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Research Report

# An immature mossy fiber innervation of hilar neurons may explain their resistance to kainate-induced cell death in 15-day-old rats

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**Abstract**

Recent studies in adult rodents have shown that mossy fibers, the axons of hippocampal granule cells, sprout into the inner molecular layer of adult rats when hilar cell death occurs following kainate-induced seizure activity. This pattern of hilar cell death and mossy fiber sprouting is not observed in young rats at 15 postnatal days of age. Since granule cells are generated postnatally, one may assume that a lack of a mature mossy fiber input to hilar neurons at 15 days of age is a possible cause for this observed difference. Neo–Timm preparations were made from rats at 5, 10, 12, 15, 20, 21, 25, 30 and 32 postnatal days of age to study the postnatal development of mossy fibers. The adult pattern of Timm-labeled mossy fiber innervation in the granule cell layer was observed by 25 days. The Timm reaction product forms large dense granules in CA3 of 15 day old rats but the hilus at this age lacks this type of large granule. Instead, the hilus displays only small labeled boutons, suggesting that mossy terminals have not yet reached a mature size. Electron microscopic preparations of the deep hilus and the subgranular zone of the hilus at 7, 12, 15, 21 and 30 days were analyzed to study the development of synapses formed by axons of granule cells. At 7 days the deep hilus showed only a few asymmetric synapses formed by the developing mossy fibers. At 12 and 15 days of age, the number of asymmetric synapses per unit area in both the deep hilus and subgranular zone was less than half that found in the adult. The density of asymmetric synapses was greater in the deep hilus than in the subgranular zone for all ages. However, the subgranular zone of the hilus reached adult levels by 21 days whereas the deep hilus attained adult values at 30 days. Retrogradely labeled commissural neurons in the deep hilus from 14 day old rats were also examined, and these cells displayed axodendritic synapses formed by immature axon terminals. Since most of the asymmetric synapses in the hilus are formed by axon terminals that have the appearance of mossy terminals, these data taken together with other data indicate that hilar neurons from 15 day old rats lack a mature mossy fiber innervation, and this may contribute to their resistance to kainate-induced cell damage.

*Key words:* Mossy fiber; Dentate gyrus; Synaptogenesis; Hilus; Basket cell; Hippocampus

## 1. Introduction

The development of the tri-synaptic excitatory circuitry of the hippocampus has been analyzed over the past twenty years. The first excitatory synapse in this circuit is the one formed by perforant path fibers from the neurons of the entorhinal cortex. In the dentate gyrus, these synapses are found in the outer two-thirds of the molecular layer where they terminate on the distal dendrites of granule and non-granule neurons [13,22,54]. The development of synapses in the molecular layer mainly occurs from postnatal day 4 to 25 [11]. The maturation of this pathway coincides with the termination of granule cell formation in the dentate gyrus that occurs between postnatal days 21–25 [5]. At birth, only 15% of the granule cells are present but by day 25, 95% of the granule cells have reached their final position in the granule cell layer. Moreover, there is evidence that another 5% are generated through adult life [1,5].

The second excitatory synapse in the hippocampal circuitry involves the mossy fibers of granule cells that contact hilar neurons and pyramidal cells of the CA3 in Ammon's horn. The development of mossy fibers and their synapses with complex spines in CA3 was described in electron microscopic preparations from the rat [2,45]. On postnatal day 3, mossy fibers are

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observed in CA3 and form synapses with dendritic shafts. The large, thorny excrescences or complex spines of the proximal apical dendrites of CA3 pyramidal cells do not appear until the middle of the second postnatal week. By the end of the second week, the mossy terminals in CA3 display a mature adult-like appearance [2].

The third synapse in the tri-synaptic circuit is formed by the Schaffer collaterals of CA3 pyramidal cells with CA1 pyramidal cells. Anatomical studies of the development of this pathway have used light microscopy of intracellularly filled neurons to show axonal arbors [47]. Since the axon terminals are not readily identified in normal electron microscopic preparations, no known data are available that show synaptogenesis in this part of the tri-synaptic circuit. Since CA3 pyramidal cells are generated prenatally [5], one may assume that Schaffer collaterals establish synapses with CA1 neurons in the late fetal and early postnatal periods.

An important part of the excitatory circuitry of the hippocampus that is not included in the tri-synaptic circuitry is the granule cell input to neurons in the hilus of the dentate gyrus. The hilar neurons are generated prenatally [3] and display important commissural and associational projections with the contralateral and ipsilateral dentate gyrus, respectively [8,18,35,39,41,48, 52]. Recent studies indicate that many hilar neurons degenerate in experimental and human epilepsy, and that mossy fiber sprouting in the inner molecular layer subsequently replaces vacated synaptic targets [4,19,42,46]. In rats treated with kainic acid, such neuronal plasticity is described only for the adult dentate gyrus [6,49]. It is interesting to note that kainic acid-induced seizures do not produce hilar neuronal death or sprouting of mossy fibers in the immature brain [7,25,44]. Thus, 15 day old rats showed no cell loss, however, 3–4 week old rats with such seizures displayed supragranular sprouting. From experimental preparations of adult rats, Represa et al. [30] showed that a substantial number of kainic acid binding sites is associated with the mossy fibers. Together, these results suggested that a mature mossy fiber innervation of principal cells in the hilus is necessary for their vulnerability to kainate-induced excitotoxic damage.

Since previous studies have not examined synaptogenesis in the hilus, we have made a light and electron microscopic study of the development of mossy fiber synapses with hilar neurons. Timm-stained preparations were used to assess the light microscopy of mossy fibers and both quantitative and qualitative electron microscopy and retrogradely labeled commissural neurons were utilized in the ultrastructural analysis of mossy terminals and their synapses. The data obtained from this study indicate a delayed maturation of mossy fiber synapses in the hilus as compared to CA3. Also, these results show that at 15 postnatal days of age, the number of asymmetric, mossy terminal synapses per unit area in both the deep hilus and subgranular zone was less than half that found in the adult. The lack of a mature mossy fiber innervation of hilar neurons at this age may provide the structural basis for their resistance to kainate-induced excitotoxicity.

## 2. Materials and methods

### 2.1. Timm staining in the developing dentate gyrus

The animals selected for this study were Sprague–Dawley rats at the following ages: 5, 10, 12, 15, 20, 21, 25, 30, 32 and 90 days old. These time points were selected for analysis because a previous study showed that the development of synapses in the molecular layer of the dentate gyrus mainly occurs between 4 and 25 postnatal days [11]. The day that the rats were born was denoted as postnatal day 0. All of the animals were anesthetized using a mixture of Ketaset/Rompum and transcardially perfused with a buffered 4.9% sodium sulfide solution (pH 7.4). The animals were subsequently perfused with a solution of 3% paraformaldehyde and 1% glutaraldehyde in a phosphate buffer (pH 7.4). After the perfusion the brains were left in situ for 2 h at 4°C before being dissected from the cranium and placed in the perfusion fixative at 4°C for no more than 48 h. Blocks of tissue from these brains containing the entire hippocampal structure were sectioned in the coronal plane on a Vibratome 1000 at 40 $\mu$m. During the course of sectioning the hippocampus, three 100 $\mu$m thick sections were obtained from the rostral, medial, and caudal parts of the hippocampus for electron microscopy (see below). The 40 $\mu$m sections were mounted on gel-coated slides and labeled using the neo-Timm method outlined by Danscher et al. [12]. The neo-Timm staining was done only for the light microscopic preparations. The slides were placed in the neo-Timm staining solution that contained 5.1 g citric acid, 4.7 g sodium citrate, 3.4 g hydroquinone, 0.17 g silver nitrate and 120 ml gum arabic and was brought to a total of 200 ml with double distilled water. The slides were then incubated at 37°C in a dark chamber. The developing process was checked periodically over a 30–60 min period or until the desired staining had been reached. This was usually when three distinct layers of the molecular layer were observed [32]. After two 5 min rinses in water, the reaction was stopped by placing the sections in a 1% sodium thiosulfate solution for 10 min. The sections were then rinsed in water for 10 min, counterstained with Cresyl violet, dehydrated with ethanol, and cleared in xylene before being coverslipped with DPX mountant. The sections were analyzed using an Olympus BH-2 microscope, and camera lucida line drawings of the dentate gyrus were made using a Zeiss microscope with a drawing tube.

