Spontaneous Impulse Activity of Rat Retinal Ganglion Cells in Prenatal Life

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The existence of spontaneous neural activity in mammalian retinal ganglion cells during prenatal life has long been suspected. This activity could play a key role in the refinement of retinal projections during development. Recordings in vivo from the retinas of rat fetuses between embryonic day 17 and 21 found action potentials in spontaneously active ganglion cells at all the ages studied.

ELECTRICAL ACTIVITY IS THOUGHT to affect the pattern of neural connectivity in the development and regeneration of the nervous system (*1*). It has been proposed that spontaneous impulse activity plays this role in the prenatal development of the visual system in mammals (*2*). Several experiments have shown that early in development repeated intraocular or intracranial injections of tetrodotoxin, which blocks action potentials, strongly affect the pattern of synaptic connections in the visual system (*3*). However, spontaneous impulse activity of retinal ganglion cells has never been recorded in mammals during prenatal life, when retinal projections develop (*4*) and when light-evoked activity is absent because the retina is immature. To investigate whether retinal ganglion cells are active early in development, we have recorded from the retinas of rat fetuses between embryonic day 17 (E17) and day 21 (E21).

Pregnant Long-Evans rats were anesthetized with intraperitoneal (ip) injections of urethane (1.25 g per kilogram of body weight) at known times of gestation. One fetus was gently removed from the maternal uterus, keeping the umbilical cord intact to maintain maternal blood supply. The fetus was then laid just above the mother, in a dish provided with a slot in the bottom for the umbilical cord (Fig. 1A). The fetus was then given a dose of urethane (0.5 g per kilogram, ip) and a small bipolar stimulating electrode was placed in the superior colliculi, which at these ages are just below the skull, through a small slit behind the transverse sinus. The fetus was then paralyzed [pancuronium bromide (0.005 to 0.01 ml), injected intramuscular] and completely covered with a low melting point wax, maintained at 38°C. The wax covering one eye was carefully removed, the eyelids opened, and a small hole was made in the center of the cornea with an electrocoagulator. Glass micropipettes filled with 3*M* NaCl (10 to 15 megohms) were lowered through the lens to reach the retina. Single unit activity in the retina was recorded extracellularly, conventionally amplified, and stored on an analog recorder. Fetal body temperature and electrocardiogram were monitored continuously. No recordings were obtained when the heart rate was below 300 beats per minute. For each fetus the experiment lasted for about 2 hours after the surgery. Two to three fetuses from the same mother were used (*5*). No attempts were made to record from fetuses younger than E17.

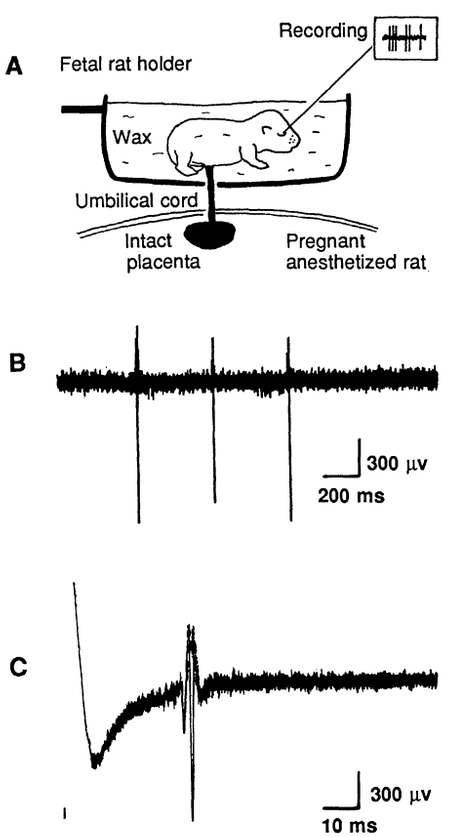

Fig. 1. Spontaneous activity of rat retinal ganglion cells during prenatal life. (**A**) Diagram of the recording apparatus. The fetus, in a low melting point wax bath, is in contact with the maternal circulation by means of the intact umbilical cord. Fetus and mother are anesthetized. (**B**) Sample of the spontaneous activity of a retinal ganglion cell in an E19 rat fetus. The cell was recorded for 7 min. (**C**) Antidromical activation of the same cell as in (B) by electrical stimulation of the superior colliculi. Bar below the trace indicates the onset of the stimulus.

