SYNAPTIC PLASTICITY WITHIN THE INTERPEDUNCULAR NUCLEUS AFTER UNILATERAL LESIONS OF THE HABENULA IN NEONATAL RATS¹

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Abstract

In rats, synaptic terminations of the medial habenular (MH) afferents to the interpeduncular nucleus (IPN) are just beginning to form at the time of birth. The alterations in synaptogenesis resulting from unilateral MH lesions placed in neonatal rats were demonstrated with the autoradiographic and degeneration methods after the operated animals had reached maturity. The distribution of S synapses, *en passant* MH afferents to the central IPN, is normally 95% ipsilateral to their MH of origin in spite of the fact that their axons cross through the contralateral half of the IPN one or more times. The lesions result in a symmetrical distribution of S synapses from the remaining MH. The density of this projection on each side is equal to the normal ipsilateral density of termination.

Crest synapses, present in intermediolateral IPN bilaterally, consist of two endings which contact a narrow dendritic process. Normally, the two endings arise one from each MH at 90% of crest synapses, and two from the same MH at 10%. After unilateral MH lesions in neonates, approximately 96% of crest synapses are formed by two axons from the one remaining MH. At only 4% of crest synapses one or both processes is heterologous, that is, of non-MH origin.

These findings demonstrate that synapse formation on IPN neurons preferentially favors MH afferents, both normally and when left-right interaction is precluded by a neonatal unilateral lesion. Left-right interactions normally occur at both the level of the left and right sides of the IPN with regard to S synapses and individual left and right habenular afferents at crest synapses. Plasticity at crest synapses is considerably greater in neonates compared to adult rats. Control of these phenomena can be understood as the interaction of multiple factors. These may include specific determinants localized to the presynaptic axons, particularly prior to synaptogenesis and competition for synaptic space, especially with regard to S synapses and perhaps involving synapse elimination. There is also evidence for postsynaptic determinants both in the separation of synaptic types into separate subnuclei and in the formation of crest synapses in the IPN regardless of the presence of normal or reduced numbers of MH afferents, or their total absence.

The interpeduncular nucleus (IPN), situated in the ventral midbrain, receives afferents from each medial habenula (MH) via the habenulo-interpeduncular (H-IPN) tract, which occupies the core of the fasciculus retroflexus of Meynert (Herkenham and Nauta, 1979).

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Individual MH axons repeatedly cross the IPN (Ramon y Cajal, 1909; Herrick, 1948; Lenn, 1976; Herkenham and Nauta, 1979; Kemali et al., 1980). This pattern involves two or more crossings of the midline by these axons. Ramon y Cajal (1909) and others therefore assumed that *en passant* synapses are formed on both sides of the IPN, resulting in a convergence of the bilateral habenular input. However, a recent electron microscopic study of degenerating terminals in the IPN following tract lesions demonstrated that most MH neurons project to the ipsilateral part of the IPN throughout the mediolateral extent of the ventral two-thirds of the IPN, apparently at middle levels rostral-caudally (Murray et

al., 1979). These contacts, which have been termed S synapses, are formed *en passant* between horizontal MH axons containing spherical synaptic vesicles and small dendrites at asymmetrical contact zones (Lenn, 1976). Their distribution at middle and caudal levels of the IPN corresponds to the central subnucleus in which they are the predominant synaptic type (Hamill and Lenn, 1981). Except for occasional incomplete S synapses already present on the day of birth, they are formed in the first 4 postnatal weeks in the rat (Lenn, 1978a).

The crest synapses of the IPN also arise from the horizontal MH axons and are found in bilaterally symmetrical zones (Murray et al., 1979), recently termed the intermediolateral (IML) subnuclei (Hamill and Lenn, 1981). The crest synapses have been observed as early as 8 days postnatally and show their full diversity of configuration 1 week later (Lenn, 1978a). They are virtually all formed jointly by two MH axons which synapse on opposite sides of either sheet-like dendritic processes, which envelop the axons to form synaptic glomeruli, or a dendritic crest which is 75 to 80 nm in width. Their synaptic contacts are asymmetrical, generally parallel and coextensive, and are characterized by a presynaptic accumulation of spherical, agranular synaptic vesicles (Lenn, 1976, 1978a). All of the presynaptic MH processes forming S and crest synapses are thus of one type, and all form similar asymmetrical contacts. These axons have been shown in serial sections to occasionally form both types of synapse, probably near the border of central and IML subnuclei. Identification therefore is not possible when such a process is seen in a section which does not pass through its contact. However, crest synapses are all

located within IML subnuclei, and the available serial section material indicates few if any S synapses within this subnucleus. The two axons forming individual crest synapses are normally paired so that one comes from each MH in 90% of cases, both from the ipsilateral MH in 5%, and both from the contralateral MH in 5% (Lenn, 1976; Murray et al., 1979; Lenn et al., 1982).

Electron microscopic study of the IPN showed that S synapses were reduced in number and delayed in their time of appearance following destruction of one MH in neonatal rats (Lenn, 1978b). Crest synapses were also seen despite the fact that the normal pairing of one afferent from each MH was precluded by the lesion. These findings imply that the axons of the remaining MH form additional S synapses with IPN neurons which normally would have received input from the destroyed MH. The crest synapses in these cases were also assumed to have been formed by two axons from the one remaining MH, a major change from normal. Following bilateral MH lesions in neonatal rats, S synapses were not formed, whereas crest synapses were found after 1 month of age. The presynaptic processes at these crest synapses are of course from nonhabenular sources. This could be appreciated in some cases by differences in vesicle shape or packing. The postsynaptic morphology of these heterologous crest synapses including the parallel contacts and marked dendritic narrowing was not distinguished from that at normal crest synapses formed with H-IPN axons.

