Target-controlled differentiation of axon terminals and synaptic organization

(hamster/synaptic glomeruli/plasticity/development/synapses)

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ABSTRACT These experiments investigate the processes regulating the morphological differentiation of synaptic connections. Electron microscopy showed that the terminal boutons and synaptic complexes of retinal afferent axons in the main thalamic visual nucleus, the dorsal lateral geniculate nucleus, differ in their morphology from those of ascending afferent axons in the main thalamic somatosensory (ventrobasal) nucleus. Developing retinal ganglion cell axons in hamsters were made to project permanently to the ventrobasal nucleus, rather than to the lateral geniculate nucleus. With respect to most of the ultrastructural features examined, the terminals and synaptic complexes of mature, anterogradely labeled retino-ventrobasal axons more closely resembled those of normal somatosensory afferents to the ventrobasal nucleus than they did those of normal retinofugal axons within the lateral geniculate nucleus. These results suggest that the ultrastructural differentiation of axon terminals and synaptic complexes is regulated largely by the target environment, although some features appear to be intrinsic to the afferent axons themselves.

It is increasingly evident that patterns of axonal projection and the morphology and biochemistry of synaptic connections in the vertebrate central nervous system are determined by trophic interactions between presynaptic neurons and their targets, but the extent and details of these important processes are not well understood (1–3). A variety of observations suggest the role of postsynaptic neurons or their immediate environment (e.g., glia, extracellular matrix) in the morphological differentiation of presynaptic elements during normal development (4). For example, mossy fibers originating from widely scattered parts of the central nervous system all have the same structural type of terminal that synapses in a stereotyped way with the dendrites of cerebellar granule cells (4, 5). Furthermore, both mossy and climbing fibers, which originate from different neuronal populations, contact multiple cerebellar cell types. Both fiber types make terminals with morphologies that are unique to each of their targets, and both fiber types make the same kind of terminal when synapsing on the same cell type (5–7). Though retinal ganglion cell (RGC) axons projecting to the primary visual nucleus of the thalamus, the dorsal lateral geniculate nucleus (LGd), are collaterals of RGC axons projecting to the superior colliculus (SC) (8), the morphologies of RGC axon terminals differ in the LGd and SC (refs. 9–12; G.C., unpublished data). Similarly, auditory nerve fibers have multiple collaterals, each of which projects to a different division of the cochlear nucleus and each of which has a distinct terminal morphology (13–15).

Axon terminals frequently participate in synaptic complexes called “glomeruli”—regions containing numerous neuro-

nal elements engaged in multiple synaptic contacts and isolated from areas of simpler neuropil by sheets of astrocyte cytoplasm. Despite the preceding data from normal animals, the hypothesis that the differentiation of axon terminals and their synaptic complexes is under the control of their target neurons or their environment requires experimental testing. This can be done when a population of axons projects abnormally to a novel target. We and others (9, 11, 12, 16, 17) show that the synaptic complexes of ascending, specific sensory axons in the LGd differ from those in the primary somatosensory (ventrobasal) thalamic nucleus (VB). Therefore, in this study we induced RGC axons in newborn hamsters to form permanent, functional, abnormal retinal projections to VB (18–20). When the hamsters were adults, the RGC axon synaptic complexes were anterogradely labeled and examined by electron microscopy to determine if the VB had influenced their differentiation. Morphological features of retino-VB axon synaptic complexes that more closely resemble corresponding features of normal somatosensory afferents to VB than corresponding features of normal retino-LGd axons are likely to be determined by the target. Conversely, features of the synaptic complexes of normal retino-LGd axons that are not shared with normal somatosensory afferents to VB but that are conserved by retino-VB axons are likely to be intrinsically determined by the presynaptic neurons.

