 0.64 |
| P27        | 110.49 ± 39.9  | 59.36 ± 19.5  | 101.38 ± 55.2 | 0.54 | 0.92 |

Data show enrichment of [³H]-imipramine binding in the growth cone fraction, and are shown relative to [³H]-imipramine binding in the LSS as a function of developmental age. The concentration of [³H]-imipramine was 6 nM; nonspecific binding was estimated in the presence of 1 μM norzimelidine. Each experiment was carried out in triplicate. The values represent means of three to five independent experiments. The C band was the fraction obtained at the 1.0-2.55 M sucrose interface. Means were compared statistically by an ANOVA.

* [³H]-imipramine binding in the IGC fraction was significantly enriched over that of the LSS (p < 0.05).

Figure 4. Photographs of computer-assisted video images of sections through E20 rat brain. The densities of [³H]-imipramine (A, B) or [³H]-paroxetine (C-F) binding sites are represented in pseudocolor. Black, background; red, high density; purple, low density. A and B, Adjacent sections incubated with 10 nM [³H]-imipramine in the absence (A) or presence (B) of 1.0 μM norzimelidine. The brain has been sectioned in an oblique plane, so the right half of each section is anterior to the left. Note that [³H]-imipramine binding is displaced by norzimelidine. [³H]-imipramine binding sites are concentrated in deeper layers of the frontal cortex. Less dense, but still specific, binding of [³H]-imipramine is observed in the anterior olfactory area, olfactory tubercle (OtU), and the cingulate cortex (Cg). P comprises frontal cortex; Ins, insular cortex; PrF, piriform cortex; Acc, accumbens nucleus; CPu, caudate putamen. C-F, [³H]paroxetine binding sites in sections of fetal rat brain (E20). Sections were incubated with [³H]-paroxetine (1.0 nM). Adjacent sections (illustrated in F) were incubated with [³H]-paroxetine (1.0 nM) and fluoxetine (30 μM). C. A coronal section reveals a high density of [³H]-paroxetine binding sites in the cingulate cortex (Cg), anterior septum (S), and anterior olfactory area (Aoa). D, A coronal section reveals a high density of [³H]-paroxetine binding sites in the lateral region of the posterior hypothalamus (P) where the fibers of the median forebrain bundle (mfb) are located. E, A mid sagittal section reveals a high density of [³H]-paroxetine binding sites in the brainstem and diencephalon (D). A particularly high level of [³H]-paroxetine binding sites is present in the region of the raphe nuclei (arrow). A lower density of binding sites is observed in the frontal and posterior poles of the cerebral cortex (arrowheads). F, The binding of [³H]-paroxetine is almost completely displaced by fluoxetine. Cb, cerebellum; DR, dorsal raphe; T, tegmentum; Th, thalamus.
tosomes isolated from adult rat brains. These data suggest that 5-HT, but not 5-HIAA, is retained in growth cones.

**Depletion of endogenous 5-HT by reserpine**

The sensitivity of endogenous stores of 5-HT to depletion by reserpine was used as an indicator of the presence of vesicles that sequester 5-HT. The membrane of such vesicles can be assumed to contain the amine carrier (Liu et al., 1992), inhibition of which is known to be the action of reserpine. In the adult rat brain, a single injection of reserpine (5 mg/kg) causes a maximal depletion of 5-HT (~90%) within 4 hr (Carlsson, 1965). This effect is stable for at least 36 hr, after which the level of 5-HT begins to recover. In the present experiments, therefore, reserpine (5 mg/kg) was administered to pregnant dams 16 hr before they were killed. Although at E15 reserpine significantly \( p < 0.001 \) lowered the 5-HT level in the LSS fraction (Fig. 9A), reserpine failed to deplete 5-HT from the IGC fraction (Fig. 9B). In contrast to the results obtained at E15, at E20 \( p < 0.02 \) and P3 \( p < 0.02 \) reserpine significantly reduced the levels of 5-HT in IGC fractions (Fig. 9B). No reserpine-induced change in the levels of 5-HIAA in IGC fractions could be detected at any age until P3, at which time a significant rise \( (8.7 \pm 0.6 \) pmol/mg protein to 31.3 \( \pm 4.6 \) pmol/mg protein; \( p < 0.0001 \)) was detected. These data indicate that a reserpine-deletable pool of 5-HT develops in serotonergic neurons by E15 and, between E15 and E20, appears in their growth cones.