### 2.2. Electron microscopy of synapses in the developing hilus

For electron microscopy, the rostral 100 $\mu$m sections of the hippocampal formation were used from two rats at each of the following ages: 7, 12, 15, 21, 30 and 90 postnatal days. The sections were postfixed in 1.0% osmium tetroxide for 30–60 min, dehydrated in ethanol, and embedded in Medcast. Semi-thin 2 $\mu$m sections were sectioned on a MT 5000 Sorvall ultramicrotome and then stained with 0.05% Toluidine blue. Serial thin sections were cut in the silver interference color range, mounted on formvar-coated slot grids, then stained with lead citrate and uranyl acetate and examined with a Philips CM10 electron microscope. Electron micrographs encompassing an area of 50 $\mu m^2$ were taken of the neuropil of both the subgranular zone of the hilus and the deep hilus. For this study, the subgranular zone consisted of a region 30 $\mu$m subjacent to the

granule cell layer, whereas the deep hilus was located more than 50 μm below the granule cell layer. For the quantitative study of developing synapses, fifteen electron micrographs were taken of the subgranular zone and deep hilus of each animal at each age except for the 7 day old animals because they rarely displayed synapses in the hilus. From these electron micrographs counts of both symmetric and asymmetric synapses were made. The variation between the two animals at the same age was small in that the difference was less than 20% at the younger ages and less than 10% at the older ages. The following criteria were used to determine the presence of a synapse and its type: symmetric or asymmetric. A symmetric synapse was identified as one that contained elongated or flattened vesicles at the presynaptic membrane of the axon terminal. Round vesicles were used to identify asymmetric synapses. To be considered a synapse, the vesicles had to cluster at the presynaptic membrane of the axon terminal. Also, a postsynaptic density for the membrane of the postsynaptic profile had to be evident. This density is more prominent for the asymmetric synapses.

### 2.3. Mossy fiber innervation of developing hilar commissural neurons

Three 14 and two 30 postnatal day rats were anesthetized with Nembutal (approx. 50 mg/kg) and perfused with 0.9% saline followed by 4% paraformaldehyde solution in 0.1 M phosphate buffer. The fixed brain was then removed from the skull, and the caudal portion of the forebrain was separated from the rostral portion by making a cut with a razor blade in the coronal plane at the mid-septo-temporal level of the hippocampus. The two hemispheres rostral to the cut were not separated from each other to maintain the continuity of the ventral hippocampal commissure. DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) crystals of approximately 0.1–0.2 mm in diameter were placed in the dentate gyrus of one hemisphere. Brains were kept in 4% paraformaldehyde and incubated for 3 to 4 weeks at 37°C. Following the incubation period 60 μm coronal sections were obtained using a Vibratome. The sections were stored in a 2% paraformaldehyde solution in incubation trays at 4°C. The sections were examined and photographed with a fluorescent microscope to determine the presence or absence of labeled commissural neurons in the hilus of the dentate gyrus.

Sections that had labeling of neurons in the hilus of the dentate gyrus were selected for photo-oxidation and subsequent electron microscopic examination. These sections were treated at 20 min intervals, first with a rinsing solution of 0.05 M Tris, and then with a diaminobenzidine (DAB) solution (10 mg DAB sonicated with 10 ml, pH 7.6, 0.05 M Tris). The DAB soaked tissue sections were individually placed under the strong ultraviolet light of either a 20× or 40× objective of a Zeiss microscope to convert the DiI fluorescence into a dark brown reaction product. This electron dense reaction product allowed for the subsequent identification of labeled fibers in electron microscopic preparations [24]. These photoxidized sections were processed for electron microscopy using a routine protocol that included postfixation with osmium tetroxide, dehydration in alcohols and embedding in Medcast. Thin sections from the hilus of the dentate gyrus were prepared and examined with a Philips CM10 electron microscope to determine the presence and morphology of synapses formed with labeled dendrites.

## 3. Results

### 3.1. Light microscopy of mossy fiber development in the dentate gyrus

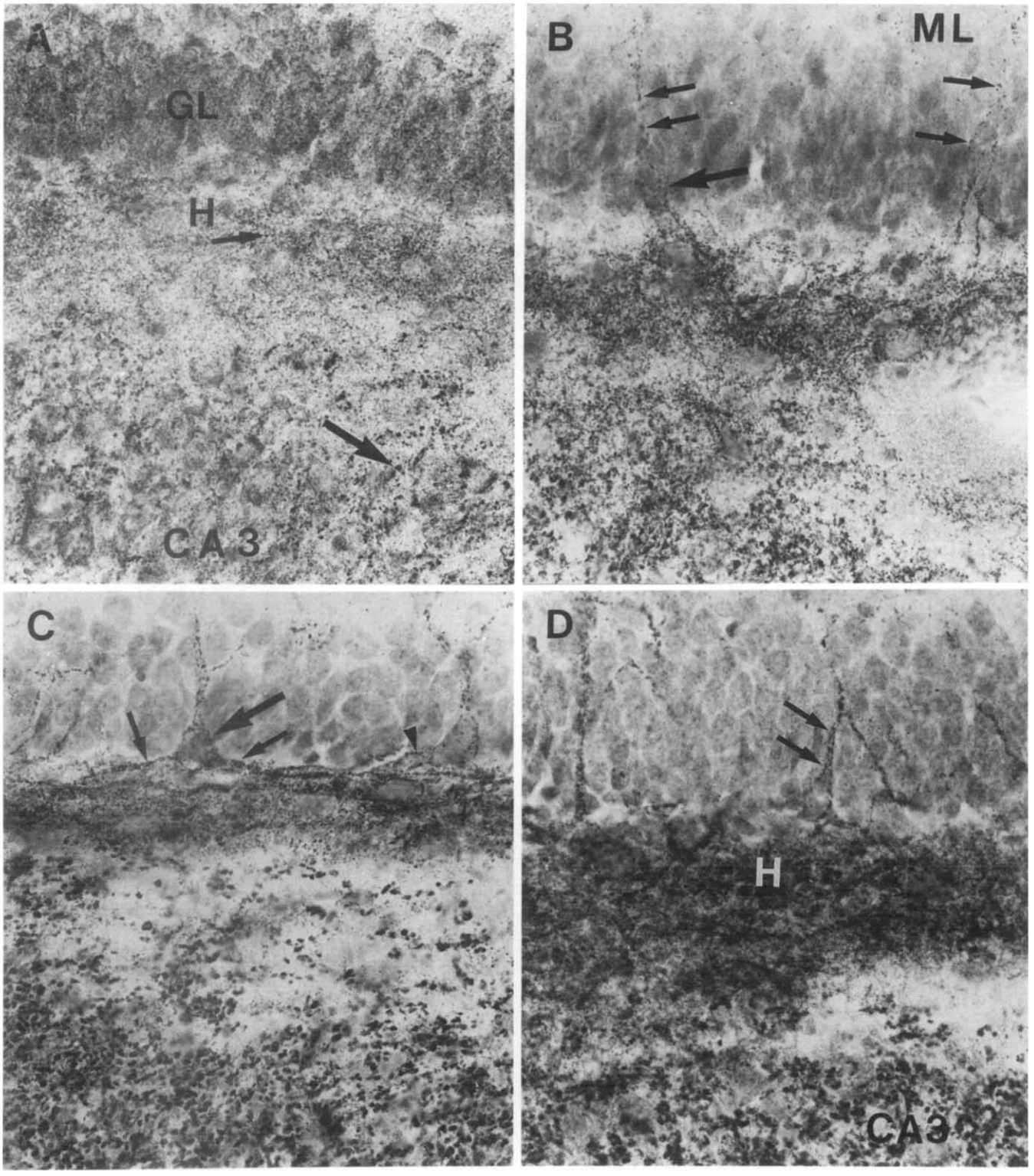
Timm-stained mossy fibers were absent inside the granule cell layer of 5 day old rats. The first intragranular mossy fibers were observed in the septal pole of 10 day old rats (Fig. 1). They appeared at the border between the granule cell layer and hilus in both supra- and infrapyramidal blades (Fig. 1A) and the peak (Fig. 2A) of the dentate gyrus. At 15 days of age, a well-developed plexus of small mossy fibers was associated with non-granule cell somata and dendrites in the granule cell layer (Fig. 1B). The boutons formed a plexus that often branched in the granule cell layer and appeared to follow the apical dendrites of large, non-granule cells into the inner molecular layer (Figs. 1B and 2B). This plexus was most common at the septal pole at 15 days but was infrequently observed in the more temporal sections of the dentate gyrus (Fig. 3). By 21 days, the intragranular mossy fiber plexus increased in density in the temporal half of the dentate gyrus but the septal pole still remained more dense. By 21–25 days, the development of the mossy fiber plexus was prominent, reaching an adult pattern in both the supra- and infrapyramidal blades of the granule cell layer (Figs. 1C and 2C). The adult pattern is recognized by a marked increase in the number of Timm-stained boutons associated with dendrites in the granule cell layer when compared to the 10 and 15 day old animals. This pattern of boutons can be so numerous that the complete outlining of basket cells can be observed in the granule cell layer, as described in adult rats [32]. The 30 day preparations also showed an adult appearance (Figs. 1D and 2D).

