An In Vitro Model of the Kitten Retinogeniculate Pathway

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Guido, William, Fu-Sun Lo, and Reha S. Erzurumlu. An in vitro model of the kitten retinogeniculate pathway. J. Neurophysiol. 77: 511–516, 1997. An organotypic explant coculture method is described for the developing retinogeniculate pathway of the cat. Retinal explants and thalamic slices containing the dorsal lateral geniculate nucleus (LGN), derived from early postnatal kittens, can be grown in serum-free culture medium for several days. In such cultures, retinal ganglion cells (RGCs) and LGN neurons retained their age-specific morphological features and developed functional connections. Labeling of RGCs and their processes with DiI showed that all three major classes of RGCs (α/, β/, γ/) were present in cocultured retinal explants. Retinal axons readily regenerated into thalamic slices and, over time, developed arbors within the LGN. Retrograde labeling from the LGN traced the origin of these axons almost exclusively to α-cells in the retina. In vitro intracellular recordings indicated that LGN cells maintained their basic electrophysiological properties in coculture. Current injection generated action potentials, and, at hyperpolarized levels, it led to low-threshold Ca2+ spiking. Regenerated retinal axons also formed functional connections with LGN neurons. Electrical stimulation of the retinal explant elicited excitatory postsynaptic responses (EPSPs) in LGN cells. Drop application of specific glutamate antagonists indicated that EPSPs had both N-methyl-D-aspartate (NMDA) and non-NMDA receptor components. The morphology of the LGN neurons was examined after intracellular injections of biocytin during recording. Labeled cells were very similar to those of early postnatal kittens. Although, in general, they had relatively small soma and simple dendritic branching patterns, a few could be recognized as X- or Y-cells. Thus the coculture model can be used to assay the regenerative propensity of different types of RGCs during development.

INTRODUCTION

The cat visual pathway from the retina through the lateral geniculate nucleus (LGN) of dorsal thalamus, to visual cortex serves as an important model for elucidating developmental mechanisms underlying the formation of ordered neural connections (see Cramer and Sur 1995; Garraghty and Sur 1993; Goodman and Shatz, 1993; Shatz 1990a,b, 1996; Shatz and Sretavan 1986). This pathway develops from a crudely wired, relatively undifferentiated network, into a highly organized sensory system. In the retina, physiologically and morphologically distinct cell types, namely α-, β-, and γ-cells, establish connections with similarly distinct relay neurons of the LGN, namely X, Y, and W-cells (see Sherman 1985). Presently, little is known about the cellular changes, particularly in the membrane properties and synaptic responses of developing LGN cells, during the establishment of parallel pathways. We have used explant cocultures of kitten retina and thalamic slices containing LGN to investigate the structural and electrophysiological features of retinogeniculate connectivity. The experimental approach described here circumvents many of the technical difficulties associated with in vivo experiments and provides a model in which morphological and electrophysiological experiments can be done in parallel with pharmacological manipulations to explore cellular mechanisms underlying the establishment and refinement of the retinogeniculate pathway.

METHODS

Cocultures

Kittens between 0 and 2 days of age were killed with halothane. The brains and eyes were removed and placed in sterile Gey’s balanced salt solution (GIBCO) supplemented with 6.5% D-glucose (GBSS, kept at 4°C). The retina from each eye was dissected out in GBSS and divided into wedge-shaped explants. The diencephalon containing the LGN was excised and immersed into oxygenated artificial cerebrospinal fluid (ACSF; containing (in mM) 124 NaCl, 2.5 KCl, 2 CaCl2, 1 MgSO4, 26 NaHCO3, 1.25 NaHPO4, and 10 dextrose; pH = 7.4) and transferred into a temperature-controlled slice recording chamber. Cultures were kept at an interface of warm (34°C) humidified air (95% O2-5% CO2) and ACSF. The boundaries of the LGN and retina were readily identifiable under a stereoscope with fiber optic illumination (Fig. 1 F). The inserts then were placed in six-well culture plates. The cultures were kept in fresh serum-free medium (Brewer and Cotman 1989; Collazo et al. 1992) in a humidified CO2 incubator at 33°C for 3–5 days.