METHODS

Permanent retinal projections to VB (18–20) were surgically induced in eight newborn Syrian hamsters. Under hypothermia anesthesia, ablations of two of the principal targets of RGC axons, the SC and LGd, were made. Heat lesions of the SC were made bilaterally, whereas the LGd was ablated unilaterally by making a heat lesion of the ipsilateral occipital cortex [because of the cortical lesion, the LGd undergoes rapid, retrograde degeneration (21)]. The ipsilateral VB was made an alternative target for RGC axons by making a midbrain hemisection to cut its ascending somatosensory afferents. When the hamsters were young adults (two each at 7, 10, 11, and 14 weeks of age) they were anesthetized with ether (inhalation) or Nembutal (60 mg · kg⁻¹, i.p.) and the eye contralateral to the deafferented VB was intravitreally injected with horseradish peroxidase (HRP, Sigma type VI; 5 µl, 70% in saline). After 24 hr (*n* = 2) or 48 hr (*n* = 6) the

Abbreviations: RGC, retinal ganglion cell; LGd, dorsal lateral geniculate nucleus; SC, superior colliculus; VB, ventrobasal thalamic nucleus; HRP, horseradish peroxidase; TMB, tetramethylbenzidine; R-boutons, axon terminal boutons containing clear, round synaptic vesicles and pale mitochondria; LR-boutons, large axon terminal boutons containing clear, round synaptic vesicles and dark mitochondria; F-boutons, axon terminal boutons with flattened synaptic vesicles; P-boutons, presynaptic dendritic appendages with pleiomorphic synaptic vesicles.

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FIG. 1. (A) Glomerulus in the LGd contralateral to the HRP-injected eye of a normal adult hamster. The glomerulus (wrapped in astrocyte cytoplasm, a) consists of: six small- to medium-sized R-boutons, two heavily labeled (R1, R2) with electron-dense Hanker-Yates reaction product, two lightly labeled (R3, R4), and two apparently unlabeled (R5, R6); one P-bouton (P) that is postsynaptic (double-headed arrow) to R5; a presumptive relay cell dendrite (D) that is postsynaptic (arrows) to R1 and R3, in filamentous contact (arrowheads; refs. 16 and 27) with R4 and gives rise to two small appendages (*) that invaginate R2. The multiple R-boutons in a single glomerulus of the LGd cannot be completely accounted for as multiple lobes of single, very large amorphous terminals: (i) short series of 10–20 sections only rarely reveal any tendency of these boutons to fuse with one another; (ii) extremely large R-boutons are never seen in single sections, although this should sometimes occur if they were present in significant numbers. [The presence of multiple R-boutons in single glomeruli has been shown in the cat LGd (28)]. The mitochondria of the R-boutons are pale with dilated irregular cristae. (B) Glomerulus in the VB of a normal hamster. The glomerulus (en-sheathed in astrocyte cytoplasm, a) contains a single, very large presumptive ascending somatosensory axon terminal (LR-bouton, LR) that is invaginated by ten appendages in the plane of this section: five appendages are clearly postsynaptic to the LR-bouton, and four appendages clearly arise from presumptive relay cell dendrites (D1, D2) that are also in filamentous contact (arrowheads) with the LR-bouton. The dark mitochondria with narrow regular cristae in the LR-bouton contrast with the mitochondria of the R-boutons in A and C. Two F-boutons (not illustrated) are contiguous with the large-diameter dendrite D1, opposite the LR-bouton. (C) Glomerulus in the VB contralateral to the HRP-injected eye of an operated adult hamster. The glomerulus (surrounded by astrocyte laminae, a) contains a single, very large R-bouton (R) that originates from a retinal axon, as demonstrated by TMB reaction product (arrows; upper and lower double-headed arrows denote reaction product enlarged in left and right insets, respectively). The R-bouton is invaginated by eleven appendages in this section; seven appendages are postsynaptic to the R-bouton and two appendages arise from the presumptive relay cell dendrite D1. Dendrites D1 and D2 are also in filamentous contact (arrowheads) with the R-bouton. Note the pale mitochondria with dilated irregular cristae in this R-bouton and their resemblance to those of the R-boutons in A. An F-bouton (not illustrated) is apposed to the large-diameter dendrite D1 opposite the R-bouton. (Scale bar = 1 μ m for A–C and 0.5 μ m for insets to C.)

animals were reanesthetized and perfused with 1% paraformaldehyde/1.25% glutaraldehyde in 0.1 M Millonig's phosphate buffer, pH 7.3 (23).