**SBP immunoreactivity in brain fractions**

SBP immunoreactivity in rat brain fractions at E15, E20, P3, and P27 was assayed on immunoblots using monospecific polyclonal antibodies directed against the ~45 kDa form of SBP. One major band of protein, corresponding in electrophoretic mobility to 43 kDa, reacted with these antibodies (Fig. 10). An additional minor band with an electrophoretic mobility corresponding to 32 kDa was also immunoreactive. Both of these bands were detectable in LSS and IGC fractions as early as E15. Immunoreactivity in each of these fractions was more intense, however, at E20 and P3. At these ages, the immunoreactivity of the IGC fraction appeared to be greater than that of the LSS. In contrast, while the immunoreactivity of the 43 and 32 kDa bands was also present at P27, that of the IGC fraction was now less than that of the LSS fraction or of synaptosomes (or the crude P, fraction from which synaptosomes are derived). SBP immunoreactivity was also found in synaptosomes isolated from mature brains.

The distribution of the 32 kDa band in subcellular fractions appeared to be similar to that of the major immunoreactive 43 kDa band. The 32 kDa material also cross-reacted with three
other polyclonal antibodies and one monoclonal antibody (Liu et al., 1990) prepared against ~45 kDa SBP (data not shown). These observations suggest that the 32 kDa minor band may be a product of the degradation of the 43 kDa protein. The amount of SBP immunoreactivity present in the fractions was estimated from the immunoblots with 125I-protein A. Separate determinations were made for the 43 and 32 kDa bands. Values were normalized to that detected in the LSS fraction at E15 (the lowest detected) in each experiment. The sum of the immunoreactivities of the 32 and 43 kDa bands was found to yield relative values similar to those of the 43 kDa band itself. We therefore considered the 32 kDa band to be derived from the 43 kDa material and added the immunoreactivities of each to estimate the total SBP immunoreactivity present in each frac-

Figure 7. EM radioautographs of an IGC fraction (obtained from a rat brain at P3) that was incubated with 3H-5-HT. A–C. Note that the radioautographic silver grains are found over membrane-enclosed cytoplasmic fragments that resemble growth cones. The distribution of radioautographic silver grains is analyzed in Table 4. Scale bars, 0.5 μm.
Table 4. $^{3}$H-5-HT radioautographic grain distribution in IGC fraction from rat brain at P3

| Structure                | Grains expected (random distribution) | Grains observed | RSA       |
|--------------------------|---------------------------------------|----------------|-----------|
| $^{3}$H-5-HT             |                                       |                |           |
| Growth cones             | 144                                   | 778            | 144.69    | 1.93      |
| Space                    | 152                                   | 65             | 49.80     | 0.43      |
| Non-growth cones         | 64                                    | 17             | 34.52     | 0.27      |
|                          |                                       |                | $\Sigma = 209.01$ | $p < 0.0001$ |
| $^{3}$H-5-HT + fluoxetine (25 μM) |                                       |                |           |
| Growth cones             | 75                                    | 50             | 8.33      | 0.67      |
| Space                    | 58                                    | 92             | 19.93     | 1.6       |
| Non-growth cones         | 19                                    | 10             | 4.26      | 0.52      |
|                          |                                       |                | $\Sigma = 32.53$, | $p < 0.01$ |

Data show statistical analysis of the distribution of silver grains in EM radioautographs. IGC fractions incubated with $^{3}$H-5-HT alone are compared with material incubated with $^{3}$H-5-HT + fluoxetine. Grain distribution was compared with that of random circles by means of $\chi^2$. The relative specific activity (RSA) is defined as the percentage of grains associated with a component divided by the percentage of random circles associated with the same component. Areas occupied by the different components were estimated by point count planimetry. For the fractions incubated with $^{3}$H-5-HT alone, 360 silver grains, 1176 circles, and 3075 points were included in the analysis. For the fractions incubated with $^{3}$H-5-HT in the presence of fluoxetine, 152 silver grains, 873 circles, and 1740 points were included in the analysis.