In the present study, alterations in the development of S and crest synapses within the IPN have been examined in detail. Unilateral lesions of the MH were again made in neonatal rats, prior to the normal time of syn-

TABLE I
Summary of rats studied

	Neonatal Lesion, Rt. MH ^a	Adult [³ H]Leucine Injection		Adult Lesion		Survival Time (hr)
		Lt. MH ^b	Rt. MH	Lt. MH	Rt. MH	
Crest Synapse Study						
Lesioned						
R.633-634	x			x		6
R.640-647	x			x		20
R.613-614, 635-636	x			x		24
R.620-621, 624	x			x		36
R.629-630	x			x		40
R.615-617	x			x		48
R.631	x			x		60
R.570-571	x			x		120
Quantitative EM Distribution of S synapses						
Control						
R.650-651				x		48
R.652				x	x	48
Lesioned						
R.641	x			x		20
R.642	x			x		36
R.615	x			x		48
Axoplasmic Transport Studies						
Control						
R.599			x			24
R.609-612		x				24
Lesioned						
R.600, 604-608	x	x				24

^a Rt., right.

^b Lt., left.

aptogenesis. A second experimental manipulation, either a lesion of or an injection of isotope into the remaining MH, was added after synaptogenesis to directly demonstrate the induced synaptic plasticity.

Materials and Methods

Lesions. Mid-term pregnant Sprague-Dawley rats were obtained from a commercial source (Simonsen Laboratories) and observed daily until delivery. The 41 animals studied are listed in Table I. Each pup underwent initial placement of a right MH lesion when its weight was between 8.5 and 9.5 gm, which was reached on day 2 or 3 with day 1 being the day of birth. Under deep hypothermia anesthesia, the animals were placed in a holder, and an insulated electrode was placed stereotactically (Mosko and Moore, 1978). With the bregma as zero

point, three lesions were made in the right MH with coordinates: AP-1.4, -1.9, and -2.4; L-0.7; V-3.3. A current of 1 to 2 mA for 15 sec/lesion placement was delivered from a Grass S-5 stimulator. After 28 to 30 days' survival, these animals were re-anesthetized with pentobarbital (3.5 mg/100 gm of body weight) for the stereotaxic placement of a second lesion of the remaining left MH. Using ear bars as zero point, three lesions were again induced using 1 to 2 mA for 15 sec at coordinates: AP+2.3, +2.8, and +3.3; L+0.5; V+0.56. Animals without neonatal lesions served as controls. Some of these received identical unilateral or bilateral MH lesions as adults, with 48 hr of survival prior to sacrifice. Serial sections through the habenula were used to verify both neonatal and adult lesion placement (Fig. 1).

Electron microscopy (EM). Following survival times of 6 to 120 hr after the adult lesion, these animals were anesthetized and cooled (26°C rectally). Paraformaldehyde (1%), glutaraldehyde (1.25%), and CaCl_2 (0.10%) in 0.1 M sodium cacodylate buffer were perfused via the aorta under 140 to 160 torr pressure (Lenn and Beebe, 1977). This was immediately followed by 10 ml of 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.10% CaCl_2 in the same buffer, perfused over 5 to 10 min. The brains were left undisturbed *in situ* for 1 hr, removed from the cranium, and stored in the stronger fixative. On the following day, three coronal blocks were cut through the IPN area, washed in 0.2 M sodium cacodylate buffer containing 0.2 M sucrose (600 mOsm), treated with 2% osmium tetroxide in the same buffer for 2 hr, block stained with maleate-buffered uranyl acetate, and embedded. Rostral, mid, and caudal IPN were identified on 1- μm sections stained with toluidine blue. Routine and serial thin sections were stained with uranyl acetate and lead citrate. Micrographs were taken at original magnifications of $\times 4,000$ to $\times 30,000$.

Figure 1. Line drawing of a coronal section from a 29-day-old rat. A neonatal lesion of the right habenula (Hb, arrow) was followed 28 days later by a left habenular lesion (stippled area). The black spot within the latter represents the area of total necrosis. Magnification $\times 9$.

Figure 2. Coronal section of midbrain region in a 29-day-old rat. Arrows indicate bilateral tissue biopsies of the IPN made with chilled and blunted 22 gauge hypodermic needles (300 μm frozen section). Magnification $\times 12$.

Quantitative EM distribution of S synapses. Rats 615, 624, and 641 were studied 20, 36, and 48 hr, respectively, after the second, adult MH lesion. An equal number of random pictures were taken in each pair of symmetrical, corresponding grid windows on the left and right sides of the midline at rostral, mid, and caudal levels of the IPN. These levels were defined grossly by the flattened ventral contour of the IPN rostrally, the curved ventral contour and penetrating vessels in the midlevel, and the pons caudally. All degenerating S synapses were counted on the resulting 642 micrographs. Four hundred thirty-four photographs were obtained and examined in an identical manner from control rats 650 and 651 with unilateral left MH lesions, and from rat 652 with bilateral lesions. The Student's *t* test was used to evaluate these and the scintillation counting experiments.

[³H]Leucine scintillation counting. Nine 29-day-old rats with a neonatal lesion of the right MH were anesthetized and the left MH was stereotaxically injected (coordinates: AP+2.3; L+0.5; V+0.56) with 1.5 μ l of [³H]leucine neutralized with NaOH (New England Nuclear; specific activity 51.5 Ci/mmol). On the following day, each animal was re-anesthetized, and its brain was quickly removed and frozen. Tissue samples from the left and right habenula and from the left and right sides of midlevel IPN were removed from 300- μ m frozen sections with a 22G punch (Fig. 2). Neonatal lesion placement was confirmed at this time. The tissue samples were solubilized and assayed by liquid scintillation counting. Results are expressed as left to right ratios of activity in the habenula and IPN. A calculated distribution allowing for contralateral uptake and transport of isotope was

Figure 3. A horizontal axon traversing the right half of the figure forms an *en passant* S synapse (solid arrow) with a dendrite. A crest synapse of normal morphology (open arrow) is formed by two presynaptic terminals containing spherical vesicles. The two synaptic contacts are asymmetrical, coextensive, and largely parallel to one another. Six hours' survival. Magnification $\times 35,000$.