We found spontaneously active cells in fetal retinas at all ages studied (E17 to E21). Our procedure for the experiment was as follows. Once a single cell was isolated, we recorded from it for several minutes. To ascertain that the activity was originating in a retinal ganglion cell, the optic fibers in the superior colliculi were then electrically stimulated (1 to 8 Hz, 1-ms square pulse, 1 to 10 mA) and the unit was antidromically activated. Twenty-two well-isolated units were recorded from for several minutes, and their patterns of discharge were analyzed. Most of these cells could be antidromically activated by stimulating the superior colliculi (*6*). In many preparations we recorded only mass activity. A sample of the spontaneous discharge of a retinal ganglion cell recorded in an E19 fetus is shown in Fig. 1B. This cell was antidromically activated by electrical stimulation of the superior colliculi (Fig. 1C). The latency of the response was 40 ms. Action potentials recorded from retinal ganglion cells of prenatal animals had longer time constants than action potentials in adult animals and were more variable in amplitude.

Average frequencies of discharge were around one action potential per second. Relatively long periods of silence alternated with periods of an average discharge rate of about four to five "spikes" per second. The average shortest interspike interval observed was 50 ms, whereas the longest interval was 29 s (Table 1). Examples of discharge pat-

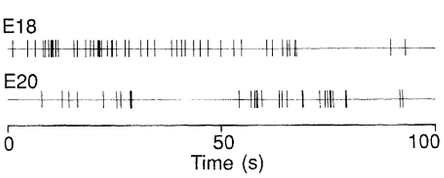

Fig. 2. Examples of the spontaneous activity of prenatal rat retinal ganglion cells. The upper trace is a 100-s segment from a cell recorded for 10 min in a fetus at E18. The lower trace is a 100-s segment from a cell recorded for 6 min in a fetus at E20. Each bar indicates the occurrence of an action potential.

Table 1. Characteristics of the discharge pattern of prenatal rat retinal ganglion cells. Animals were allowed to mate for 12 hours in the laboratory colony so that each fetal age is determined with a day of error. For E17, $n = 1$; E18, $n = 5$; E19, $n = 8$; E20, $n = 4$; E21, $n = 4$.

Response	Firing rate*	Shortest interspike interval (ms)	Longest interspike interval (s)
Average	0.9	50	29
Minimum	0.05	9	1.3
Maximum	4	174	108

*Spikes per second.

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terns are shown in Fig. 2 for two cells, one recorded on E18, the second on E20. The discharge patterns of most units were irregular. Some units, however, showed periodic patterns of discharge when monitored for 10 min (*7*).

We report that mammalian retinal ganglion cells are spontaneously active in prenatal life. In this time period the axons of ganglion cells reach the superior colliculus and the lateral geniculate nucleus (*4*) and functional synapses are formed (*8*); during the same time period projections are refined and many erroneous connections are eliminated (*4, 9*). Impulse activity in ganglion cells may play a key role in these events.

REFERENCES AND NOTES

1. See for a review E. Frank, *Trends Neurosci.* **10**, 188 (1987).
2. By "spontaneous activity" we mean any impulse activity in retinal ganglion cells that is not evoked by light, independent of its origin.
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4. Although there are many papers on this subject, a description of the prenatal development of rat retinal projections can be found in S. M. Bunt, R. D. Lund, P. W. Land, *Dev. Brain Res.* **6**, 149 (1983).
5. M. Fitzgerald, *Nature* **326**, 603 (1987).
6. Failure to activate some units could also be due to the fact that at E17 to E21 these axons still have not reached the superior colliculi.
7. Experiments are in progress to study the variation of firing patterns with age, and to find out whether neighboring ganglion cells have correlated activity.
8. C. J. Shatz and P. A. Kirkwood, *J. Neurosci.* **5**, 1378 (1985).
9. See P. Crespo, D. D. M. O'Leary, W. M. Cowan, *Dev. Brain Res.* **19**, 129 (1985) for a discussion of the refinement of rat retinal projections during prenatal life. In the rat ganglion cell death and projection refinement continue in the first 10 days of postnatal life. Preliminary experiments in postnatal animals show neural activity in the retina during this same period.

10. We thank M. Fitzgerald and P. W. Land for advice; A. Fiorentini and M. P. Stryker for helpful discussions on the manuscript; and B. Margheritti, M. Antoni, V. Alpigiani, M. Benvenuti, D. Moriconi, A. Bertini, and P. Taccini for technical assistance.

