Use of a Culture System to Identify Possible Causes of Abnormal Retinal Development

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The aim of this study was to apply a recently developed cell culture system to the problem of identifying possible etiologies of altered retinal maturation. Using techniques of electrophysiology and neuropharmacology, it was possible to monitor the release of acetylcholine at synapses formed by cultured retinal neurons derived from the fetal rat. The major finding of this study was that the functional development of cholinergic retinal neurons of the fetus could be altered if the mother rat had been injected with a synthetic glucocorticoid hormone or stressed by cold exposure during a critical period in pregnancy. Thus, the maturation of at least certain fetal retinal neurons appears to be influenced by factors affecting the mother. The culture system described here provides a potentially useful approach to the identification and investigation of possible causes of abnormalities in the functional development of the mammalian retina. Invest Ophthalmol Vis Sci 25:691-697, 1984

An important goal of investigative ophthalmology is to identify possible causes of abnormal retinal development. This is a challenging task, in part, since there are relatively few methods for assaying the function of fetal retinal neurons. The objective of this paper was to apply a recently developed culture system to the study of the pathophysiology of cholinergic retinal neurons derived from the retina of the fetal rat. Cholinergic retinal neurons appear to be a subpopulation of amacrine cells in the mammalian retina. The distribution of choline acetyltransferase (CAT) activity, CAT-like immunoreactivity, acetylcholinesterase histochemical staining, and α-bungarotoxin binding in the rat retina are consistent with this localization of cholinergic neurons.

Previously, a cell culture system consisting of retinal and muscle cells proved useful in physiologic studies of transmission across synapses formed by cholinergic neurons of immature retinas. By recording postsynaptic responses, it has been demonstrated that cholinergic neurons dissociated from the perinatal rat retina form functional synapses in culture with striated muscles cells. Myotubes are used as postsynaptic targets since their membranes have areas with a high density of cholinergic receptors and because their response to acetylcholine has been well studied both in vivo and in culture. Also, their relatively large size permits prolonged intracellular monitoring of postsynaptic responses.

The retina-muscle culture system appears to be well suited for the identification and investigation of factors influencing the maturation of synaptic transmission by cholinergic retinal neurons. As described previously, early in the functional development of cholinergic retinal neurons, there is a period in which the release of acetylcholine occurs spontaneously, but cannot be evoked by a depolarizing stimulus such as potassium or the putative excitatory neurotransmitter, glutamate. This early development stage is followed by the emergence of acetylcholine release that is stimulus-evoked and dependent on extracellular calcium.

The experiments presented here were prompted by my earlier observation that the addition of glucocorticoid hormones to the growth medium of retina-muscle cocultures markedly accelerated the functional maturation of cholinergic retinal neurons. Specifically, glucocorticoids precociously induced in cholinergic neurons derived from the fetal rat retina the capability of releasing acetylcholine at synapses in response to excitatory stimulation. This hormonal effect in culture occurred at physiologic concentrations and, based on pharmacologic experiments, involved glucocorticoid receptors, acted at the transcriptional level, and required protein synthesis. Experimental findings indicated that glucocorticoids may influence the maturation of neuronal mechanisms that couple the ability to respond to a depolarizing stimulus with the capability of releasing acetylcholine.

A specific aim of this present study was to assess systematically whether changes in the hormonal status of the mother could alter the maturation of cholinergic neurons.
Materials and Methods

Detailed methods for the preparation and maintenance of cell cultures as well as for the electrophysiologic assay have been described. Sprague-Dawley rats were obtained from a timed pregnancy colony (Taconic Farms; Germantown, NY). Pregnancy was determined by using sperm smears. The first sperm-positive day was counted as embryonic day-1. Primary cell cultures of striated muscle cells were prepared from hindlimb tissue of postnatal day-1 rat pups (postnatal day-0 is the day of birth). Muscle tissue was treated with trypsin, mechanically dissociated, preincubated in plastic culture dishes, and plated on 35-mm collagen-coated dishes. From the second to the fourth day, the cultures were exposed to cytosine arabinoside (1 µM) to reduce the number of dividing cells. Between 3 and 5 days in culture, myoblasts fused into myotubes that were 200–400 µm in length.