Figure 4. A normal synaptic terminal containing large numbers of dense core vesicles and pleomorphic, largely spherical vesicles (arrow). Six hours' survival. Magnification $\times 35,000$.

Figure 5. A crest synapse (arrow) is formed by one apparent MH terminal of normal structure above and a presynaptic terminal (H) which is considered heterologous due to its content of pleomorphic vesicles within a light cytoplasmic matrix. The synaptic contacts are parallel, minimally asymmetrical, and not coextensive. Six hours' survival. Magnification $\times 35,000$.

plotted and used for graphic solution in recalculating the raw data from the control animals.

[^3H]Leucine autoradiography. A stereotaxic injection of concentrated [^3H]leucine into the left MH was made in rat 599, a normal control, and rat 600, which had a verified lesion of the right MH at 2 days of age. After 24 hr survival, serial coronal sections through the H-IPN region were prepared for autoradiography with NTB-2 emulsion, developed in D-19, and stained with thionin. Total silver grain counts were made across the entire width of the midlevel IPN in these two animals using an eyepiece radicle at $\times 1250$.

Results

Lesions

The neonatal lesions completely destroyed the right MH nuclei (Fig. 1; Fig. 1 in Lenn et al., 1979). By the time of sacrifice the region of the lesion was well healed. The needle track was visible as a linear scar in overlying cerebral cortex and hippocampus, or these structures were mildly distorted. There was minor injury of the parafascicular and lateral nuclei of the thalamus. The fasciculus retroflexus was absent on the side of the lesion in each case. The adult lesions each destroyed 90 to 100% of the MH or of the origin of the fasciculus retroflexus (Fig. 1). There was little if any damage to other structures, except for the electrode tract through overlying cerebrum and hippocampus.

EM observations

The extent of degenerative changes in the IPN in adult rats with a neonatal MH lesion correlated with survival time after the second MH lesion. Thus after 6 hr survival no degenerating endings were found. The presynaptic terminals forming S and crest synapses were of normal orientation and diameter, contained spherical synaptic vesicles, and formed asymmetrical *en passant* contacts with dendritic processes (Fig. 3). The vesicles consisted of a main group with profiles varying from 40 to 60 nm in diameter and occasional dense core vesicles of 70 to 100 nm in diameter. Boutons of slightly increased axoplasmic density containing aggregates of spherical vesicles were no different from occasional endings in normal IPN and are therefore not considered to be degenerating. The dendrites postsynaptic to S synapses were likewise of normal morphology, having an electron-lucent cytoplasm, mitochondria with parallel cristae, neurotubules, and occasional multivesicular bodies.

Synaptic terminals containing large numbers of dense core vesicles and pleomorphic, largely spherical vesicles were occasionally seen in these animals (Fig. 4). In general, either the dense core vesicles were situated near the periphery of the terminal with the pleomorphic vesicles contained in the center, or the two groups were adjacent, but separate. The origin of these terminals, which may be concentrated dorsolaterally in the IPN (Murray et al., 1979), is unknown. Astrocytic profiles containing glial filaments were observed, although their morphology was not indicative of a reactive state. Degenerative debris was not observed at this survival time. All of these findings are consistent with previous observations of normal adult IPN (Lenn, 1976).

Crest synapses. At 6 hr survival, most crest synapses were formed by two endings which had the morphology of H-IPN terminals (Fig. 3). Occasional crest synapses were encountered in which one of the presynaptic terminals contained pleomorphic vesicles with diameters of 30 to 40 nm (Fig. 5). The other ending forming each of

Figure 6. A crest synapse (arrow) is formed by one terminal (H) which is probably heterologous based on its small spherical vesicles, whereas the second terminal is more ambiguous. It has some characteristics of MH endings, but the vesicles are more pleomorphic and the contact is less asymmetrical than most MH endings. Sixty hours' survival. Magnification $\times 32,000$.

Figure 7. A crest synapse (arrow) is formed by two heterologous terminals (*H*) as judged by their vesicles. The terminal on the *right* contains small spherical vesicles of morphology similar to those seen in Figure 6 (*H*), while that on the *left* contains pleomorphic vesicles. The two synaptic contacts are not coextensive and are parallel over only part of their extent. Sixty hours' survival. Magnification $\times 35,000$.

Figure 8. A crest synapse (large arrow) is formed by a normal appearing MH afferent and a heterologous afferent (*H*) as judged by its pleomorphic vesicles. The latter also makes a symmetrical synaptic contact (small arrow) with a neuronal soma (*S*). Sixty hours' survival. Magnification $\times 30,000$.

these crest synapses contained spherical vesicles of 40 to 60 nm in diameter and was indistinguishable from the MH endings which normally form crest synapses. The differences in morphology of the former endings suggest

that they are non-MH in origin, or heterologous. Although minimally asymmetrical, parallel, and opposing a narrow dendritic process, the synaptic contacts at some of these crest synapses were not coextensive. Within both terminals mitochondria of normal size and appearance were found. Some crest synapses at longer survival times were formed by either two heterologous endings or endings which were not definitely identified (Figs. 6 and 7). In one case an apparently heterologous ending which formed a crest synapse paired with an MH ending also synapsed separately with a cell body. This has not been observed in normal IPN (Fig. 8). At occasional crest synapses, observed at most survival times, one contact was formed with an apparent H-IPN ending, whereas the other contact was juxtaposed to the external surface of a myelin sheath (Fig. 9), or occasionally an oligodendroglial process (Fig. 10).