As was done with SBP, values were normalized to that detected in the LSS fraction at E15 (the lowest detected) in each experiment. These data, which include P15, are summarized in Table 6. Note that, as was also observed in studies of SBP immunoreactivity, synaptophysin immunoreactivity increased considerably between E15 and P27; the most pronounced increase again occurred postnatally (the increase between E20 and P27 is ~ ninefold). On E15, E20, and P3 the IGC fraction contained a significantly higher level of SBP immunoreactivity than did its corresponding LSS fraction. The amount of SBP-immunoreactive material in the IGC fraction declined relative to that in the LSS at P27. At P27 serotonergic synaptogenesis is largely complete (Lidov and Moliver, 1982a); therefore, at P27 and later, SBP should be found in synaptosomes instead of growth cones. At P27 SBP was found in synaptosomes, where its level was about 70 fold higher than that detected in the reference LSS fraction from E15 fetal brain.

Syntophysin immunoreactivity in brain fractions

Syntophysin (p38) immunoreactivity in rat brain fractions at E15, E20, and P15 was assayed on immunoblots using monoclonal antibodies (Jahn et al., 1985). One major band of protein, corresponding in electrophoretic mobility to ~38 kDa, reacted with these antibodies (Fig. 11). An additional minor band with an electrophoretic mobility corresponding to ~76 kDa was also immunoreactive. Syntophysin immunoreactivity was detectable in LSS and IGC fractions as early as E15 (inset of Fig. 11). Immunoreactivity in each of these fractions was more intense, however, at E20. At E15 and E20 the immunoreactivity of the IGC fraction appeared to be greater than that of the LSS. In contrast, syntophysin immunoreactivity in the IGC fraction at P27 was less than that of the LSS fraction and that of synaptosomes (which could be obtained at this age). The level of syntophysin immunoreactivity in fractions of developing brain was estimated by dot immunobinding.
Figure 9. Effect of reserpine (5.0 mg/kg) on the level of endogenous 5-HT. A, LSS fraction; B, IGC fraction. At E15 reserpine depleted 5-HT from the LSS, but not the IGC fraction. After E20 reserpine depleted 5-HT from both fractions. Values represent means ± SE of three independent experiments, each carried out in duplicate.

GAP43 immunoreactivity in brain fractions

The immunoreactivity of GAP43 ("neuromodulin") in IGC fractions at E15, E20, P3, and P27 was assayed on immunoblots using monoclonal antibodies (Goslin et al., 1988, 1990). For comparison, synaptosomal fractions from P3 and adult brains were also assayed. GAP43 was analyzed because it is known to be associated with axonal growth and to be present in axonal growth cones (Meiri et al., 1986; Skene et al., 1986). One band of protein, corresponding in electrophoretic mobility to ~57 kDa, reacted with these antibodies (Fig. 12). GAP43 immunoreactivity was found in the IGC fraction at days E15 and E20. At both of these ages, GAP43 immunoreactivity was enriched in the IGC fraction relative to the LSS fraction. In contrast, very little GAP43 immunoreactivity could be found in either the IGC or synaptosomal fraction at P27.