Timm-stained boutons were infrequently observed in the hilus of 5 day old rats even though they were present in the stratum lucidum of the CA3 region. A substantial number of mossy fibers was found in the hilus of 10 day old rats (Figs. 1A and 2A) which is consistent with the previous study by Gaarskjaer [17]. However, virtually all of them were relatively small compared to the large mossy fibers associated with the pyramidal cells in the CA3 region at this age (Fig. 1A). The difference in the size of the Timm-stained boutons between the hilus and CA3 region was observed in both septal and temporal sections of the dentate gyrus. By 15 days, the number of Timm-labeled boutons in the hilus increased (Figs. 1B and 2B), and many larger size boutons were found in the deep hilus, the region closest to the CA3 region. At 21 days, basal dendrites of apparent pyramidal basket cells were preferentially outlined by small Timm-labeled granules in the subgranular zone of the hilus (Fig. 1C). Timm-labeled boutons in the hilus were highly concentrated at 30 postnatal days of age (Figs. 1D and 2D). Many large Timm-stained boutons were found in the deep hilus but few were located in the subgranular zone.

### 3.2. Electron microscopy of the development of asymmetric synapses in the hilus

In the earliest examined preparations from the 7 postnatal day old rats, large swollen axon terminals

formed synapses with small dendritic profiles in the deep hilus (Fig. 4A). These axon terminals displayed only a few synaptic vesicles that were clustered near the active zone. The remaining portion of these terminals displayed large vesicles that are often observed in growing axon tips [29]. A large extracellular space was also present. In contrast, the next examined age, 12 postnatal days, did not show an excessive extracellular space (Fig. 4B). In addition, the axon terminals forming asymmetric synapses at this age were not swollen.



Instead, they displayed a compact appearance with many synaptic vesicles spread throughout the terminal. Although these terminals formed asymmetric synapses with dendrites (Fig. 4B), many immature dendrites lacked synapses. At 15 days, many immature dendrites were found again in the deep hilus (Fig. 5A), and they were infrequently found to be postsynaptic to axon terminals that formed asymmetric axodendritic synapses (Fig. 5A). Also, axon terminals at 15 days were usually small and packed with round synaptic vesicles. At 21 days, spines were often associated with dendrites in the deep hilus and axospinous asymmetric synapses were frequent (Fig. 5B). In addition, the dendrites displayed more mature features, such as numerous cisternae of granular endoplasmic reticulum. By 30 days, the neuropil of the deep hilus was dominated by axospinous asymmetric synapses formed by mature-appearing mossy fibers (Fig. 5C).

The subgranular zone of the hilus is defined as a region of the hilus within 30 $\mu$m of the granule cell layer. It displayed a similar pattern of development as that described above for the deep hilus. At 12 postnatal days, the dendrites in this region showed immature features. Portions of the dendrites lacked organelles but a few adjacent axon terminals nevertheless formed synapses with the dendrites (Fig. 6A). The dendrites in 15 day old preparations appeared to have more organelles and the axon terminals that formed asymmetric synapses displayed only a few synaptic vesicles clustered at the active zones (Fig. 6B). By 21 postnatal days, the neuropil in the superficial hilus resembled that found in the 30 day and adult preparations. Dendrites appeared to be mature, and axon terminals with many round vesicles formed both axospinous and axodendritic asymmetric synapses (Fig. 6C,D).

A quantitative electron microscopic analysis of asymmetric synapses in the hilus was made to determine when adult values of mossy terminal synapses are obtained. Data for the deep hilus, a region that is 50–100 $\mu$m below the granule cell layer are shown in Fig. 7. At 12 days, about 20% of the adult number of asymmetric synapses per unit area are present in the deep hilus. By 15 days of age, about 50% of the adult value is found. The number of asymmetric synapses per unit area reaches adult values at 30 days of age for the deep hilus. The axon terminals in the deep hilus formed markedly more axospinous synapses (79.5%) than axodendritic synapses (20.5%) at 30 postnatal days (Table 1). However, the percentage of axospinous synapses was fewer at earlier ages; 31% at 12 days, 56% at 15 days, and 68% at 21 days (see Table 1). Thus, as expected from the qualitative description, the number and relative frequency of axospinous synapses in the deep hilus increases with age.

The development of asymmetric synapses in the subgranular zone of the hilus was different from the deep hilus. Adult values for asymmetric synapses were reached by postnatal day 21 (Fig. 7). It is important to note that the values in the subgranular zone for the 12 and 15 day animals were less than 50% of the 21 day old animals. There are fewer asymmetric synapses per unit area in the subgranular zone as compared to the deep hilus. For example, at postnatal day 30, the mean number of asymmetric synapses per unit area was 5.9 for the subgranular zone whereas the deep hilus had 11.6 asymmetric synapses per unit area. The subgranular zone of the hilus had a greater percentage of axodendritic synapses than the deep hilus at each examined age (see Table 1). The deep hilus and the subgranular zone displayed more asymmetric synapses than symmetric synapses for all ages (Fig. 7). In fact, the number of symmetric synapses per unit area remained relatively constant throughout the examined postnatal ages.

### *3.3. Light and electron microscopy of photo-oxidized, DiI-labeled, commissural projection neurons in the hilus*

DiI placements into one side of the hippocampus labeled the commissural projection neurons of the contralateral dentate gyrus of 14 day old rats. However, no retrograde labeling of neurons was found in the 30 day old preparations because myelination of the commissural pathway may have prevented the diffusion of DiI along the axolemma. All retrogradely labeled neurons in the 14 day old rats were located in the hilus (Figs. 8A–D). Although some labeled somata were found adjacent to the granule cell layer (Fig. 8D), none of the