In vitro recording

After 3–5 days in culture, each coculture was lifted off the microporous membrane by immersion into oxygenated artificial cerebrospinal fluid (ACSF; containing (in mM) 124 NaCl, 2.5 KCl, 2 CaCl2, 1 MgSO4, 26 NaHCO3, 1.25 NaHPO4, and 10 dextrose; pH = 7.4) and transferred into a temperature-controlled slice recording chamber. Cultures were kept at an interface of warm (34°C) humidified air (95% O2-5% CO2) and ACSF. The boundaries of the LGN and retina were readily identifiable under a stereoscope with fiber optic illumination (Fig. 1 F). In vitro, intracellular recordings were conducted using sharp-tipped glass electrodes filled with 4 M KAC or a 2% solution of biocytin dissolved in 2 M KAC (final impedance 80–110 MΩ). All neuronal activity was recorded through a high-impedance amplifier with bridge balanced circuitry, then digitized (10–20 kHz), and stored directly on computer. Membrane levels were controlled by injecting DC current (continuously and/or step-wise manner) through the recording electrode. Current-voltage relationships were examined at membrane levels between −45 and −90 mV by injecting a series of square wave current pulses (100–300 ms, ± 1 nA, 0.1 step) to reach steady state. Responses to these current step protocols also were used to determine the presence of any voltage dependent conductances and to explore the firing pattern of LGN cells.
To generate synaptic responses in LGN, teflon-coated bipolar stimulating electrodes (Pt-Ir) were placed in the retinal explant. Excitatory postsynaptic potentials (EPSPs) were evoked at relatively low stimulus frequencies (0.2–1 Hz) using brief (100–300 μs) current pulses (0.1–0.5 mA) over a wide range of membrane potentials. Drop application (5 μl) of selective glutamate antagonists, DL-2-amino-5-phosphonovaleric acid (DL-APV; 1 mM), and 6,7-dinitroquinoxaline-2,3-dione (DNQX; 250 μM) were used to determine whether EPSPs were comprised of N-methyl-D-aspartate (NMDA) and/or non-NMDA receptor components, respectively (cf., Scharfman et al. 1990).

**Morphological analyses**

Some of the cocultures were fixed with 4% paraformaldehyde in phosphate buffer and small crystals of carboxycyanine dye, DiI (Molecular Probes) were placed either in the retina or in the LGN. The first labeling approach allowed us to visualize retinal axons growing into the thalamus and to examine if all classes of retinal ganglion cells (RGCs) survived and differentiated in coculture. The second labeling approach enabled us to determine whether certain types of RGCs could regenerate their axons into the LGN. To compare the morphology of cocultured RGCs with that seen in normal retinae, we prepared retinal whole mounts from 2-day-old kittens and labeled the RGCs with DiI. All dye implanted preparations were kept at 35°C for 3–14 days, after which the labeled neuronal profiles were analyzed with a Nikon Diaphot microscope equipped with epifluorescence or by using a Leica confocal laser-scanning microscope.

During intracellular recording, some LGN cells were filled with biocytin by passing a small amount of positive and negative current through the recording electrode. Later, the explants were immersion fixed in buffered 2.5% paraformaldehyde and 0.25% glutaraldehyde. Biocytin-filled LGN cells then were visualized by using the ABC method (Horikawa and Armstrong 1988). Labeled cells were drawn using a drawing tube attached to a Nikon Labophot microscope.

**RESULTS**

Placement of DiI in the retina yielded retrograde labeling of a stream of retinal ganglion cells whose axons passed through the dye site. The same procedure also led to anterograde labeling of retinal axons that regenerated into the cocultured thalamic slice. Figure 1 illustrates the arrangement of retinogeniculate cocultures (Fig. 1F) as well as various labeled RGCs and their axonal processes (Fig. 1, A–E, G, and H). Within the retina, morphologically different types of ganglion cells could be visualized at similar eccentricities (Fig. 1, A–D). A comparison of DiI-labeled retinal whole mounts from 2-day-old kittens (data not shown) and retinal explants cocultured with LGN for 5 days indicated that all types of retinal ganglion cells survived and maintained class-specific morphology (Fig. 1, A–E) (cf., Montague and Friedlander 1991). Some of these cells had medium size soma and small, bushy dendritic trees (Fig. 1D), typical of β-cells. Others had large soma and widely dispersed dendritic trees characteristic of α-cells (Fig. 1, A–C, and E). There were also cells with smaller soma and sparsely branched, but long dendrites resembling γ-cells (not shown). Another morphological characteristic of ganglion cells in coculture and in normal retinal wholemounts was the presence of extensive dendritic and somal spines, a transient feature of the developing cat retina (Ramoa et al. 1988).