Brains were cut coronally at 70–100 μ m on a Vibratome, and alternate sections were processed by modified Han-

ker-Yates (22, 23) and tetramethylbenzidine (TMB) (24, 25) methods. In the TMB procedure, sections were incubated in TMB and sodium nitroferricyanide solutions according to Mesulam (25), except that citric acid-ammonium acetate buffer, pH 6.0, was used in place of sodium acetate buffer, pH

3.3. The TMB method labeled more retinofugal axon terminals than did the Hanker-Yates method. However, the "ghost-like" crystalline reaction product of the TMB method was more difficult to see (26). Hanker-Yates- and TMB-treated sections were placed for 1 hr in 2% OsO₄ in Millonig's buffer, stained *en bloc* with 2% uranyl acetate, dehydrated in ethanols, and embedded in Epon/Araldite. Regions of the VB in which labeled RGC axons and terminals were visible were cut from the sections and remounted onto blank resin stubs. Semi-thin sections were cut, stained with toluidine blue, and traced with a *camera lucida* in order to clearly define the borders of the VB by cyto- and myeloarchitectonic criteria. Ultrathin sections (silver-to-light gold interference color) were then cut and examined by electron microscopy. Control sections through the LGd and VB were similarly prepared from five normal adult hamsters (one injected, 24-hr survival time; four uninjected) and six normal neonatal hamsters (1.5–2 days old; all injected; *ca.* 24-hr survival time).

RESULTS

We examined normal retino-LGd projections, normal ascending somatosensory projections to the VB, and anomalous retino-VB projections. In all three instances, the specific sensory afferent terminals participate in glomeruli (Fig. 1). We compared these glomeruli with respect to seven features listed in Table 1.

In both normal and operated hamsters, RGC axons and their terminals are readily identified by their content of HRP reaction product (Fig. 1A and C). As previously reported (9), in the normal LGd virtually all axon terminals containing round synaptic vesicles and pale mitochondria (R-boutons) are of retinal origin, and R-boutons are the only bouton type labeled following intraocular HRP injection (Fig. 1A). Ascending somatosensory axons projecting to VB originate in the spinal cord, dorsal column nuclei, and trigeminal nuclei (ref. 29; D.F., unpublished data). In the rat, these axons terminate in large boutons containing round synaptic vesicles and dark mitochondria (LR-boutons) (16, 17). LR-boutons in the normal hamster VB (Fig. 1B) nearly certainly originate from the same structures as do those in the rat. In operated hamsters, abnormal, retino-VB axons terminate in R-boutons, that like those in the normal LGd, have round synaptic vesicles and pale mitochondria (Fig. 1C).

Normal retino-LGd projections and ascending somatosensory projections to the VB also differ with respect to all six of the other features examined (Fig. 1; Table 1). With respect to all these features, abnormal retino-VB projections

more nearly resemble normal ascending somatosensory projections to the VB than normal retinal projections to the LGd (Fig. 1; Table 1). The synaptic glomeruli formed by normal retino-LGd axons are situated on intermediate and proximal (but not the most proximal) dendritic regions but not on somata. Normal ascending somatosensory and abnormal retinal afferents to the VB form glomeruli positioned on proximal dendrites (including the most proximal dendritic regions) and on neuronal perikarya. Normal glomeruli in the LGd contain multiple R-boutons that make asymmetric synapses on dendritic shafts and appendages, whereas glomeruli in the VB contain only a single presynaptic ascending somatosensory or retinal afferent terminal. (We cannot say whether the multiple R-boutons in a LGd glomerulus arise from the same or different axons). Normal glomeruli in the LGd include presynaptic dendrites and dendritic appendages (P-boutons); in the VBs of normal and operated hamsters, P-boutons are rare. Some glomeruli in the normal LGd are also affiliated with F-boutons that contain flattened synaptic vesicles and make symmetric synapses on dendritic shafts and lie predominantly inside the glial capsule. In normal and operated VBs, virtually all glomeruli are affiliated with F-boutons, but the F-boutons are predominantly outside the glial capsule. Normal R-boutons in the LGd are small to large sized, with long axes of ≈ 0.5 – $6.0 \mu\text{m}$, whereas normal ascending somatosensory and abnormal retinal axon terminals in the VB are medium to very large sized, with long axes commonly 2– $10 \mu\text{m}$ and occasionally up to $15 \mu\text{m}$. In single sections, normal R-boutons in the LGd are usually invaginated by 0–4 dendritic appendages, which are seen to be postsynaptic to those boutons in the same or nearby sections; R-boutons rarely synapse on more than six appendages. In single sections through the VB of normal and operated hamsters, LR-boutons and R-boutons, respectively, are usually invaginated by 4–8 dendritic or somatic appendages, which are seen to be postsynaptic to those boutons in the same or nearby sections; LR- and R-boutons in the VB occasionally synapse on 10–16 appendages (see also ref. 16), and we have seen up to 34 postsynaptic appendages per bouton.