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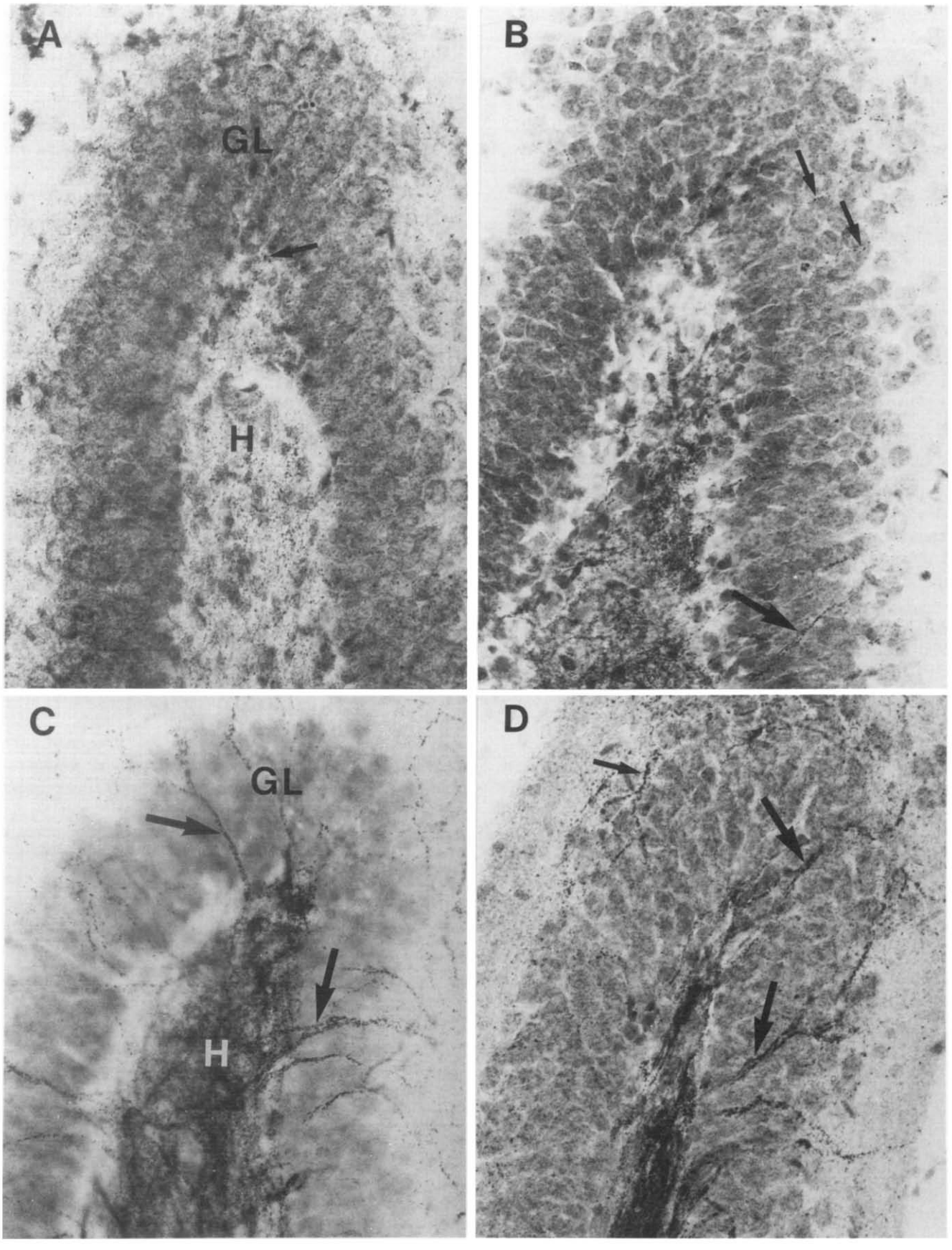
Fig. 1. Photomicrographs of the midportion of the suprapyramidal blade of the dentate gyrus from 10 (A), 15 (B), 21 (C) and 30 (D) postnatal day old rats. A: shows a 10 day old preparation where the hilus (H) appears undeveloped because it lacks the large, Timm-labeled mossy fiber granules that are seen in the CA3 region (large arrow). The hilus (H) displays only small, Timm-labeled granules (small arrow). No basket cells or apical dendrites of basket cells are outlined by mossy fibers in the granule cell layer (GL). B: shows a fusiform basket cell body (large arrow) outlined by Timm-labeled mossy fibers from a 15 day old preparation. Also visible are the apical dendrites that are outlined by small, Timm-labeled granules representing intragranular mossy fibers (small arrows). Some of these granules can be seen in the inner third of the molecular layer (ML). C: shows a section of the dentate gyrus from a 21 postnatal day old rat that displays a pyramidal basket cell (large arrow) outlined by Timm-labeled, intragranular, mossy fiber axons. The basal dendrites (small arrows) appear outlined in the subgranular zone of the hilus. A horizontal basket cell soma is outlined in a similar way except it displays only one main basal dendrite (arrowhead). D: shows Timm-labeled intragranular mossy fiber axons concentrated on the apical dendrites of basket cells (arrows) from a 30 day old rat. The larger Timm-labeled mossy fiber granules associated with the CA3 region are now also visible in the hilus (H). Magnification for A–D is 500 ×.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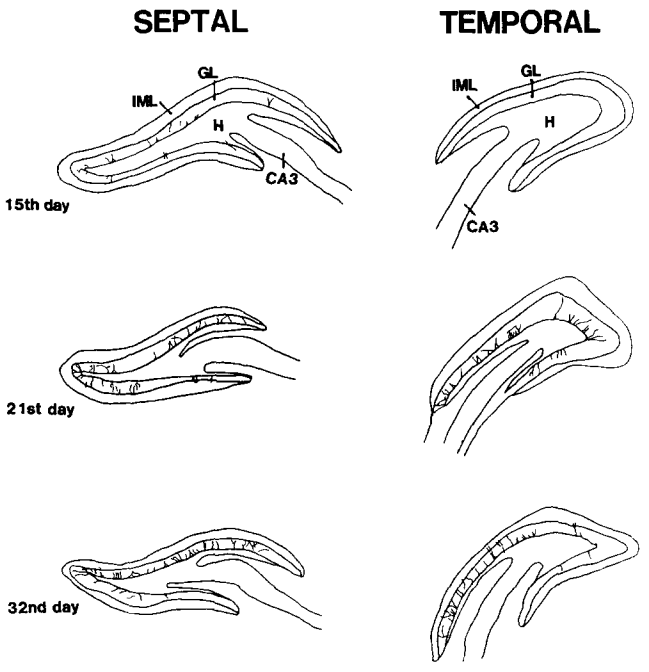


Fig. 3. Camera lucida drawings of the light microscopic results of intragranular mossy fiber labeling from 15, 21 and 32 postnatal day old rats. Septal sections of the hippocampus were identified by the presence of the striatum and habenular nuclei in the same section. Temporal sections contained the superior colliculus and medial geniculate body in the same coronal sections [28]. At 15 days, intragranular mossy fibers start to develop in the septal pole of the dentate gyrus but have not appeared in temporal sections. By 21 days, the concentration of intragranular mossy fibers has increased in the septal pole and they are now present in significant numbers in the temporal pole. In the 32 day old preparation, the concentration of intragranular mossy fibers reached adult levels in both septal and temporal poles [32].

basket cell types or other large non-granular cells were labeled inside the granule cell layer where these somata were described to occur [31,38]. The somata and dendrites of labeled hilar cells did not display spines due to the failure of DiI to enter spines or similar dendritic appendages.

Electron microscopy of DiI-labeled hilar cells revealed large and medium-sized neurons. The somata of the large cells that were located in the deep hilus had smooth round nuclei and a perikaryal cytoplasm containing numerous organelles (Figs. 9A,B). The reaction product from the photo-oxidation method was localized to cisternae of the granular endoplasmic reticulum

Table 1
Percentage of axodendritic and axospinous synapses in the developing hilus

| | 12 Day | 15 Day | 21 Day | 30 Day |
|---|---|---|---|---|
| Deep hilus | | | | |
| Axodendritic | 69.4% | 44.3% | 32.3% | 20.5% |
| Axospinous | 30.6% | 55.7% | 67.7% | 79.5% |
| Subgranular zone of the hilus | | | | |
| Axodendritic | 81.5% | 67.2% | 45.8% | 46.5% |
| Axospinous | 18.5% | 32.8% | 54.2% | 53.5% |

and portions of the Golgi complex (Fig. 9B). Such labeled organelles were found in the dendrites of commissurally labeled neurons where axodendritic synapses were found. The axon terminals that formed these synapses frequently had only a few synaptic vesicles at the active zone with a region of the terminal lacking vesicles (Fig. 9C). In addition to the axodendritic synapses, small axon terminals with round synaptic vesicles formed axospinous synapses with unlabeled spines that were adjacent to the labeled dendrites (Fig. 9D). In these preparations, it was difficult to determine whether these spines arose from the labeled dendrites because the spines were not found in continuity with the dendrites and the spines lacked DiI-labeled organelles. These DiI data show that the commissural cells at 14 days of age have projections to the contralateral hippocampus but lack an innervation by mature mossy fibers.

## 4. Discussion

In this analysis, three different anatomical methods were used to analyze the development of the mossy fibers in the dentate gyrus of the rat. First, the neo-Timm method was used to examine the light microscopic distribution of mossy fibers. Next, electron microscopic preparations of the hilus from various ages were examined for synapse development. Lastly, retrogradely labeled commissural neurons with DiI were analyzed for their ultrastructural features and the features of axon terminals that formed synapses with them. Together, these preparations provide insight into the development of the mossy fiber connections in the dentate gyrus.

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Fig. 2. Photomicrographs of the peak of the dentate granule cell layers (GL) from 10 (A), 15 (B), 21 (C) and 30 (D) postnatal day old rats. The orientation of each is the same with medial being at the top. The most medial part of the hilus (H) is located between the two blades. A: shows fine Timm-labeled granules in the hilus of a 10 day old preparation. A few appear at the base (arrow) of the granule cell layer (GL) in the peak. B: by 15 days, the labeling in the hilus is greater. Note that intragranular Timm-labeled fibers outline dendrites near the peak (large arrow) and also occur in patches (small arrows). C: the 21 day preparation shows dense labeling of Timm fibers in the hilus (H) and numerous outlined dendrites (arrows) passing through the granule cell layer (GL). D: the 30 day sections show a similar labeling pattern in the peak as the 21 day ones. However, the boutons appear to be denser and larger. Some Timm-labeled puncta outline intragranular dendrites (large arrows) whereas others are grouped together in the inner molecular layer (small arrow). Magnification for A–D is 500 ×.