It was also possible to visualize anterograde labeling of retinal axons in our coculture preparations. After 24 h in vitro, axons tipped with growth cones were observed crossing the retina-LGN border. After 3 days in culture, axons of RGCs began branching and their growth cones became simpler and smaller (Fig. 1G). With longer culture periods (5 days), retinal axons formed arbors within the borders of LGNs (Fig. 1H). Terminal branches often were beaded, each ending in a small growth cone. We were not able to determine if the terminal arbors were restricted to eye-specific layers of the LGN. During perinatal development, lamina organization can be revealed only by the arrangement of retinal arbors arising from each eye and not by the cytoarchitecture of the LGN (see Shatz 1990a).

In cocultures where the dye was placed in the LGN, it was possible to determine whether RGCs were able to regenerate their axonal processes into LGN. Often it was possible to follow single retinal ganglion cell axons from the LGN, across the retina-LGN border to the parent cell body several millimeters away from central retina (Fig. 1E). This labeling approach revealed that only specific classes of RGCs innervated LGN explants. The majority of labeled cells were of the α class (Fig. 1, A–C, and E). Rarely, isolated γ-cells (data not shown) were observed to cross into the LGN explant. However, we saw no evidence of retrogradely labeled β-cells. DiI labeling in the retina clearly indicates that β-cells are present in cocultured retinal explants (Fig. 1D), but retrograde labeling experiments failed to show axons of these cells in the LGN.

Next we examined the morphological and electrophysiological properties of cocultured LGN cells. We recorded the voltage responses of 31 LGN cells in coculture, and of these, 7 were labeled with biocytin. Examples of two labeled LGN neurons are shown in Fig. 2A. Cocultured LGN cells resembled those of early postnatal kittens (Friedlander 1982; Guido and Lo 1995; Mason 1983). Often these cells had large soma and lacked extensive dendritic branching (Fig. 2A, cell 2). Some also could be recognized as immature X and Y-cells. For example, cell 1 (Fig. 2A), resembled a Y-cell with its large soma and radially oriented dendritic field (Friedlander et al. 1981).

Examples of the voltage responses of LGN cells to intracellular current injection are shown in Fig. 2, B–D. The responses of cells in coculture were very similar to those of
postnatal kittens (Guido and Lo 1995; Pirchio et al. 1990). Cells responded to depolarizing current pulses with wide, overshooting action potentials (Fig. 2B). Strong depolarization also could generate a train of spikes that showed moderate frequency accommodation (Fig. 2D). Depolarization from a hyperpolarized state triggered low threshold Ca\(^{2+}\) spikes (Steriade and Llinas 1988). Typically, these were recognized as a triangular depolarization with one to two action potentials riding their crest (Fig. 2C). Like their normal postnatal counterparts, LGN cells in coculture were missing some key membrane properties that do not emerge until postnatal day 14–21 (Guido and Lo 1995; see also Ramoa and McCormick 1994). Most notable was the lack of the mixed cation conductance \(I_{\text{H}}\) (McCormick and Pape 1990). Strong hyperpolarizing current pulses failed to activate a “depolarizing sag” indicative of \(I_{\text{H}}\) (Fig. 2, B and C).

Finally, we tested whether the retinal axons growing into LGN formed functional connections, or merely reflected axon-substrate interactions. Electrical stimulation of the retinal explant activated EPSPs in 21 of 29 cells tested. Of our biocytin-filled cells (\(n = 7\)), three could be activated by electrical stimulation of the retinal explant and these either resembled immature Y cells or cells that could not be readily classified (see Fig. 2A). Unfortunately, our small sample of biocytin-filled cells precludes us from determining whether both X and Y cells form functional connections. EPSPs were of sufficient amplitude to generate action potential firing...
as well as low threshold spiking (Fig. 2, E and F). Drop application of selective glutamate antagonists also revealed that EPSPs had both NMDA and non-NMDA components (Fig. 2G). Stimulation of the retinal explant (top) evoked a large amplitude, long-lasting EPSP. Drop application of the NMDA antagonist APV (1 mM) reduced the amplitude of the late component, leaving the early, fast component intact. The late, slow component showed a strong voltage dependency (data not shown), and was greatest in amplitude at more depolarized membrane levels. Subsequent application of the non-NMDA antagonist DNQX (250 µM) diminished the remaining component.