At survival times of 20 hr or longer after the second lesion, degenerative changes allowed definite identification of MH afferents and, thereby, direct demonstration of afferent pairing at crest synapses (Figs. 11 and 12). At 20 hr survival, the majority of crest synapses were still normal in appearance. Of the 54 crest synapses found in this material, 33 were formed by two endings which were not degenerating but had the morphological characteristics of H-IPN terminals. One ending was degenerating at 18 of these crest synapses, while the nondegenerating endings in these cases also appeared to be of MH origin (Fig. 11). At three crest synapses both presynaptic endings were degenerating and therefore definitely arose in the left MH.

Figure 9. A crest synapse formed by an MH terminal on one side and juxtaposed to the myelin sheath of an adjacent axon on the other. Two postsynaptic densities (open arrows) within the dendrite underlie the portion of its membrane which is opposed to the myelin sheath. The MH terminal contact is opposite but not coextensive with one of these. Thirty-six hours' survival. Magnification $\times 50,000$.

The optimal survival time for this experiment was 36 hr, since degenerating endings were much more common than at shorter and longer survival times. Of the 121 crest synapses observed at this survival time, 48 from one animal (rat 620) were observed in serial sections. The results for these 48 were consistent with those for the entire sample. Eighty-one of these crest synapses (67%) showed both presynaptic terminals degenerating (Fig. 12), whereas 29 (24%) had one degenerating ending. All of the nondegenerating endings at these 29 crest synapses appeared to be of MH origin, although at 48 hr survival this was not so (Fig. 13). Of the remaining 11 crest synapses without either ending degenerating, both endings appeared to be H-IPN afferents at three, one of the endings appeared heterologous at five, and both endings appeared heterologous at three.

These data can be used to estimate but not precisely calculate the detection rate of the EM degeneration method in this material. The detection rate (DR) has recently been defined as the number of degenerating endings per total number of endings of the class, in this case H-IPN afferents (Lenn et al., 1983). The possible values of DR at 20 hr survival are from 24 to 100%. At the optimal survival time, 36 hr, the range is much narrower, from 79% (191 degenerating endings of 242

endings at the 121 observed crest synapses) if all nondegenerating endings are actually H-IPN afferents, to 100% if all H-IPN axons are detected as degenerating. Therefore, using only the 36-hr data, DR is estimated at 82%. This estimate rests on the following considerations: if our morphological identification of the nondegenerating endings as H-IPN and nonhabenular is completely accurate, DR is 83% (191 degenerating of 191 degenerating + 40 nondegenerating H-IPN endings); if some endings are misidentified in both groups, heterologous as H-IPN and vice versa, which is likely, the range of possible DR is approximately 80 to 95%; it is readily calculated that the estimated number of crest synapses with two heterologous endings is unchanged for DR from 83 to 90%; and finally, the estimate of H-IPN to heterologous pairing at DR of 85% or greater is over 10%, which exceeds the observations by much more than any likely error of identification based on morphological criteria. By using the estimate of DR thus derived, 82%, left-left H-IPN afferent pairing is calculated as occurring at 96% of crest synapses after neonatal lesion of one habenula. The remainder are divided between left H-IPN to heterologous pairings and probably slightly fewer heterologous to heterologous pairings. Without pretending undue precision for this estimation, a marked alter-

Figure 10. A crest synapse (arrow) formed by an MH terminal on one side and juxtaposed to a glial process (A) on the other. The parallel, asymmetrical synaptic contact zones are not coextensive. Ribosomes, neurotubules, and dark matrix suggest that A is an oligodendrocyte process. Thirty-six hours' survival. Magnification $\times 50,000$.

Figure 11. One of the endings at a crest synapse (arrow) is a definite MH afferent identified by the fact that it is undergoing dense-type degeneration. The other nondegenerating ending appears to be of MH origin by its morphology. Twenty hours' survival. Magnification $\times 35,000$.

ation in synaptogenesis of left H-IPN afferents is apparent.

The present experiment shows that crest synapses have been modified in a number of ways by the neonatal

lesion. Pairing of left and right MH afferents which predominate normally is of course absent. Instead, 96% are formed by two H-IPN endings of axons from the one remaining MH (Figs. 3, 11, 12, and 14). At 3% an H-IPN

Figure 12. Both endings at a crest synapse (arrow) are undergoing degeneration after the adult left MH lesion. The asymmetrical synaptic contacts are no longer coextensive due to differential shrinkage of the degenerating terminals. A multivesicular body is present within the dendrite (asterisk). Thirty-six hours' survival. Magnification $\times 35,000$.

axon is paired with a heterologous, that is non-H-IPN, ending of unknown origin (Figs. 5, 8, and 13). Approximately 1% are formed by two heterologous endings (Fig. 7) or by one unidentified, probably MH ending and juxtaposed by a myelin sheath (Fig. 9) or oligodendroglial process on the other side (Fig. 10).

The degeneration of synaptic profiles observed at crest synapses was of the electron-dense type (Figs. 11 to 14). Some of the asymmetrical synaptic contacts remained parallel and coextensive, in spite of degeneration of the presynaptic process. However, in many cases the contact became wavy or irregular in contour, probably as a result of differential shrinkage. Although many normal crest synapses are partially surrounded by the postsynaptic dendrite, serial sections showed that some degenerating synaptic profiles were largely or completely engulfed by a dendrite. In addition, dendritic cytoplasm often interdigitated into the presynaptic process, at times dividing the contact region and possibly leading to fragmentation of the degenerating ending (Fig. 14). Coated vesicles were observed to be invaginating into the dendrite in the region of synaptic contacts formed with degenerating presynaptic profiles (Fig. 14B). Degeneration debris was also present in astrocyte cytoplasm. It therefore appears that both astrocytes and dendrites contribute to the removal of the degenerating endings.