### 4.1. Hilar mossy terminals mature later than CA3 mossy terminals

The appearance of large, Timm-stained mossy fibers occurs relatively early in the CA3 region where light microscopic preparations show them clearly in 10 day old rats [2,17]. However, the hilus does not show such large Timm-stained boutons at this age. In fact, they first appear in the hilus at 15 days and are common in the deep hilus of 21 day old rats. Along with the light microscopic data, electron microscopic observations show that the mossy terminals in the CA3 region reach maturity at 15 days of age (unpublished observation and [2]). However, large-size, mature mossy terminals

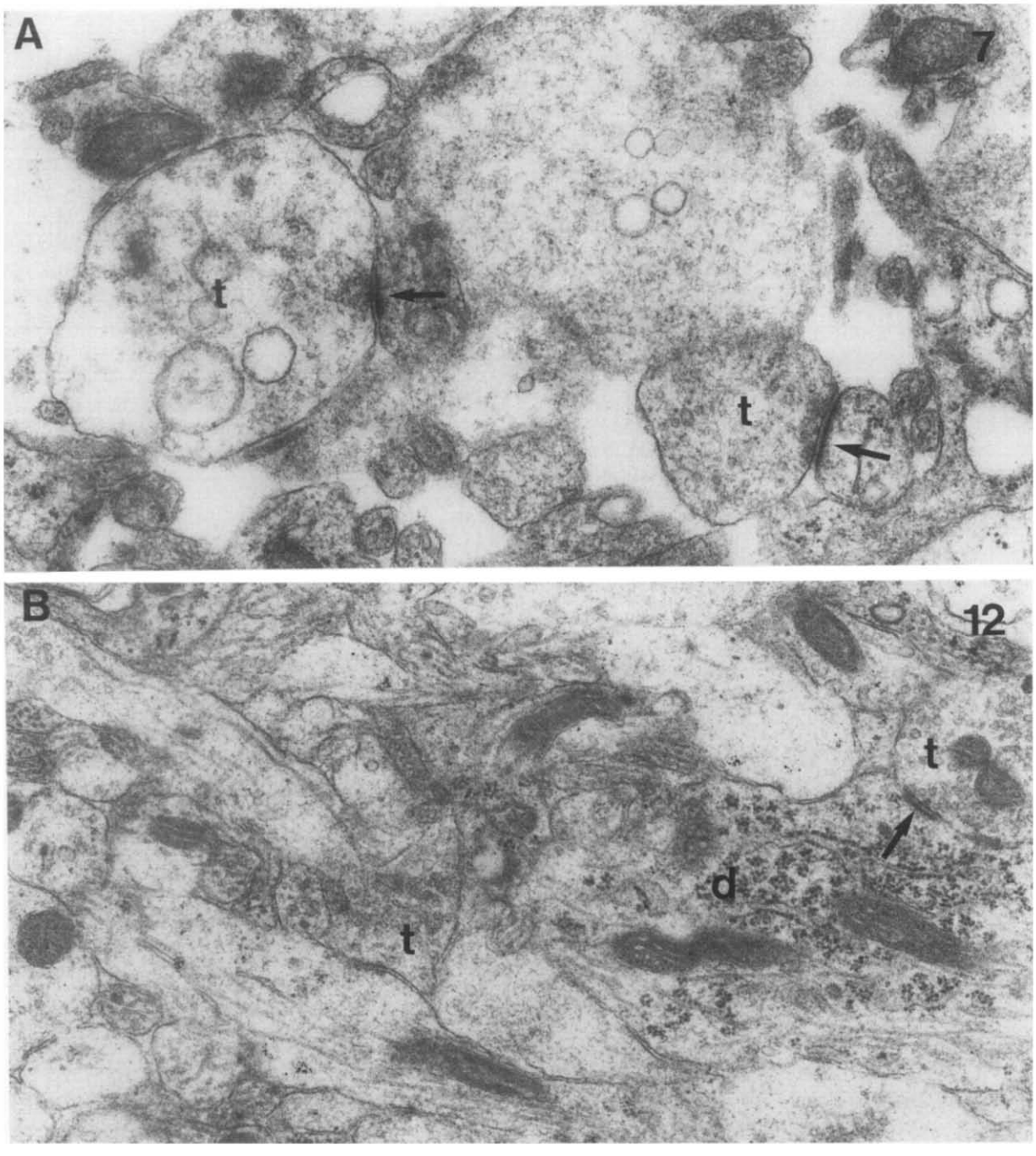



Fig. 4. Electron micrographs of the neuropil in the deep hilus from 7 (A) and 12 (B) day old rats. A: shows two swollen axon terminals (t) that contain a small number of round vesicles that are clustered near asymmetric synapses (arrows) with small dendritic profiles. The excessive space between the neuronal profiles is probably due to the immaturity of the hilus at this age. B: shows two axon terminals (t) that display more synaptic vesicles than the 7 day old preparation. One of these forms a synapse with a proximal dendrite (d) that was identified by the large number of ribosomes associated with cisternae of granular endoplasmic reticulum. Magnification for A is 31,000 × and for B is 29,000 ×.

usually are not found in the hilus until 30 days. Therefore, the mossy terminals in the CA3 region mature prior to those in the deep hilus, indicating that granule cells make mature connections with pyramidal cells before they do with hilar neurons.

Additional support for the immature innervation of the hilus at 15 postnatal days is derived from the electron microscopic study of hilar asymmetric synapses. Routine electron microscopic preparations were used for this quantitative study because the tissue