Dissociated retinal cells were prepared using modifications of previously described methods. Neural retinas derived from rats ranging in ages from embryonic day 18–21 (full-term is 22 days) were exposed for 17 min to 0.05% trypsin (crystallized ×3, Millipore; Bedford, MA), dissociated by pipetting the tissue 13 times through a 10-ml pipet (Falcon No. 7551; Becton, Dickinson & Co; Oxnard, CA) and then added (5 × 10^6 cells per dish) to 5–8-day-old muscle cultures. The growth medium (medium A) for the retina-muscle cocultures was 90% basal medium of Eagle supplemented with Earle’s salts (GIBCO; Grand Island, NY) and 10% dialyzed fetal bovine serum (MA Bioproducts; Walkersville, MD). Retina-muscle cocultures were incubated without movement at 36°C in a humidified atmosphere of 5% CO₂/95% air.

For electrophysiologic experiments, cultures were examined at ×312 magnification in a chamber on the stage of an inverted microscope equipped with phase-contrast optics. The temperature was maintained at 32°C ± 0.5; pH was kept at 7.4. All assays were done in medium A. Intracellular recordings from muscle cells were made with 3 M KCl-filled glass micropipets, which had tip resistances of 30–60 MΩ. The application of glutamate near retinal neurons was achieved by the microiontophoresis of this putative neurotransmitter from micropipets (tip resistance of 5–20 MΩ) that were filled with 0.5 M sodium glutamate (Sigma; St. Louis, MO) in distilled water at pH 8.

Muscle cells innervated by retinal neurons were detected by the presence of spontaneous postsynaptic potentials as described previously. When an innervated muscle cells was found, the ability of glutamate to evoke synaptic input to the myotube was assayed. This was done by microiontophoretically (5 nA) applying glutamate at approximately 10-µm intervals along the entire length of an innervated muscle cell and determining whether there was a site where glutamate could affect the synaptic activity of the muscle cell. If the rate of postsynaptic potentials was increased reproducibly two-fold or more during periods of glutamate application, a myotube was classified as having synaptic input that could be evoked by glutamate. In all cases in which evoked synaptic activity was detected, the glutamate-filled micropipet had been positioned near one or more retinal neurons located adjacent to or on the muscle cell being examined. Glutamate applied microiontophoretically does not alter the response of muscle cells to microiontophoretically applied acetylcholine, induce changes in the membrane potential of myotubes nor influence intracellular recordings in muscle cells lacking spontaneous synaptic activity. These control experiments were not influenced by whether or not the muscle cells were derived from rats previously exposed to dexamethasone or whether or not they were maintained in dexamethasone-containing medium. Currents passed through micropipets similar to those used for glutamate application, but filled with NaCl at pH 8 do not affect synaptic activity in myotubes.

Dexamethasone (Sigma) and progesterone (Sigma), 17β-estradiol (Sigma), and testosterone (Sigma) were dissolved in ethanol at stock concentrations of 20 mg/ml. Cold stress was induced by housing rats in a room maintained at 4°C.

Results

Dexamethasone Injection

Rats at various stages of pregnancy were injected subcutaneously in the nape of the neck with dexamethasone (5 mg/kg) or vehicle only. Twelve hours later, dissociated retinal cells from the fetuses were added (5 × 10^6 cells per dish) to muscle cultures. After 1 day of coculture, myotubes innervated by retinal neurons were assayed to determine if their synaptic input could be evoked by the putative excitatory neurotransmitter glutamate (Fig. 1). In control experiments, neurons from embryonic day-18 retinas spon-
taneously released acetylcholine at synapses with muscle cells. However, this synaptic input could not be modified (0 of 20 sampled myotubes) by the microiontophoretic application of glutamate. The percent of innervated muscle cells with evocable synaptic input remained low in the control cultures until after embryonic day-20. With neurons from embryonic day-21 retinas, 75% of the innervated myotubes exhibited stimulus-dependent synaptic activity. If pregnant rats received an injection of dexamethasone, this developmental time course for the fetal neurons could be accelerated. Specifically, after hormone administration, 26% of the myotubes innervated by embryonic day-18 retinal neurons had glutamate-evoked synaptic input. The percentage increased to 59% when neurons from embryonic day-19 rats were used.