The retino-VB projection in operated adult hamsters is due to the stabilization and sprouting of a normally transient retino-VB projection that is already present in newborn hamsters (30). In order to ascertain the developmental status of the VB neuropil at the time of surgery and during the period when the retino-VB axons develop their synaptic connections, we examined the VB neuropil in six normal hamsters, all 1.5–2 days old. At this age (Fig. 2) axons have neither been

Table 1. Comparison of normal retino-LGd, normal ascending somatosensory-to-VB, and abnormal retino-VB projections with respect to seven features of terminal morphology and glomerulus synaptic organization

Structural category	Normal visual afferent terminals in LGd	Normal somatosensory afferent terminals in VB	Abnormal visual afferent terminals in VB
Glomerular position	Intermediate and proximal (not most proximal) dendrites	Proximal (including most proximal) dendrites and perikarya	Proximal (including most proximal) dendrites and perikarya
Glomerular organization	Multiple specific sensory axon terminals per glomerulus	Single specific sensory axon terminal per glomerulus	Single specific sensory axon terminal per glomerulus
	P-boutons common	P-boutons rare	P-boutons rare
	F-boutons sometimes present; predominantly inside capsule	F-boutons usually present; predominantly outside capsule	F-boutons usually present; predominantly outside capsule
Specific sensory axon terminal	Small- to large-sized	Medium- to very large-sized	Medium- to very large-sized
	Few appendages per specific sensory axon terminal	Many appendages per specific sensory axon terminal	Many appendages per specific sensory axon terminal
	Pale mitochondria with dilated cristae	Dark mitochondria with narrow cristae	Pale mitochondria with dilated cristae

The seven features include the position of the glomerulus on the postsynaptic neuron, the number of specific sensory axon terminals per glomerulus, the presence of presynaptic dendritic appendages (P-boutons), the presence and position of terminal boutons with flattened synaptic vesicles (F-boutons), the size of the specific sensory axon terminals, the number of postsynaptic neuronal appendages contacted by each terminal, and the mitochondrial morphology of the terminals.

FIG. 2. Electron micrograph of typical neuropil in the VB of a postnatal day-2 hamster. At this age axons have neither been enwrapped by glia in complexes containing multiple neuronal elements, nor invaginated by dendritic appendages. Axonal profiles often contain clear round synaptic vesicles (some vesicle-containing axons are indicated by arrows) and make simple synaptic contacts (double-headed arrow) with small dendritic elements. F-boutons are extremely rare at this age, and none are seen in this field, although they would be common in a comparable field in mature VB neuropil. This tissue was processed by the Hanker-Yates method following an injection of HRP into the contralateral eye in an experiment (reported elsewhere) designed to label retino-VB axon terminals; small electron-dense membranous profiles are accentuated as a result of this treatment. (Calibration bar = 1.0 μ m.)

partitioned by glia into complexes containing multiple neuronal elements, nor been invaginated by dendritic appendages, two characteristic features of mature VB neuropil. Axonal profiles often contain clear round synaptic vesicles and make simple synaptic contacts with small dendritic elements. F-boutons are extremely rare at this age, although they are common in mature VB neuropil.

DISCUSSION

The present results demonstrate that the ultrastructural differentiation of axon terminals and synaptic complexes is regulated largely by the target environment, although some features appear to be intrinsic to the afferent axons themselves. The principal difference between the retinorecipient regions of the VB in operated hamsters and the VB of normal hamsters is that in operated hamsters, LR-boutons of ascending somatosensory axons are largely replaced by R-boutons, which probably originate exclusively from retino-

fugal axons. With this exception, the constituents and synaptic organization of glomeruli are unchanged from those of the normal VB (Table 1). Thus, the morphologies and synaptic relationships of abnormal R-boutons in the VB are identical to those of normal LR-boutons in the VB, but differ from those of normal R-boutons in the LGd, with respect to all the features considered here, except mitochondrial morphology.