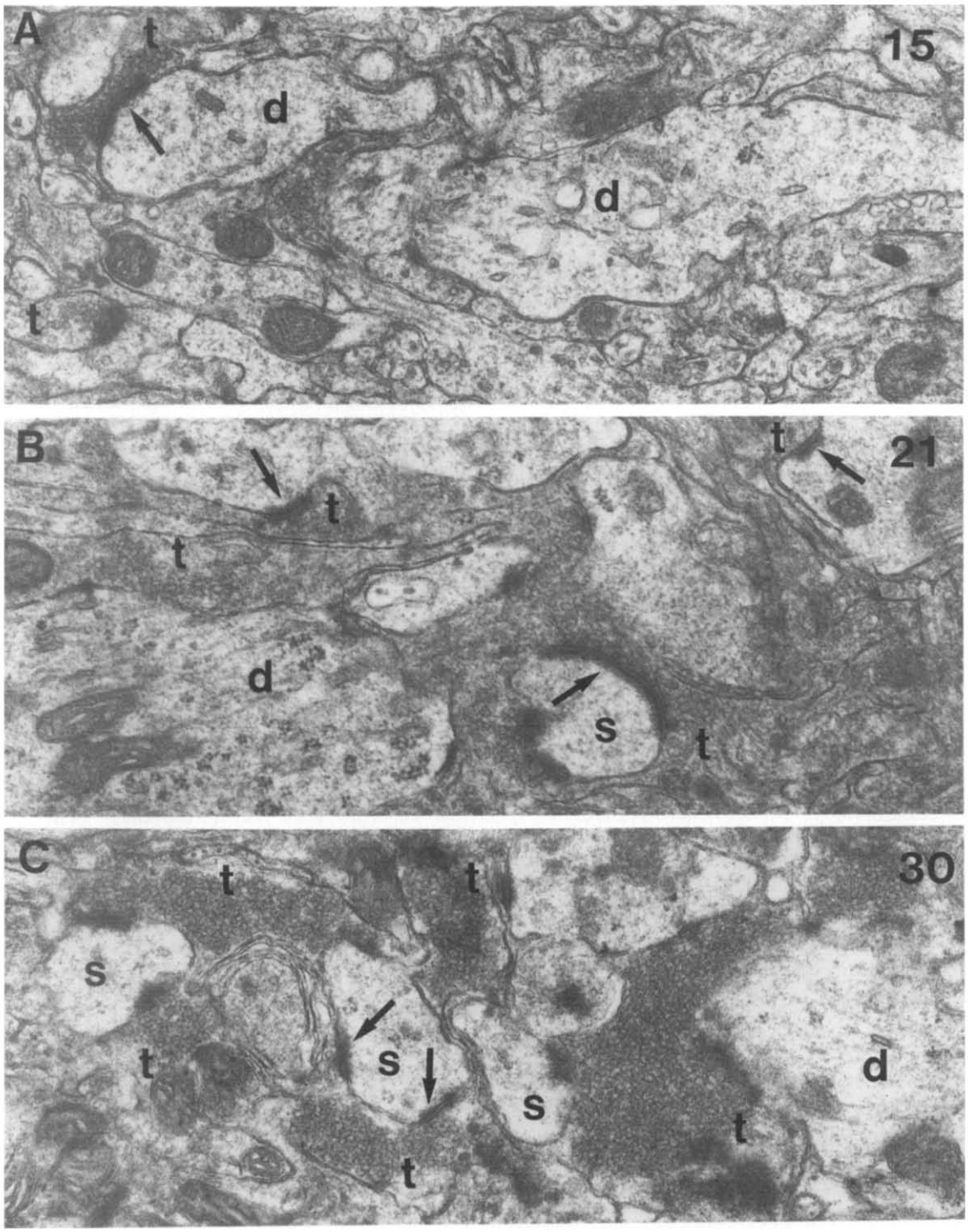
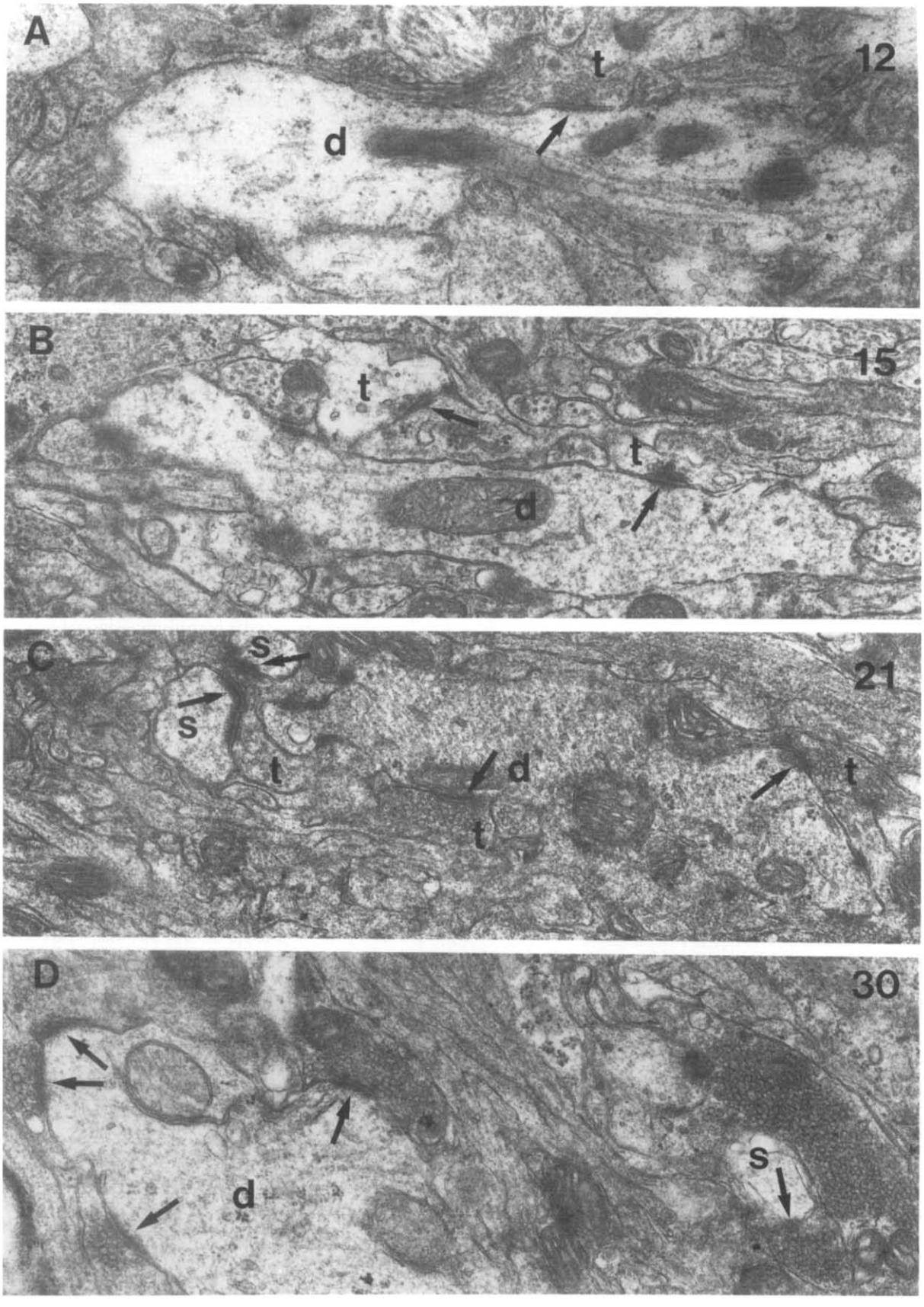



Fig. 5. Electron micrographs of the neuropil in the deep hilus from 15 (A), 21 (B) and 30 (C) day old rats. At 15 days, only a few asymmetric synapses (arrow) appear which are formed by small axon terminals (t) filled with round synaptic vesicles and dendritic shafts (d). B: shows an increase in the number of asymmetric synapses (arrows) as compared to A. Most synapses are with spines (s) but some involve dendrites (d). In C, the neuropil is filled with large-size axon terminals (t) that are packed with round synaptic vesicles. These axon terminals form asymmetric synapses (arrows) mainly with spines at this age. Magnification for A–C is 30,000 ×.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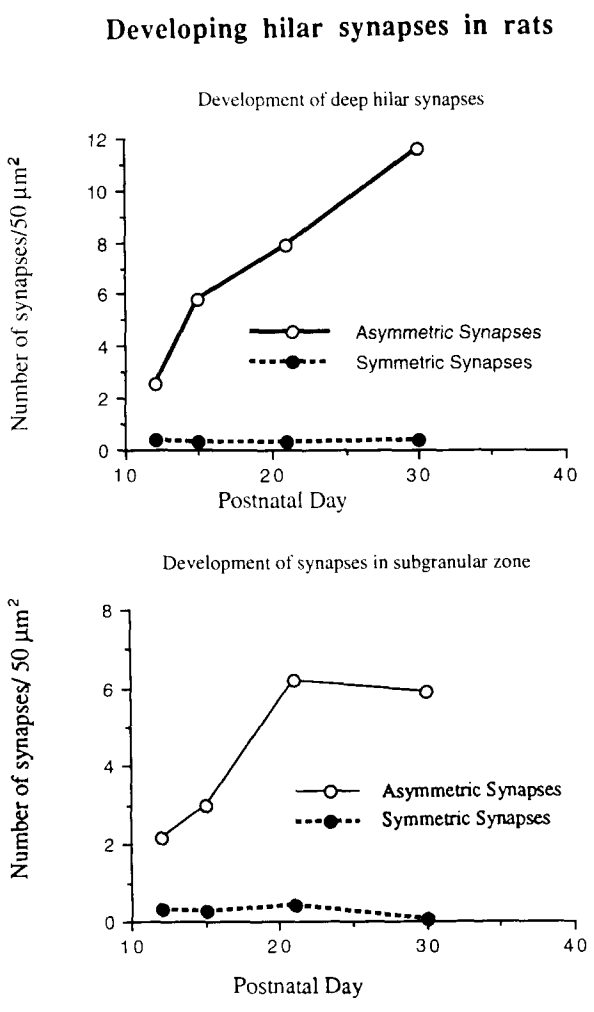


Fig. 7. Two graphs of the quantitative synapse data obtained from the deep hilus and subgranular zone. Note that the deep hilus at 15 postnatal days displays less than 50% of the asymmetric synapses per unit area than that at 30 postnatal days. The subgranular zone of the hilus reaches a mature number of synapses per unit area by 21 postnatal days.

preservation is better than that found in Timm preparations. Also, previous Timm and intracellular labeling studies have shown that small hilar axon terminals with round synaptic vesicles and asymmetric synapses are collaterals of mossy fibers [9,32]. Since most of the axon terminals that form asymmetric synapses in the adult hilus have the features of mossy terminals, it is assumed for this discussion that substantial numbers of these axon terminals in the developing hilus are mossy terminals. This assumption is consistent with the fact that the hilar region of the dentate gyrus receives only a sparse innervation from septal and commissural projections. The results from the developing hilus show only small axon terminals with variable numbers of round synaptic vesicles forming synapses prior to 15 days of age. These presumed, developing mossy terminals in the hilus at 15 days of age were less mature in their appearance than those in the CA3 region at this age [2]. Furthermore, Fig. 7 shows that the percentage of hilar asymmetric synapses that are assumed to be formed mainly by mossy terminals at 15 days is less than 50% of the 30 day value. Therefore, both the qualitative and quantitative synapse data exhibit the relative immaturity of hilar synapses formed by mossy terminals in 15 day old rats.