Discussion

The primary objective of this study was to determine whether specific characteristics of mature serotonergic axon terminals are present in the growth cones of these neurons prior to synaptogenesis. We thus attempted to determine whether or not the plasma membrane of the growth cones of developing serotonergic neurons contains the specific 5-HT transporter and whether or not the growth cones store 5-HT in vesicles of the synaptic type. The 5-HT transporter is found specifically in

| Developmental age | LSS | IGC | P3 | Synaptosome |
|------------------|-----|-----|----|------------|
| E15              | 1.0 | 3.5 ± 0.9* | —  | —          |
| E20              | 3.5 ± 0.4 | 16.9 ± 1.1* | —  | —          |
| P15              | 10.4 ± 1.7 | 23.6 ± 2.7* | 15.4 ± 0.1 | 19.0 ± 0.7 |
| P27              | 31.0 ± 0.7 | 21.5 ± 0.3* | 59.7 ± 10.5 | 68.3 ± 8.2 |
| Adult            | —   | —   | —  | —          |

Data show SBP immunoreactivity in brain fractions was analyzed by measuring the amount of 125I-protein A bound to SBP-immunoreactive bands on Western blot. Values were normalized to that of the LSS fraction at E15 within each experiment. Means ± SE of three independent experiments are given. The data were evaluated by means of Student's t test.

* Significantly (p < 0.05) different than the corresponding LSS fraction.
serotonergic neurons (Ross, 1982; Blakely et al., 1991; Hoffman et al., 1991). In contrast, the amine transporter in the membranes of serotonergic synaptic vesicles is not 5-HT specific, and is common to neurons that utilize a monoamine as their transmitter (Liu et al., 1992). The synaptic vesicles of serotonergic neurons differ, however, from those of other types of monoaminergic neuron in that only the synaptic vesicles of serotonergic neurons contain 45 kDa SBP (Jonakait et al., 1979; Tamir and Gershon, 1979; Gershon et al., 1983; Gershon and Tamir, 1984, 1985; Barasch et al., 1987; Kirchgessner et al., 1988; Tamir et al., 1990). In order to obtain growth cones for study, developing rat brains were homogenized and IGC fractions were prepared. The IGC fraction was characterized first; subsequently, markers were evaluated in the IGC fraction for the specific plasma membrane 5-HT transporter and for serotonergic synaptic vesicles.

The IGC fraction was characterized by EM because there are no accepted biochemical markers for growth cones. Preparations obtained from postnatal animals were found to be morphologically similar to the prenatal preparations described earlier (Plenninger et al., 1983; Lockerbie, 1990). The IGC fractions were highly enriched in cellular fragments, the appearance of which resembled that of growth cones in situ (Cheng and Reese, 1985; Bray and Hollenbeck, 1988). The absence of internal ribosomes and arrays of intermediate filaments as well as the relative paucity of GFAP immunoreactivity suggested that the cellular fragments were primarily derived from axons and not glia. Since bundles of microtubules were also lacking in the IGC cellular fragments, the fragments are probably derived mainly from the terminal ends of axons and not their shafts. The relatively high phospholipid-to-protein ratio found in the IGC fraction is also consistent with the idea that the fraction contains growth cones, which are rich in internal membranes. These membranes, moreover, were not derived from either the endoplasmic reticulum or the Golgi apparatus, since markers for these organelles (rough microsomes and galactosyltransferase activity) were impoverished in the IGC fraction. Furthermore, the experimental result (see discussion below), that the neuronal

Table 6. Synaptophysin immunoreactivity in rat brain subcellular fractions during development

| Developmental age | Relative binding of 125I-protein A to subcellular fractions |
|-------------------|-----------------------------------------------------------|
|                   | LSS   | IGC   | P2    |
| E15               | 1.0 ± 0.2 | 3.4 ± 0.4* | - |
| E20               | 4.6 ± 0.3 | 7.5 ± 0.7* | - |
| P15               | 16.0 ± 1.6 | 20.7 ± 2.6 | 25.3 ± 2.9 |
| P27               | 22.2 ± 1.9 | 10.4 ± 0.5* | 30.2 ± 3.8 |
| Adult             | -     | -     | 44.4 ± 2.5 |

Synaptophysin immunoreactivity in brain fractions was analyzed by measuring the amount of 125I-protein A bound in a dot immunobinding assay. Values were normalized to that of the LSS fraction at E15 within each experiment. Means ± SE of three independent experiments are given. The data were evaluated by means of Student's t test.