Quantitative EM distribution of S synapses

It is well established that S synapses in the IPN arise from the horizontal axons of the H-IPN afferents (Leranth et al., 1975; Lenn, 1976; Hattori et al., 1977). In the present study, numerous degenerating S synapses oc-

curred following the adult MH lesions in both control and experimental animals. This was evident by 20 hr survival, taking the form of typical dense-type degeneration similar to that described above for the crest synapses. The degenerating S synapses were qualitatively similar at all survival times studied, namely, 20, 24, 36, 40, and 48 hr.

To quantitate the degeneration, two parameters were measured, density of degeneration, since this allows for any change in the volume of IPN resulting from the neonatal lesions, and left-right ratios. As can be seen in Table II (Figs. 15 and 16), the density of degenerating S synapses was the same on both sides of the IPN in the bilateral control and three experimental animals, and on the ipsilateral side in the two unilateral controls (all $p > 0.1$). The density was less only on the side contralateral to the MH lesion in the two unilateral controls ($p < 0.01$). Although three survival times were studied (see Table I), the magnitude of the difference, 10- to 50-fold, the similarities of the values at the three survival times, and the statistical significance support the conclusion that S synapses are normally ipsilateral. Furthermore, the experimental animals show that the neonatal lesions have induced the intact left MH to form S synapses in such a way that they are of normal density in the right half of the IPN without diminution in the density of S synapses in the left half of the IPN.

Axoplasmic transport studies

The change in H-IPN afferent distribution induced by a unilateral neonatal lesion was also demonstrated by scintillation counting of tissue samples and by light

Figure 13. A crest synapse (arrow) composed of a degenerating, electron-dense terminal with a swollen organelle in the center (asterisk), and a heterologous terminal (H) as judged by its pleomorphic vesicles. The synaptic contacts appear to be sectioned somewhat tangentially, but this does not negate their poor match of length and position caused by shrinkage of the degenerating ending. Forty-eight hours' survival. Magnification $\times 35,000$.

microscopic autoradiography. Table III shows that the H-IPN tract projects predominantly to the ipsilateral half of the IPN in normal rats as measured by scintillation counting. However, in agreement with the quantitative EM data, the neonatal lesion resulted in a bilaterally symmetrical distribution of the transported radioactivity. Due to the uptake of isotope by the noninjected habenula in the control animals, which on average was 20% of the ipsilateral habenular uptake, the raw scintillation counts must be corrected. Whereas the raw data showed a 64 to 88% ipsilateral projection to the IPN (mean 70%), the actual projection was calculated by a graphic solution of the effect of isotope uptake in both habenulae to be 95% ipsilateral. For the experimental animals the mean left-right ratio in the IPN was 1.07, and of course, no correction for contralateral MH uptake was needed in the absence of the neonatally destroyed right habenula. The difference between control and experimental groups is significant ($p < 0.01$).

Grain counts on light microscope autoradiographs were consistent with the above finding, giving the following left-right ratios: normal, 1.62 (680/419), and neonatal unilateral lesion, 0.97 (1269/1230).

Discussion

Development of the IPN prior to synaptogenesis. The neurons of the IPN arise in the ventricular zone in the floor of the mesocoel on embryonic day (E) 11 to E15 in the rat (Hanaway et al., 1971) and embryonic stages 17 to 21 in the rhesus monkey (E36 to E42) and in humans (Lenn et al., 1978). They migrate ventrally on

each side of the raphe and then spread laterally in the roof of the pontine flexure. The habenulae are already present and the H-IPN tract grossly reaches the IPN at about the same time. Radial glial fibers have been demonstrated in this region in *Amblystoma* (Herrick, 1948). They may guide the migration of the neurons or H-IPN axons, as they do, for example, in the developing cerebellum (Rakic, 1971). The topographic relationship of subregions of the MH to subnuclei of the IPN (Herkenham and Nauta, 1979; Hayakawa and Zyo, 1982) may be reflected within the H-IPN tract prior to synaptogenesis in the IPN. At least in the adult rat habenular axons are sorted within the fasciculus retroflexus with the lateral habenular axons in a surrounding annulus and the MH axons as the core (Herkenham and Nauta, 1979). If such order is present early in development, axon sorting may be important in the development of the H-IPN system, as has been suggested in the retinotectal pathway (Easter et al., 1981).

The IPN neurons have reached their definitive location and the nucleus is discernible by E45 (stage 23) in the monkey and by E18 in the rat. The H-IPN axons enter the nucleus from the rostral pole. Within the IPN, they turn medially and course horizontally across the nucleus. No plausible mechanism or guidance has been proposed for this phylogenetically stable feature (Herrick, 1948; Kemali et al., 1980). Nonetheless, in the rat these horizontal axons are in place prior to synaptogenesis, grouped within the IPN into fascicles of up to 300 axons in the rat (Lenn, 1978a). They traverse the IPN in the adult as far as the lateral border of the

Figure 14. Crest synapse (arrows) are each formed by two MH afferents undergoing dense-type degeneration. Dendritic cytoplasm can be seen interdigitating into one of the degenerating terminals (asterisks), dividing the synaptic contact zone into two parts. In addition, in B, a coated vesicle is seen evaginating from one synaptic contact zone. Thirty-six hours' survival. Magnification: A, $\times 35,000$; B, $\times 35,000$.

TABLE II
Effect of habenular lesions on quantitative EM distribution of S synapses

	No. of Degenerating Endings		Density of Degenerating Endings/ μm^2		Left-Right Ratio
	Left	Right	Left	Right	
Controls					
Unilateral Adult Lesions Only					
Rat 650	168	3	1.76×10^{-3}	3×10^{-5}	
Rat 651	230	40	2.35×10^{-3}	4.1×10^{-4}	
Means	199	21	2.06×10^{-3}	2.2×10^{-4a}	9.36
Bilateral Adult Lesions Only					
Rat 652	353	302	3.21×10^{-3b}	2.75×10^{-3b}	1.17
Experimental					
Unilateral Neonatal plus Unilateral Adult Lesions					
Rat 615	693	701	4.81×10^{-3}	4.87×10^{-3}	
Rat 624	593	724	3.19×10^{-3}	3.89×10^{-3}	
Rat 641	504	442	2.67×10^{-3}	2.34×10^{-3}	
Means	597	622	3.56×10^{-3b}	3.70×10^{-3b}	0.96
			3.63×10^{-3}		

^a $p < 0.01$ (left versus right).