Another line of support for the immature innervation of hilar neurons is derived from the ultrastructural analysis of axon terminals that form synapses with DiI-labeled commissural neurons in the 14 day preparations. The DiI results of the distribution and types of labeled hilar neurons are consistent with previous studies that used retrograde tracing methods [8,16,35,41, 48,52,53], but they did not reveal classic basket cells as reported recently in a Fluoro-Gold retrograde tracing study in adult rats [18]. Many of the labeled cells in the electron microscopic preparations are probably not GABAergic hilar neurons because most of the DiI-labeled neurons had somal features similar to mossy cells [34]. Axosomatic synapses were rarely observed for these neurons. However, many immature appearing axon terminals formed axodendritic synapses with the proximal dendrites of labeled somata at 14 days. The paucity of synaptic vesicles and the numerous puncta adharens are features of developing synapses [51]. In addition, axospinous synapses in the neuropil adjacent to labeled dendrites were formed by small axon terminals that had the features of the immature axon terminals observed in the deep hilus of 15 day electron microscopic preparations used for the quantitative analysis. These observations are consistent with our previous Golgi study [34] that showed mossy cells from 15 day old preparations having only a few developed patches of thorny excrescences on their dendrites along with many dendritic portions both proximally and distally that lacked them. The fact that the labeled cells are commissural neurons with a substantial contralateral projection as evidenced by the anterograde labeling pattern in the inner molecular layer (Fig. 8B)

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Fig. 6. Electron micrographs of the neuropil in the subgranular zone of the hilus from 12 (A), 15 (B), 21 (C) and 30 (D) day old rats. In A, a dendrite (d) displays a few microtubules and is electron lucent. The only axon terminal (t) in the field forms an asymmetric synapse (arrow). By 15 days (B), the dendrites (d) have more microtubules and two asymmetric synapses (arrows) are formed by axon terminals (t). In C, synapses (arrows) are formed by axon terminals (t) with both spines (s) and dendrites (d). The electron density of the matrix substance of the dendrite is enhanced whereas the spines appear electron lucent. By 30 days (D), a similar distribution of axospinous and axodendritic synapses is found as that in the 21 day old specimen. Magnification for A and D is 30,000 × and for B and C is 34,500 × .

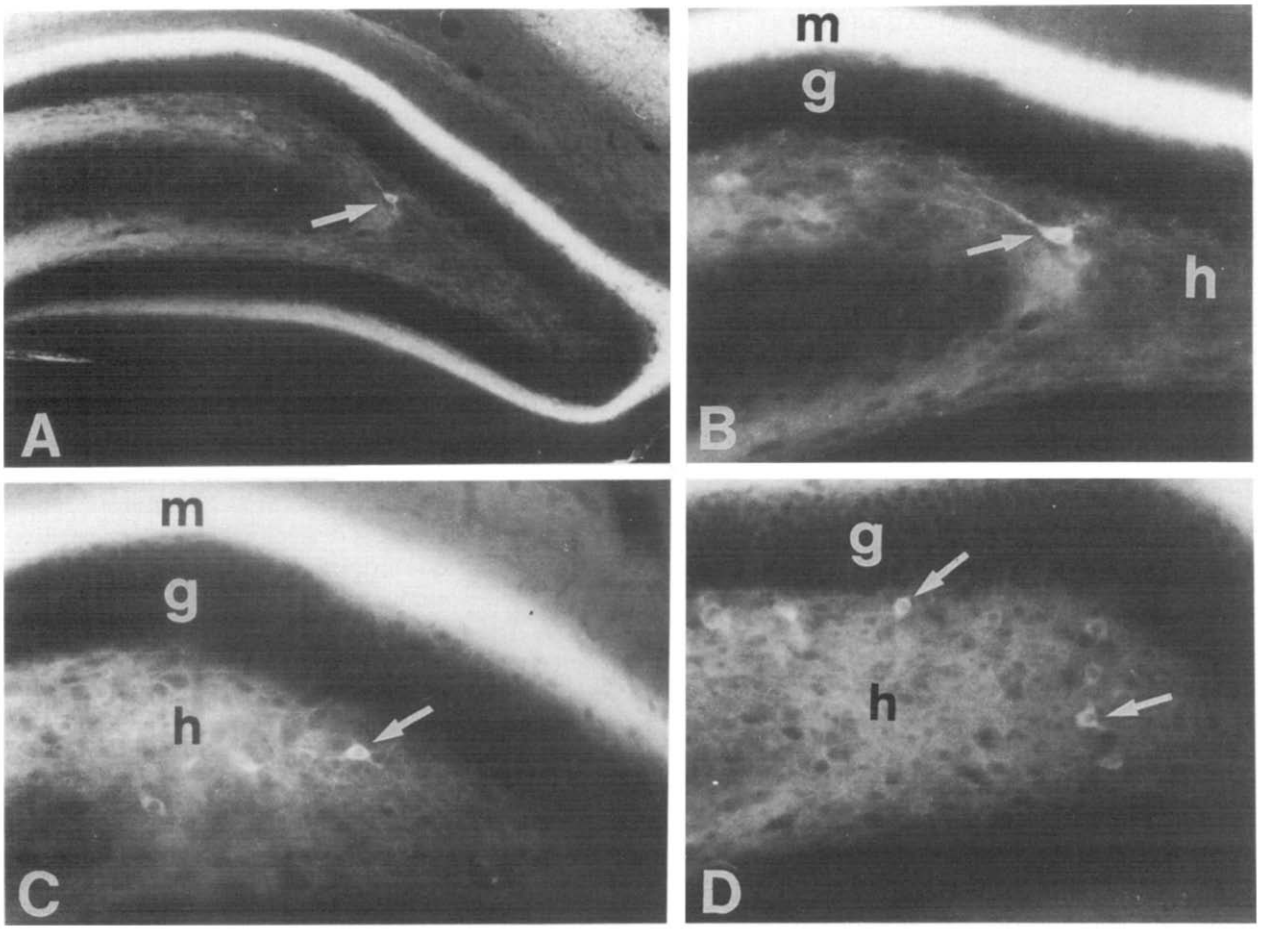


Fig. 8. Fluorescent photomicrographs of transported DiI in the dentate gyrus of 14 day old rats following contralateral placements. A: shows a labeled neuron (arrow) in the hilus. B: is an enlargement of this neuron (arrow) to show its multipolar soma and a prominently labeled dendrite in the hilus (h). Note that the inner molecular layer (m) contains many anterogradely labeled commissural axons whereas the granule cell layer is virtually free of labeled fibers. C and D: show two other sections with retrogradely labeled somata (arrows) in the hilus (h) and anterogradely labeled axons in the inner molecular layer (m). A few labeled fibers are visible in the granule cell layer (g). Magnification for A is 75 × and for B–D is 150 ×.

indicates that these cells have a well-established efferent projection. Therefore, these data indicate that hilar neurons, including the deep ones with commissural projections: (1) lack a mature mossy fiber innervation in 15 day old rats, and (2) undergo significant morphological development after establishing their long projections.