* Significantly (p < 0.05) different than the corresponding LSS fraction.
5-HT transporter is concentrated in the IGC fraction, also indicates that this fraction is primarily derived from neurons. The enrichment, relative to the LSS, of GAP43 found in IGC fractions at E15 and E20 supports the conclusion based on morphological evidence that growth cones are concentrated in IGC fractions. The loss of GAP43 from the IGC fraction at P27 is consistent with the idea that growth cones are no longer present in the fraction at this age. Finally, when mature synaptosomes were added to homogenates they did not sediment in the IGC fraction. The development of mature synapses, therefore, is unlikely to confound studies that utilize IGC fractions to investigate the properties of growth cones. It was thus concluded that IGC fractions are suitable for the study of axonal growth cones.

The binding of 3H-imipramine or 3H-paroxetine was used to detect the 5-HT transporter. The sites to which 3H-imipramine binds with high affinity and Na+ dependence have been shown to detect the 5-HT transporter. Specific binding of 3H-imipramine was detected at E15, the earliest age tested, and at all ages thereafter. The $B_{max}$ for 3H-imipramine binding increased as a function of age, while the $K_d$ did not change; therefore, development is evidently associated with an increase in the number of binding sites and not with a change in binding affinity. Only a single class of high-affinity binding sites was observed, the Hill coefficient was close to 1, and the values for $K_d$ (1.5–2.65 nm) were not significantly different from values reported for 3H-imipramine binding in adult rat (~4 nm; Marcusson et al., 1986, 1988) or human brain (2.8 nm; Bäckström and Marcusson, 1987). The similarity of the properties of 3H-imipramine binding in the developing brain to that of adults suggests that 3H-imipramine binding is a marker for the 5-HT transporter in developing as well as adult brain.

Specific high-affinity 3H-imipramine binding sites were observed to be enriched in the IGC fraction at E15 and E20, ages that precede the formation of serotonergic synapses in the forebrain (Lidov and Molliver, 1982a). Measurement of the binding of 3H-imipramine in fractions was supplemented by the radioautographic localization of 3H-imipramine and 3H-paroxetine binding sites in sections of brain. E20 was selected for detailed radioautographic study because it is a time when serotonergic axons have been reported to be present in the cortex, but have not yet begun to form synapses (Lidov and Molliver, 1982a). At E20, therefore, the cortex should contain growth cones, but not synapses. As a result, the observation, even at the light microscopic level, of 3H-imipramine and 3H-paroxetine binding sites in the cortex at E20 would suggest that growth cones contain the 5-HT transporter. Qualitatively similar results were obtained with 3H-imipramine and 3H-paroxetine, although with 3H-paroxetine more than 90% of the total binding was found to be specific. The pattern of 3H-paroxetine binding sites observed by radioautography corresponded closely to the distribution of serotonergic axons previously described by histo-fluorescence (Seiger and Olson, 1973) or immunohistochemistry (Lidov and Molliver, 1982a). As expected, at E20 3H-paroxetine binding sites were most concentrated within the region of the median raphe, where serotonergic terminals are highly concentrated (Lidov and Molliver, 1982b; Kückelmeier et al., 1988). In addition to the raphe, however, significant 3H-imipramine and 3H-paroxetine binding was also observed more rostrally, in locations that included the cerebral cortex. These observations are consistent with the idea that 3H-imipramine/3H-paroxetine binding sites, and thus the 5-HT transporter, are present on or in the growth cones of serotonergic axons.

Since serotonergic neurons are among the earliest neurons of the rat brain to differentiate and extend axons (Olson and Seiger, 1972; Seiger and Olson, 1973; Lauder and Bloom, 1975), it was anticipated that subcellular fractions prepared from brains of fetal animals early in development would contain proportionally more serotonergic elements than would similar fractions prepared from brains at later ages. As development proceeds and nonserotonergic neurons come to comprise an increasing proportion of the brain parenchyma, the degree to which the content of subcellular fractions is derived from serotonergic elements would be expected to diminish. With respect to the IGC fraction, this relative decline would accentuate that caused by the formation of serotonergic synapses. Synaptosomes, when they were added to homogenates, did not cosediment with growth cones. Neither of these considerations, however, seems able to account fully for the extensive enrichment of 3H-imipramine binding sites in the early IGC fractions at E15 and E20, which was noted relative to the LSS fractions prepared from the same brains and from which the IGC fractions were derived. This enrichment suggests that, as in synaptosomes (Rehavi et al., 1983; Briley, 1985), 3H-imipramine binding sites are more concentrated in growth cones than they are in other regions of serotonergic neurons.