**DISCUSSION**

We present an experimentally accessible, in vitro model that captures the dynamic features of the developing retinogeniculate pathway. Both retinal ganglion and LGN cells survive and maintain their age-specific characteristics in explant cocultures. Retinal axons sprout and elaborate new terminal endings in the LGN. The electrophysiological properties of developing LGN cells also are preserved in coculture (Guido and Lo 1995). Stimulation of retinal explants reveal that LGN cells form functional connections with regenerated retinal axons. Evoked EPSPs of LGN cells have both non-NMDA and NMDA components and are of sufficient magnitude to generate activity in the form of action potentials and low threshold Ca\(^{2+}\) spikes.

Studies on the developing vertebrate sensory pathways indicate that coordinated activity before- and postsynaptic elements contribute to the emergence and refinement of precisely ordered neural connections (see Cramer and Sur 1995; Goodman and Shatz 1993; Shatz 1990b). Although activity plays an important role in shaping some aspects of retinogeniculate organization, little is known about underlying cellular mechanisms. NMDA receptor-mediated activity seems essential for some aspects of retinogeniculate organization such as the formation of “on/off” sublaminae (Hahn et al. 1991). However, other aspects of organization, such as the formation of eye-specific laminations (Smetters et al. 1994) and LGN dendritic morphology (Dalva et al. 1994) are not regulated by such activity. Other signals, particularly in the form of retrograde messengers such as nitric oxide (Cramer et al. 1995; Kratz et al. 1994), or metabotropic glutamate receptors (McCormick and Von Krosigk 1992), as well as the expression of neurotrophins by target cells (Allendoerfer et al. 1994; King et al. 1993) also may participate in the establishment and refinement of retinogeniculate connectivity. The coculture model described in this study offers a simple and relatively easy means to systematically manipulate these factors. Because the cultures are grown in a chemically defined, serum-free medium, various neurotrophins, agonists or antagonists to candidate transmitter substances, or neuromodulators can be added to the culture medium to assess their effects on cellular differentiation and development of retinogeniculate connectivity.

The kitten retinogeniculate pathway is assembled primarily during prenatal life. By the time of birth, RGC axons have established adult-like arbor patterns in eye-specific layers of the LGN (see Garraghty and Sur 1993; Shatz and Srevatan 1986). Thus in our cocultures using explants from postnatal kittens, the growth of retinal axons into thalamic explants and establishment of synaptic connectivity reflects a regenerative event rather than ab initio form of connectivity. Our Dil labeling experiments show that mostly α-cells regenerate axons into thalamus, arborize and make functional connections with LGN cells. Several possibilities singly or in combination could account for this class-specific regeneration in culture. First, during neurogenesis, β-cells differentiate earlier than α-cells and their axons arrive in the LGN prior to those of α-cells (see Garraghty and Sur 1993; Shatz 1990a; Shatz and Srevatan 1986). As development proceeds, later arriving α-cell axons begin expanding their arbors while β-cell arbors are pruned (see Sur 1988). Based on this developmental sequence, it is conceivable that the β/X-cell pathway might actually lose its regenerative propensity and ability to reinnervate LGN cells around the time of birth. Because our cocultures were prepared from early postnatal tissue perhaps we simply missed the period in which β-cells are capable of regenerating. Coculture experiments that use retinal explants from fetal kittens, or perhaps from newborn ferrets, would help to resolve this issue. Second, the rate of axonal regeneration and elongation may be substantially slower in
β-cells. Extending the culture period (beyond 5 days) might enable the axons of β-cells to regenerate and grow into LGN explants. Third, these differences may reflect the more “malleable” nature of the α/Y-cell pathway. Competitive interactions between α and β cell axons in exacting terminal space in the LGN are well noted (see Garraghty and Sur 1993; Shatz 1990a; Shatz and Sretavan 1986; Sur 1988). Experiments which alter these competitive interactions (e.g., monococular eye lid suture or monococular enucleation) have a profound and preferential effect on the development of the α/Y cell pathway (Friedlander et al. 1982; see Garraghty and Sur 1993; Sherman and Spear 1982; Sur 1988). Within this context, it is also worth noting that mature α/Y-cells seem to show a greater regenerative capacity than other retinal ganglion cell types. Experiments in which transected optic nerve is sutured to a peripheral nerve reveal that α-cells more readily regenerate their axons into a peripheral nerve graft (Watanabe et al. 1993). Taken together with the present results, it would appear that the intrinsic biological properties of α/Y-cells differs substantially from other ganglion cell types.

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REFERENCES