### *4.2. Functional considerations for the normal and epileptic dentate gyrus*

The light microscopic Timm-labeling and the quantitative electron microscopic data provide support for a developmental gradient for mossy fibers in the hilus. At 21 postnatal days, the subgranular zone of the hilus shows a preferential outlining of basal dendrites by Timm-labeled terminals and the number of asymmetric synapses per unit area reaches the same value as that of the 30 day old rats. In contrast, the deep hilus displays only 70% of the adult value for the number of asymmetric synapses at this age. Consistent with these data are immunocytochemical results that show the distribution of embryonic neural cell adhesion molecule in 15 and 30 day old rats [21]. This molecule is assumed to play a role in the development of neural connections

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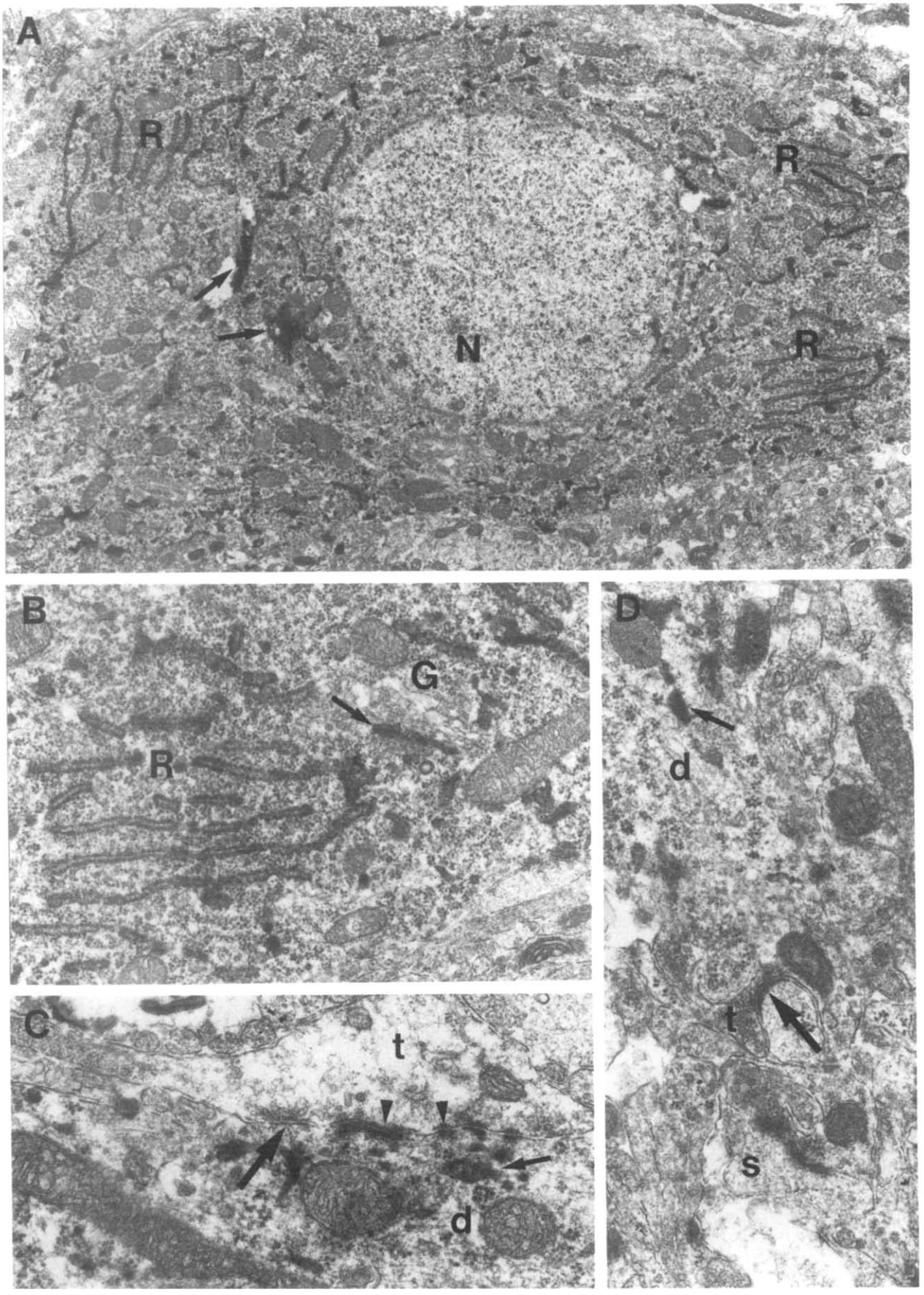
Fig. 9 Electron micrographs of the DiI labeled neuron in the deep hilus indicated in Fig. 8A,B. A: shows the soma of this neuron with a large round nucleus (N). Electron dense reaction product occurs in the perikaryal cytoplasm (arrows) and is associated with the membranes of the rough endoplasmic reticulum (R). B: shows an enlargement of the Nissl body in the lower right corner of A. Note the electron density associated with the membranes of the rough ER (R) and labeling of cisternae of one side of the Golgi complex (G). In C, two axon terminals (t) form asymmetric synapses (large arrows) with a proximal dendrite (d) of this neuron. Note the electron dense reaction product (small arrow) in the cytoplasm of the dendrite. D: shows a synapse (large arrow) formed by a small axon terminal (t) and a spine next to a distal portion of the labeled dendrite (d). Another spine (s) appears to be postsynaptic to an axon terminal. Electron dense label (small arrow) appears in the dendrite. Magnification for A is 7,500 ×, for B is 22,000 × and for C and D is 29,000 ×.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and is closely associated with the mossy fiber system. Its immunoreactivity was shown to be highly concentrated in the subgranular zone of the hilus at 15 postnatal days whereas the deep hilus displayed only labeled axons of passage that were directed toward the CA3 region. By 30 days, the immunostaining was homogeneous throughout the hilus. Taken together, these findings suggest that hilar mossy fiber connections mature faster in the subgranular zone than in the deep hilus.

This developmental gradient of hilar mossy terminal maturation may have different physiological effects on GABAergic, inhibitory interneurons and deep hilar neurons. In adults, intra- and supragranular mossy terminals innervate the proximal apical dendrites and somata of basket cells [20,32], whereas hilar mossy terminals form numerous synapses with basal dendrites of rat basket cells [31] that are mainly confined to a subgranular zone within 30 $\mu$m of the granule cell layer as determined by Golgi [23,31,40], immunocytochemical [26,33,36] and intracellular staining [37] methods. Since the proximal dendrites of these cells appear to be outlined by numerous Timm-labeled boutons and the subgranular zone of the hilus displays adult numbers of asymmetric synapses in 21 day old preparations, the mossy fiber afferent connections to GABAergic basket cells appear to be mature by this age. In contrast, the mossy cells and other commissural neurons are located in the deep hilus where the number of asymmetric synapses per unit area reaches adult levels at 30 postnatal days. Therefore, the excitatory mossy fiber afferent connection to deep hilar neurons appears to mature later than that for the GABAergic basket cells that have extensive basal dendrites in the subgranular zone of the hilus. Physiological studies of the developing dentate gyrus should be conducted to determine whether a difference exists in the maturity of mossy fiber stimulation of these two classes of cells.

The innervation of hilar neurons by mossy fibers in both the deep and superficial hilus at 15 days of age is less than 50% of the adult values. This finding suggests a lack of a mature mossy fiber innervation of hilar neurons at 15 days, an age when hilar neurons are resistant to the excitotoxic effects of kainic acid-induced seizures [7,25,44]. Mossy fibers use glutamate, an excitatory neurotransmitter, for synaptic transmission [50]. A type of glutamate receptor, the kainate type, has its greatest concentration in the mossy fiber terminal field [10]. Represa et al. [30] have indicated a presynaptic localization of kainate binding sites, a notion suggested by earlier biochemical and electrophysiological studies [14,15]. Furthermore, lesions of mossy fibers in adult rats were shown to reduce substantially the degeneration of CA3 and hilar neurons following kainate-induced seizures [27]. Thus, these studies indicate that kainate causes the release of glutamate from mossy fibers leading to an excitatory postsynaptic potential. Through this action, kainate may sustain excitatory activity, such as that associated with seizures, and this can lead to the excitotoxic death of postsynaptic hilar neurons. Since the mossy fiber innervation of hilar neurons is immature at 15 days, it is likely that kainate receptors on mossy fibers are not present in large enough quantities in the hilus to cause excitotoxicity of hilar neurons at this age. It is interesting that CA3 pyramidal cells are also resistant at this age, and the basis for this could be immature numbers of mossy terminals in stratum lucidum because granule cells are still being generated at this age [5]. Therefore, the lack of susceptibility to excitatory effects of kainate is probably due to the immature numbers of excitatory synapses on the hilar neurons at 15 postnatal days, especially the hilar neurons in the deep hilus that have extensive commissural and associational projections.

In summary, the results of this study indicate that mossy terminals in the hilus of the dentate gyrus appear immature at 15 postnatal days as compared to those in the CA3 region. Also, the number of mossy terminals per unit area in the 12 and 15 day hilus is less than half of the number observed in 30 day old rats. This immature mossy fiber innervation of hilar neurons may explain the lack of excitotoxic damage following kainate-induced seizures at young ages [7,25,44]. The findings that the number of hilar synapses in 30 day old rats is similar to adults and that excitotoxicity occurs at this age [7,25,43] are consistent with the hypothesis that a mature number of mossy fibers must be present for kainate-induced neurotoxicity. Further studies are required to show the physiological maturation of the mossy fiber connections with hilar neurons.

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