The ability of IGC fractions to accumulate 3H-5-HT itself was investigated as a function of age. This was done because, in the absence of additional information, it is impossible to judge from biochemical or radioautographic studies of 3H-imipramine or 3H-paroxetine binding whether the binding sites are internal or on growth cone surfaces; therefore, it is important to know not only whether or not the transporter is present, but whether it is functional. IGC fractions were found to accumulate 3H-5-HT specifically as early as E15. Since far more 3H-5-HT (on a molar basis) became associated with IGC fractions than 3H-imipramine, 3H-5-HT must have been transported into growth cones and not simply bound to their surfaces. The 3H-5-HT:3H-imipramine ratio was 2.6 at E15, 7.9 at E20, and 17.2 at P3. The increase in this ratio as a function of age may indicate that the internal ability of growth cones to store 5-HT increases more during ontogeny than the ability of the growth cone plasma membrane to transport 5-HT. The suggestion that growth cones themselves actually accumulate 3H-5-HT was supported by EM radioautography, which also revealed that the internalization of 3H-5-HT by these structures was inhibited by fluoxetine. These observations are consistent with the conclusions that the 5-HT transporter is responsible for the accumulation of 3H-5-HT in IGC. It can thus be concluded that the early presence of 3H-imipramine/3H-paroxetine binding sites correlates with the presence of a functional 5-HT transporter in the plasma membrane of the axonal growth cones of serotonergic neurons.

Earlier studies have shown that elements of the developing brain or subcellular fractions take up 3H-5-HT prior to the age when serotonergic synapses are formed (Yamamoto et al., 1981; Reisert et al., 1989; Ugrumov et al., 1989; Lockerbie et al., 1991); however, these prior observations did not show that the 5-HT transporter was actually present in the plasma membranes of serotonergic growth cones. Serotonergic growth cones are probably not unique in their ability to take up a neurotransmitter. Isolated growth cones have also been found to accumulate 3H-GABA or 3H-noradrenaline (Gordon-Weeks et al., 1984; Lockerbie et al., 1985). The accumulation of 3H-GABA...
by growth cones has also been confirmed by EM radioautography (Taylor and Gordon-Weeks, 1989).