^b $p > 0.1$ (each value compared left versus right and compared to mean of left side of unilateral adult lesioned animals).

contralateral IML subnucleus and then recurve to cross the nucleus one or several more times while spiraling caudally (Hamill and Lenn, 1981). Since the axons turn in relation to the penetrating arterial branches from the interpeduncular fossa, and since these vessels are present in IML subnuclei in the late fetal rat and monkey, they could have a role in the recurving of the axons. Another possible factor in the early arrangement of axons within the IPN is the caudal to rostral gradient of neuronal maturation, demonstrated in primates. Regardless, at least most of the axons are in place when synaptogenesis begins. These features, because they predate synaptogenesis, may be important in determining its course and outcome.

Synaptogenesis in the IPN. There is a period of rapid synaptogenesis in the IPN. In the monkey this is prenatal, especially between E148 and E162 for crest synapses (Lenn and Wong, 1982). In the rat, this period, which is postnatal, has been studied in normal IPN and after unilateral and bilateral MH lesions in newborns (Fig. 17). The two obvious, direct effects of the unilateral lesions studied here are that the number of H-IPN neurons sending axons to the IPN is reduced to one-half of normal, and that left-right afferent interactions cannot occur. Nevertheless, following such lesions in neonatal rats, 96% of crest synapses are formed by two MH afferents. In addition, one MH afferent participates at another 3% of crest synapses. Thus 97.5% of the presynaptic processes at crest synapses are of MH origin after such lesions, only slightly less than the almost 100% normal incidence (Murray et al., 1979; Lenn et al., 1982). This suggests a selective advantage favoring MH afferents over other possible presynaptic elements in normal development which is retained in the lesioned animals. Although random synaptogenesis would probably also result in more MH afferents at crest synapses than any other type, there are several arguments against this being the only or even predominant mechanism. There are several types of non-MH processes in the IPN (Hamill

and Lenn, 1981). With a 50% reduction in MH afferents, a random process would likely result in far more than the observed 2% increase in non-MH afferents at crest synapses. The alternative that non-MH processes cannot form crest synapses is known to be incorrect. They are formed by two heterologous presynaptic processes after bilateral MH destruction in newborn rats (Lenn, 1978b); in addition to the 4% incidence in the present study. Among possible origins of these processes are several intrinsic IPN elements and other afferents (Contestabile and Flumerfelt, 1981).

Similar considerations are raised by the effect of unilateral lesions on S synapse development. Murray et al. (1979), by plotting the location of S synapses, showed that approximately 95% of S synapses are ipsilateral to the MH of origin, with most of the remainder near the midline on the contralateral side. This result has been confirmed in the control animals of the present study by both EM degeneration and axonal transport methods. Thus, the equal density of S synapses on the two sides of the IPN observed in the present experiments after neonatal destruction of one MH is a major change. This indicates either that the contralateral S synapses are formed in addition to, rather than at the expense of, the normal ipsilateral S synapses, or that shrinkage of the nucleus and change in S synapse distribution from predominantly ipsilateral to bilateral are equal. The former is more likely, but not completely established by these data on density. A perhaps unique feature is the inferential conclusion that the additional S synapses are formed by these axons in the contralateral IPN through which they normally course without forming, or at least without retaining, S synapses in the normal adult rat. The preferential formation of synapses between H-IPN axons and IPN dendrites which characterizes crest synapse formation is again apparent. S synapses thus predominate markedly over synapses formed by other afferents in the deafferented half of the IPN as they do in the normal IPN. This is not to say, however, that there

Figure 15. Low power micrographs of the IPN in rat 650 after an adult unilateral left habenula lesion. Many S synapses are undergoing dense-type degeneration on the left side, ipsilateral to the MH lesion (A), whereas few degenerating terminals are found contralaterally (B). Magnification $\times 12,000$.

is not sprouting of other afferents as a result of the neonatal lesion, only that the sprouting of S synapses is quantitatively greater. The somatic synapses are known to sprout in the central IPN after such lesions (Lenn et al., 1979). The localization of midbrain tegmental afferents in the IPN is not yet clear, but they also appear to sprout after neonatal lesions (N. J. Lenn and G. Hamill, unpublished observations).

The preferential development of synapses between MH axons and IPN dendrites thus involves both crest and S synapses. It is most clearly shown by the increase in other pairings at crest synapses caused by decreased numbers of MH axons (Fig. 17). An important implication of these results is that the mechanisms responsible for MH preference and left-right interaction are somehow separable, since MH preference persists when left-

Figure 16. Low power micrographs of the IPN in rat 624 after a neonatal right MH lesion followed 28 days later by a left MH lesion. Degenerating S synapses are found in equal number and density on both the left (A) and right (B) sides of the IPN. Magnification $\times 12,000$.

right interaction is prevented, both in the present experiments and those in adults (Murray et al., 1979).