The morphologies of the terminals and glomeruli of retino-VB axons do not reflect intrinsically determined characteristics normally expressed by RGC axons in the SC. Normal RGC axon terminals in the SC (G.C., unpublished data) have a different morphology from those of RGC axon terminals in the LGd and VB of normal and operated hamsters, respectively, and are not enclosed in glomeruli. Furthermore, the changes in RGC axon terminals in the VB are not due merely to generalized disturbances of the retinofugal pathway caused by the neonatal lesions. In normal rodents, virtually all retinothalamic axons are collaterals of retinotectal axons (8). In most operated hamsters, there is a remnant of the LGd, due to incomplete lesions of occipital cortex. Despite the damage to the tectal collaterals of retino-LGd axons, the neuropil of the LGd remnant is similar to that of the normal LGd, and the labeled R-bouton population resembles that of normal hamsters with respect to all the features of terminal and glomerulus morphology here considered.

Does the morphology of RGC axon terminals and glomeruli in the VB reflect normal developmental processes or, alternatively, regenerative processes or plastic responses of the RGC axons to mechanical or biochemical constraints imposed by the prior differentiation of the VB? Multiple data demonstrating that at the time of surgery, both RGC axons and the VB are immature, suggest the action of developmental processes: For some time after birth, RGC axons are in growth states associated with elongation, collateralization, and the formation of synaptic connections (23, 31-33). Light microscopy shows that the retino-VB projection in neonatally operated hamsters is due to the stabilization and sprouting of a normally transient retino-VB projection that is already present at birth in normal unoperated hamsters (30, 31). Furthermore, our electron microscopic data show that within the VB at birth (shortly after which surgery is performed) and for some days thereafter, all axon terminals (whether visual or somatosensory) are immature, only a few, poorly differentiated synapses are present, and there are no synaptic complexes enclosed by glial capsules.[†] Thus, the permanent retino-VB axons differentiate at the same time and under all the same environmental influences as do normal ascending somatosensory afferents to the VB (except for the absence of the somatosensory axons themselves).

These experiments do not permit the specification of which element(s) in the VB determine how afferent axon terminals and synaptic complexes differentiate. It might be suggested that normal ascending somatosensory afferents to the VB transected by the surgery leave behind a signal that influences the differentiation of the stabilized retino-VB projections. Little is known about the development of somatosensory afferents to the thalamus in rodents. In rats, at stages of development comparable to the day of birth in hamsters, axons of the medial lemniscus have reached the thalamus, but have not ramified significantly within the VB (37). However, observations on congenitally anophthalmic mice suggest the

[†]The late appearance and slow maturation of thalamic synaptic glomeruli seem to be common phenomena. In the hamster LGd (23) and rat VB (34), glial-encapsulated glomeruli do not appear until the second postnatal week and do not take mature forms until the third postnatal week, while in the cat LGd, glomeruli do not appear until 25 days postnatally and do not mature until 40-45 days postnatally (35, 36).

action of elements other than medial lemniscus axons, or other primitive somatosensory afferent axons, in the differentiation of retino-VB axons in the present experiments. In anophthalmic mice RGC axons never reach the LGd, yet the terminals of other axons (probably corticofugal) replace those of RGC axons in the glomeruli of the LGd and take on the unique morphology and synaptic relationships of normal RGC axon terminals (38); thus, novel afferents do not require a signal from the afferents they replace in order to adopt the morphology and synaptic relationships of the normal afferents.

The results of the present experiments, in which retinal axons terminate anomalously in the VB, demonstrate that (i) the positions of synaptic complexes on their target neurons and (ii) synaptic complex morphology are determined principally under the control of the target neurons or their environment. The diversity of terminal and synaptic types impinging on a single target neuron may be due to regional variations in membrane properties across the postsynaptic neuronal surface or to temporal changes in these properties, which could create different environments for afferents arriving at different loci or at different times, respectively (4). A significant problem for future research is to understand the biochemical and molecular mechanisms by which target neurons or their immediate environment control the differentiation of so many functionally important features of their afferent connections.

Although retino-VB axons participate in synaptic complexes that resemble those of normal somatosensory rather than visual thalamic afferents, the visual receptive field properties of single neurons in the somatosensory cortex resemble, in many of their essential features, those of normal visual cortical neurons (20, 39). These observations are likely due to the fact that the morphological features examined in this study do not determine the parameters of neuronal response assayed in our visual receptive field studies. Neurophysiological examination of other parameters (e.g., the detailed temporal patterns of response of visually activated thalamic neurons) are necessary to reveal the functional consequences of retinofugal axons participating in synaptic complexes with somatosensory, rather than visual, morphologies.

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