Enrichment of vesicular markers in the IGC fraction relative to their concentration in the LSS fraction was investigated in order to determine whether growth cones contain synaptic vesicles. 5-HT and 5-HIAA were each found to be present in IGC and LSS fractions as early as E15. The ratio of 5-HT to 5-HIAA in the IGC fraction was greater than 1 at each age examined. These data suggest that growth cones retain 5-HT, but not 5-HIAA. Retention of 5-HT within growth cones is compatible with, but not proof of, the idea that these structures contain vesicles that are able to sequester 5-HT. Growth cones contain mitochondria (Landis, 1983), mitochondria contain MAO (Greenawalt, 1972; Gershon et al., 1990), and MAO is known to have developed in the fetal rat brain prior to E15 (Schmidt and Sander-Bush, 1971; Hamon and Bourgoin, 1982; Liu et al., 1987). In order to retain 5-HT, therefore, growth cones must be able to prevent its catabolism by MAO. 5-HT could be protected by sequestration within synaptic vesicles. If so, then reserpine, by inhibiting the action of the vesicular transporter, should deplete growth cone 5-HT. Since reserpine depleted 5-HT from the LSS fraction as early as E15, vesicles with an amine carrier have presumably been acquired by serotonergic neurons by E15. The failure of reserpine to deplete 5-HT in the IGC fraction at this age suggests that although these vesicles are present elsewhere in serotonergic neurons at E15, they are not present in growth cones. Unlike mature neurons, where synaptic vesicles and their proteins are concentrated in axon terminals and are sparse in perterminal axoplasm (De Camilli and Jahn, 1990), synaptic vesicles, or antigens associated with them, have been found to be distributed throughout the axons of developing neurons (Bixby and Reichardt, 1985; Mason, 1986; Chun and Shatz, 1988; Leclerc et al., 1989; Lupa and Hall, 1989; Fletcher et al., 1991). In contrast to E15, reserpine administration on E20 was effective in depleting 5-HT from the IGC fraction; thus, by E20, vesicles with an amine carrier are likely to have moved into growth cones. The developmental change between E15 and E20 could arise if serotonergic neurons become more mature or if the population of growth cone collected at E15 is different from that collected at E20 and subsequently. It is not clear why, if sequestering vesicles are absent, the IGC fraction should contain any 5-HT at all. It is possible that some cytosolic 5-HT within growth cones may be protected by binding to soluble SRP (56 and 68 kDa), which has been found in the brain (Li et al., 1990). MAO does not catabolize 5-HT that is bound to these proteins (Liu et al., 1987). More likely, the cytosol of growth cones at E15 may contain a small amount of 5-HT, which reflects a balance between opposing rates of synthesis and degradation. Such a steady state would not be altered by reserpine. This explanation is supported by the observation that when 5-HT was depleted from IGC fractions by reserpine administered at E20, the residual 5-HT concentration remaining in the fractions did not differ significantly from that observed in IGC fractions prepared from animals at E15 (see Fig. 4). Whatever the explanation for the small amount of 5-HT present in growth cones at E15, the effect of reserpine on the fetal brain is consistent with the idea that substantial numbers of vesicles of the synaptic type enter growth cones between E15 and E20.

The idea that growth cones of serotonergic neurons acquire synaptic vesicles as early as E20 is strongly supported by studies of the distribution in fractions of two markers, SBP and synaptophysin, which are found in synaptic vesicles in adult neurons. Synaptophysin is an integral membrane protein of the small synaptic vesicles that are present in all presynaptic nerve endings (Navone et al., 1986; Floum and Feist, 1989; De Camilli and Jahn, 1990). In fact, even when expressed by transfected non-neuronal cells, synaptophysin is sorted into small recycling vesicles (Johnston et al., 1989; Leube et al., 1989). Only neurons or neuroendocrine cells, however, appear to be able to sort synaptophysin-containing vesicles away from other early endosomes into a synaptic vesicle population (Linstedt and Kelly, 1991). SBP is concentrated in synaptosomes, in vesicles liberated from them in vitro (Tamir and Gershon, 1979), and in the secretory vesicles of 5-HT secreting paraneurons (Barns et al., 1987; Tamir et al., 1990; Cidon et al., 1991). SBP is also subject to fast axonal transport (Tamir and Gershon, 1979), as are other components of synaptic vesicles (Grafstein, 1977), and SBP is released from stimulated serotonergic neurons by exocytosis (Jonakait et al., 1979). The ~45 kDa form of SBP, moreover, forms a complex with 5-HT in synaptic vesicles in serotonergic nerve terminals in vivo (Gershon et al., 1983). SBP is thus a marker for synaptic vesicles derived from serotonergic neurons. The observation, therefore, that synaptophysin is enriched in IGC fractions suggests that synaptic vesicles are present in growth cones. This idea is strengthened by the enrichment of SBP found in the same fractions, which also implies that the subset of growth cones in the IGC fractions that is derived from serotonergic neurons contains synaptic vesicles. These data, taken together with the evidence, derived from studies with reserpine (discussed above), that these vesicles contain the amine carrier, support the conclusion that the growth cones of serotonergic neurons contain synaptic vesicles. The presence of small clear vesicles in electron micrographs of IGC adds further support to this conclusion.