The second major effect of the unilateral lesions in the present experiments is the elimination of left-right interactions involving the MH afferents to the IPN. The pairing of one afferent from each MH normally occurs at 90% of individual crest synapses (Lenn et al., 1982). However, after neonatal unilateral lesions of the habenula two left MH afferents are paired at 96% of crest synapses. In the case of S synapses, the left-right afferent interactions are also altered by the lesions, but in this case it is the separation of the two populations of affer-

ents from the two MH that is absent, with the afferents from the one remaining MH filling the entire central IPN with a normal density of S synapses as indicated above. Therefore, in spite of the differences, the important similarities in the control of left-right interactions for both types of synapse are emphasized. The present data do indicate that postsynaptic factors do not prevent ipsilateral afferent pairing, that is, left-left and right-right pairing at crest synapses. First of all, 10% of normal crest synapses in the adult rat are connected this way. Secondly, 96% were found to be connected this way after unilateral lesions of the habenula in neonates and 66%

TABLE III
Effect of neonatal lesions on axoplasmic transport of [3 H]leucine from habenula to IPN

	Lt. IPN	Rt. IPN	Lt./Rt. Ratio	Lt. Hab.	Rt. Hab.	Lt./Rt. Ratio
Group 1 (Controls)						
Rat 609	434.6 ^a	59.3	7.33	1055.7	160.1	6.59
Rat 610	226.5	128.5	1.76	1475.6	701.9	2.10
Rat 611	132.0	67.7	1.95	481.5	148.9	3.23
Rat 612	158.9	57.0	2.79	237.4	46.7	5.08
Means			3.46 ^b			4.25
Group 2 (Lesioned)						
Rat 604	127.9	89.0	1.44	197.1		
Rat 605	23.0	56.1	0.41	575.8		
Rat 606	61.5	88.5	0.69	60.8		
Rat 607	47.2	26.7	1.77	40.1		
Rat 608	47.4	45.0	1.05	244.7		
Mean			1.07 ^b			

^a cpm.

^b $p < 0.01$ (Lesioned versus Controls).

Figure 17. Normal and altered afferent pairing at crest synapses in four situations. A, Normal adult IPN (data from Murray et al., 1979). B, Unilateral MH lesions in neonates (data from the present study). C, Unilateral MH lesions in adult rat with long survival (data from Murray et al., 1979). D, Bilateral MH lesions in neonates (data from Lenn, 1978b). The four types of pairing are: (a.) crest synapses composed of two MH afferents, one from each MH (left-right pairing); (b.) crest synapses composed of two afferents, both from the same MH (left-left, right-right); (c.) crest synapses composed of one MH afferent and one heterologous (non-MH) afferent (left-het); and (d.) crest synapses composed of two afferents of unknown origin (het-het). Left-right pairing is 90% in normal IPN, with left-left and right-right being 5% each (A). Preventing left-right pairing prior to crest synapse formation results in 96% left-left pairing with very small numbers of other pairings (B). Destroying left MH in adults results in reinnervation with right-right pairing at 66%, fewer right-het pairings, and 3% het-het pairing (C). Preventing crest synapse formation with MH afferents by bilateral neonatal lesion is still associated with crest synapse formation, all with heterologous endings paired (D).

in adults. Left MH axons form S synapses in the right half of the central IPN. Therefore, the left-right interactions in normal IPN are best explained by control factors localized to the presynaptic axons. Axon sorting

alone is probably not sufficient since the individual axons spiral repeatedly through both sides of the IPN without evidence for the precise timing and geometry that left-right pairing would require if axon sorting were the major determinant. Synapse elimination may operate in the IPN, as has been demonstrated in the case of muscle and autonomic ganglion development (Purves and Lichtman, 1980) and the visual system (Rakic, 1976; Ferster and LeVay, 1978). The initial development of crest synapses could involve random pairing or could be more complicated, with three or more afferents related to one dendritic site. Synapse elimination would then be sufficient to produce the observed outcomes. Another possibility, that the first arriving MH axon induces a change which limits the choice of the second afferent at individual crest synapses, is unlikely. The presence of ipsilateral pairing at 10% of crest synapses may relate to aspects of IPN function, which is related to autonomic control (Cragg, 1961).

Synaptogenic processes of these types have been conceptualized as competition in other systems (Schneider, 1973; LeVay et al., 1980). This is usually conceived of as operating at the synaptic level. Thus the normal density of S synapses across the two sides of the central IPN induced by the neonatal lesions can be viewed as regulation of synaptic space in the absence of competition from the contralateral MH. This is analogous to the result of the classical experiment in which half of the retina is removed prior to regeneration in the sense that 50% of the normal habenular origin of the projection interacts with 100% of the target tissue (Attardi and Sperry, 1963). Similar experiments in the hippocampus have indicated an increased number of synapses maintaining a normal density of endings (Gall et al., 1979). Conversely, the number of boutons per axon is regulated downward in the half-tectum experiment (Murray et al., 1982). Competition also occurs with the implantation of a supernumerary eye at the time of optic nerve crush. When two eyes innervate one tectum in such animals, separate stripes of termination from each eye alternate (Constantine-Paton and Law, 1978). Experiments involving retinal lesions (Lund, 1978) and cortical ocular dominance columns in mammals have been similarly interpreted (Hubel et al., 1977). In fact the latter case

lead to the suggestion that synchronous activity decreases competition between afferents, whereas asynchronous activity increases competition (cat, Weisel and Hubel, 1965; monkey, LeVay et al., 1980). This concept could apply to the activity of the two, or possibly initially more, afferents at crest synapses in the IPN regarding the development of left-right pairing and its plasticity after MH lesions, although the generality of such mechanisms is of course unknown. If there is an upper limit to the capacity of MH axons to conserve synaptic density in the IPN by increasing the number of boutons per axon, as there is for retinal ganglion cells (Frost and Schneider, 1979), the present 50% MH lesions do not exceed it. In any case, competition perhaps acting to control synaptogenesis or via synapse elimination, like MH preference, suggests principally a presynaptic locus.