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Localization of an Arg-Gly-Asp Recognition Site Within an Integrin Adhesion Receptor

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Many adhesive interactions are mediated by Arg-Gly-Asp (RGD) sequences within adhesive proteins. Such RGD sequences are frequently recognized by structurally related heterodimers that are members of the integrin family of adhesion receptors. A region was found in the platelet RGD receptor, gpIIb/IIIa, to which an RGD peptide becomes chemically cross-linked. This region corresponds to residues 109 to 171 of gpIIIa. This segment is conserved among the β subunits of the integrins (76 percent identity of sequence), indicating that it may play a role in the adhesive functions of this family of receptors.

TWO RECENT DEVELOPMENTS HAVE aided our understanding of the molecular mechanisms of cell adhesion. First, a family of structurally and functionally related adhesion receptors has been identified (*1*). This family of receptors, the integrins, can be separated into three distinct subfamilies in which members share a common β subunit that combines with unique α subunits to create functionally distinct receptors. Second, many of the adhesive proteins that serve as ligands for the integrin receptors contain the tripeptide sequence, Arg-Gly-Asp (RGD) (*2*). This sequence has been directly implicated in the recognition of the fibronectin receptor of the VLA (very late antigens) subfamily, Mac-1 of the leukocyte adhesion proteins, and gpIIb/IIIa and the vitronectin receptor of the cytoadhesin subfamily (*3, 4*).

GpIIb/IIIa, a platelet integrin, is involved in the platelet adhesive response through its interaction with fibrinogen, fibronectin, and von Willebrand factor (*5*). An RGD recognition site mediates these interactions, because RGD-containing peptides inhibit the binding of these ligands to either intact platelets or purified gpIIb/IIIa, and RGD-containing peptides interact directly with gpIIb/IIIa (*3, 6*). Activation of platelets with an agonist, an event necessary for binding of adhesive proteins to gpIIb/IIIa, markedly and selectively enhances the chemical cross-linking of small RGD peptides to gpIIIa (*7, 8*). Here we have defined a discrete site within gpIIIa to which an RGD peptide becomes cross-linked. This region is conserved in the primary sequence of other members of the integrin family, suggesting that it could have a role in receptor function.

The RGD peptide used, Fn-7, has the structure Lys-Tyr-Gly-Arg-Gly-Asp-Ser (KYGRGDS). When ^{125}I-labeled Fn-7 was bound to stimulated platelets and then bifunctional reagents, such as bis(sulfosuccinimidyl)suberate (BS^3), were added (*8*), Fn-7 was cross-linked predominantly to gpIIIa (Fig. 1, lane 1). When the gpIIIa:Fn-7 complex was digested with either V8 protease or chymotrypsin, the radioactivity of the Fn-7 peptide migrated as two major bands. In the V8 protease digest, the major derivatives were 8 and 10 kD (Fig. 1, lane 3). A fainter doublet was variably noted at 18 and 20 kD. Fn-7 does bind and becomes cross-linked to gpIIIa on resting platelets, albeit less efficiently than on stimulated platelets (*8*). The radiolabeled fragments obtained by V8 protease digestion of gpIIIa from non-stimulated and stimulated platelets had the same electrophoretic mobility (Fig. 1, lane 5). With chymotrypsin, gpIIIa:Fn-7 from stimulated platelets yielded a major band at 23 kD and a less prominent band at 14 kD (Fig. 1, lane 7). Both proteases generated numerous proteolytic fragments; the V8 protease digest, for example, contained 37 peaks on a C 18 high-performance liquid chromatography (HPLC) column monitored at 212 nm. Thus, Fn-7 becomes cross-linked to gpIIIa in a systematic orientation such that only a limited number of radiolabeled fragments are obtained. Therefore, we undertook to localize the cross-linked Fn-7 within gpIIIa.

We cross-linked ^{125}I-labeled Fn-7 to thrombin stimulated platelets and isolated the gpIIIa:Fn-7 complex by SDS–polyacrylamide gel electrophoresis (PAGE). The eluted product was digested with chymotrypsin or V8 protease and applied to a C18 column. Radioactive peaks were pooled, separated on a 15% SDS-PAGE gel, transferred to polyvinylidene difluoride (PVDF) membranes, and subjected to NH_2-terminal sequence analysis. The 23-kD chymotryptic fragment yielded a single predominant sequence (Fig. 2, step 3). At all of the determined positions, the amino acids were identical to the NH_2-terminal sequence of gpIIIa (*9*). In the 14-kD chymotryptic fragment, a major sequence predominated for 22 cycles and coincided to residues 91–112 of gpIIIa (Fig. 2, step 3). A preferred chymotrypsin cleavage site, Leu, is in position 90 of gpIIIa. The 14-kD gpIIIa fragment should extend to residues 200–220 of the glycopro-

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