Although some previous studies of the expression of synaptophysin have reported that it develops in parallel with the appearance of mature synapses (Knaus et al., 1986; Devoto and Barnstable, 1989; Taylor et al., 1990), many other studies suggest that it is found in growth cones. Before the development of significant numbers of synapses in the rat cerebellum, for example, synaptophysin-immunoreactive vesicles were observed in growth cones (Leclerc et al., 1989). Similarly, immunoreactivity of synapsin I (which is also associated with synaptic vesicles, De Camilli et al., 1990) was demonstrated to be present in growth cones of the developing mouse cerebellum long before nerve endings and synaptic contacts mature (Mason, 1986). Synapsin I immunoreactivity in cerebellar growth cones correlated with the presence of synaptic vesicles in these structures. The extending axons of developing motor nerves contain immunoreactive synaptic vesicle markers and these are particularly prominent in growth cones (Lupa and Hall, 1989). Restriction of the immunoreactivity of vesicular proteins to nerve terminals has been reported to be a progressive feature of maturation (Mason, 1986; Chun and Shatz, 1988; Lupa and Hall, 1989). The onset of the appearance of the immunoreactivity of another synaptic vesicle protein, p65, has been reported to precede that of synapsin I and the presumed period of synapse formation in the chick brain (Bixby and Reichardt, 1985). Finally, synaptophysin and synapsin I have each been shown to be distributed in distal axons and growth cones of developing hippocampal neurons in culture (Fletcher et al., 1991).

The possibility that the enrichment of SBP and synaptophysin immunoreactivities in IGC fractions is not due to the presence of synaptic vesicles has not been completely excluded. Con-
ecvibly, these proteins are located in other components of growth cones, which may be precursors of synaptic vesicles. It has previously been suggested that synaptic vesicles may be formed (De Camilli and Jahn, 1990) from smooth membranous structures, which are abundant in growth cones (Cheng and Reese, 1985, 1987) and preterminal axons of mature neurons (Droz and Rambourg, 1982). Our observation, however, that a reserpine-sensitive amine carrier protein, which can protect 5-HT from catabolism, presumably by catalyzing its uptake into a vesicular compartment, is also present in growth cones by E20, suggests that they must contain structures that are functionally similar to synaptic vesicles. In cultured hypothalamic neurons, furthermore, synaptophysin immunoreactivity in the trans-most cisternae of the Golgi apparatus has been demonstrated to be incorporated into vesicles that look like synaptic vesicles; moreover, synaptophysin immunoreactivity is transported from cell body to axon terminals in the membrane of recognizable vesicles (Tixier-Vidal et al., 1988). Only limited synaptophysin immunoreactivity was observed to be associated with the smooth endoplasmic reticulum (Tixier-Vidal et al., 1988).

Our observation that IGC fractions contain general and 5-HT-specific synaptic vesicle markers as well as the plasma membrane 5-HT transporter before the onset of cortical synaptogenesis suggests that the growth cones of serotonergic neurons have acquired many, if not all, of the components of the neurotransmitter machinery before mature synapses are formed. The transmitter apparatus of growth cones, therefore, may serve a developmental function. The acquisition of synaptic vesicles have acquired many, if not all, of the components of the neurogenesis suggests that the growth cones of serotonergic neurons display characteristics of mature synaptic terminals. These characteristics include the plasma membrane 5-HT transporter and an internal 5-HT storage compartment that has properties in common with synaptic vesicles. These observations suggest that synaptogenesis involving serotonergic neurons may involve the reorganization of preexisting components that are already present in growth cones. This "prebaptization" of the essential elements of the presynaptic components of synapses may enable physiological activity to be rapidly established when serotonergic synaptic contacts are formed. Contact of pre- and postsynaptic components of a synapse may thus rapidly be followed by neurotransmission. Such early establishment of activity could be important in determining the ability of developing synapses to survive and persist in a competitive environment (Frank, 1987; Shatz, 1990). The presence of neurotransmitter mechanisms in growth cones is compatible with suggestions that have been made in the literature that 5-HT plays a role in neural development.

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