The third major conclusion of this study is that the postsynaptic neurons have an important role in synaptogenesis in the IPN. A postsynaptic role in the formation of the distinctive geometrical relationships which define the crest synapse has been suggested previously by the occurrence of crest synapses in the absence of MH afferents, that is, after bilateral lesions of the habenula (Lenn, 1978b). Crest synapses are formed regardless of the presence of normal numbers, reduced numbers, or the total absence of H-IPN axons within the IPN (Fig. 17). Even presumably synaptogenically inert elements, myelin and glia, are found juxtaposed to characteristic crest synapses. The presence of crest synapses with one H-IPN afferent and one heterologous process, and others with two heterologous processes in the present experiments, again indicates that MH afferents are not necessary for the formation of crest synapses. If crest synapse formation in the IPN were normally, or became, a property of other presynaptic elements independent of the dendrite simply by eliminating MH afferents, crest synapses would most likely be much more widespread throughout the brain. Whatever the mechanism of crest synapse formation, the observation that the postsynaptic IPN dendrite is the only tissue element consistently involved with these synapses strongly suggests an essential factor localized to the dendrite. Furthermore, the segregation of crest and S synapses into separate subnuclei may be explained in part by differences between IPN neurons in IML and central subnuclei, respectively. Since there is evidence that separate portions of the MH project to the subregions of the IPN, crest and S synapses are probably usually formed by separate axons, in spite of some exceptions (Lenn, 1976). Since the H-IPN axons which form crest synapses also course through at least the central subnucleus without forming crest synapses, but where they do form some S synapses instead, a postsynaptic factor, a property of IML but not central subnucleus neurons, is strongly suggested. This property of IML neurons appears to be necessary for crest synapse formation since they are not found in central, and sufficient, since crest synapses occur even in the absence of MH afferents.

Comparison to adult reinnervation. The present experiments of course differ from reinnervation studies in adults in that the lesion is prior to formation of synapses, a factor which has been associated with differences in outcome in other systems as well. For example, in some

species the tectal retinotopic map is not present prior to initial innervation (Chung and Cooke, 1978) but is retained for a time after optic nerve crush (Schmidt, 1978). Nonetheless, reinnervation has been demonstrated in many systems to be highly nonrandom (Landmesser and Pilar, 1970; Raisman and Field, 1973), as is the altered synaptogenesis in the IPN. Reinnervation, in both the central and peripheral nervous systems in adults, is complicated by a number of additional factors. These include scarring, misdirection of axons, and greater distances (Stelzner, 1982), changes in glial response (Field and Raisman, 1981), and changes such as completion of myelination and decline in the availability (Raisman and Field, 1973) or formation of postsynaptic sites (Boothe et al., 1979). Furthermore, major although subtle differences may relate to the exact mode, size, and timing of lesions in young versus adult animals, more so in disease than in experiments. As shown below, reinnervation in the IPN is similar but quantitatively less vigorous compared to altered initial synaptogenesis induced by neonatal lesions. This suggests that there is not necessarily a basic difference in the response of the nervous system to injury at increasing age, no intrinsic loss of plasticity. Perhaps "critical periods" and other age-related effects reflect quantitative and qualitative modifications conditioned by a number of factors which to various degrees are unfavorable for reinnervation.

Comparison of the present observations on the effect of neonatal lesions preceding synaptogenesis and the reinnervation after adult lesions allows an assessment of the effect of age on response to injury in this system. The functional consequences are not known, but crest synapses are especially favorable for morphological comparison at the synaptic level. For a direct comparison, the existing adult data (Murray et al., 1979) have been recalculated using DRs, as was done above for the data of the present experiments. The DR can be calculated for the normal adult IPN where it is 35% (Table 1B in Murray et al., 1979). An increase in DR after sprouting is expected since the degenerating endings, arising from half as many neurons, should degenerate more synchronously (cf. Raisman and Matthews, 1972). That this does occur is clear from the data cited (Table 1C in Murray et al., 1979), for which a DR of 35% is too low, giving a large discrepancy between number of endings and number of crest synapses. The DR for their sprouting data is actually estimated as being 50%. This estimate rests on the following considerations: the minimum possible value for DR, 42%, would obtain if all of the 3448 axons forming the crest synapses are of habenular origin ($1432 \text{ degenerating axons} / 3448 = 0.42$, or 42%); the maximum DR could be as great as 100% if all habenular axons are detected as degenerating; the calculation of the incidence of each type of afferent pairing for DRs in this range, along with the morphological data which indicate that most of the nondegenerating endings appeared to be of habenular origin, narrows the probable DR to 50%, or possibly up to 60%. This DR is half again the value of 35% in normal IPN. Using this DR, reinnervation by left habenular axons is calculated to have occurred at 66% of crest synapses, 3-fold greater than that indicated by the raw data. If the DR is actually 60%, sprouting would involve 38% of crest synapses, still more than the raw

data indicates. Thus, MH axons respond to partial deafferentation by replacing lost MH afferents at 66% or possibly less of crest synapses in adults, compared to 96% in neonates. That this result is not due to some unrecognized factor other than age is supported by the use of DR to analyze the raw data, and by the fact that the more vigorous sprouting after neonatal lesions compared to adult lesions is associated with a greater increase in DR, 82% in neonates versus 50% after adult sprouting, and 35% in normal adults. Another way of stating this age-related effect is that after sprouting in the adult, left-left pairing is 22% by raw data (Murray et al., 1979) and 38 to 66% when calculated using estimated DR, as indicated above. In contrast, the present neonatal lesions result in 96% left-left pairing.

In conclusion, the interpretation of these studies of the IPN appears to require multiple mechanisms acting together during synaptogenesis. The present data support a role for presynaptic factors, possibly including axonal interaction, synapse elimination, or other modes of competition, specific recognition based on site, and possibly side of origin of axons. Determinants localized to the synaptic sites of the postsynaptic cell are also demonstrated. The similarity of normal IPN development in several species, and in the result of habenular lesions in neonatal compared to adult rat, encourages further search for the factors controlling synaptogenesis in this nucleus.

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