Figure 2 shows that the effect of dexamethasone was dose-dependent. The one-half maximally effective dose was 0.6 mg/kg. The effects of steroids with minimal glucocorticoid activity also were tested (Fig. 3). In the 19th day of pregnancy, rats were injected (5 mg/kg) subcutaneously with testosterone, 17 β-estradiol or progesterone. Twelve hours later, dissociated neurons from the fetal retinas were added to muscle culture. When assayed after one day of coculture, the percent of innervated muscle cells with glutamate-evoked synaptic input was not significantly different from the control value (P > 0.2, Student's t-test).

It was of interest to determine whether or not progesterone could block the development effect of injecting mothers with dexamethasone, since this sex steroid has been shown to be a competitive antagonist of glucocorticoid receptors. 23 As shown in Figure 3, when rats in the 19th day of pregnancy received injections of progesterone (50 mg/kg) 15 min prior to the injection of dexamethasone (5 mg/kg), the subsequent induction of glutamate-evoked transmission at synapses between fetal retinal neurons and myotubes was reduced by more than 80%.

While the effect of dexamethasone injection was assayed routinely 1 day after the addition of retinal cells, it also was important to compare experimental and control groups at various other times in culture. For the series of experiments shown in Figure 4, subcutaneous injections of dexamethasone (5 mg/kg) or vehicle only were administered to rats in the middle of the 19th day of pregnancy. Twelve hours later, dis-
Fig. 3. Evocable synaptic transmission by fetal retinal neurons derived from mother rats that had been exposed to various hormones and/or cold stress. Rats in the 19th day of pregnancy were used in these experiments. See text for details. Each column shows the mean for two to four experiments. A mean of eight innervated muscle cells were sampled per culture. Standard deviations of greater than five are shown.

Fig. 4. Maturation with time in culture of evocable synaptic transmission. On the 19th day of pregnancy, rats were injected with dexamethasone (5 mg/kg [•]) or vehicle (O) only. Twelve hours later, dissociated retinal cells from the fetuses were added to muscle cultures (time 0 in this figure). Each point represents the mean of two to four experiments. A mean of eight innervated muscle cells were sampled per culture. Standard deviations of greater than five are shown.

Maternal Stress

The finding that the administration of dexamethasone to pregnant rats could accelerate the maturation of cholinergic neurons derived from the fetal retina raised a new question. Could other methods of altering the hormonal status of the mother affect the functional maturation of retinal neurons? One well-known cause for alterations in endogenous hormone levels, including glucocorticoid concentrations, is stress caused by ex-
posure to cold. Maickel et al.\textsuperscript{24} found that plasma glucocorticoid levels of rats are increased by two- to three-fold within 1 hr of exposure to 4°C temperatures. These high levels of hormones are maintained for at least 10 hr while the animal is in the cold. Based on these considerations, the effect of maternal cold stress on the development of evocable transmission by fetal neurons was examined.

Rats at various stages of pregnancy were placed in a cold room (4°C) for 10 hr. After this maternal stress, dissociated retinal cells from the fetuses were added (5 × 10^6 per dish) to muscle cultures. After 1 day of coculture, myotubes that were innervated by retinal neurons were assayed to determine whether or not their synaptic input could be evoked by glutamate (Fig. 5). In control cultures, neurons from embryonic day-18 retinas spontaneously released acetylcholine at synapses with muscle. But, this synaptic input could not be evoked by glutamate. When retinal neurons from embryonic day-21 rats were used, 73% of the innervated myotubes had glutamate-evoked input. Maternal stress accelerated this maturation of evocable synaptic transmission by approximately 2 days. The results of these experiments involving maternal stress (Fig. 5) were not statistically different (\(P > 0.4\), Student’s t-test) from those found when pregnant rats were injected with dexamethasone (Fig. 1).

As in the case of dexamethasone injection, the effect of maternal stress could be blocked by a prior injection of progesterone (Fig. 3). Specifically, on the 19th day of pregnancy, rats were injected subcutaneously with 50 mg/kg of progesterone 15 min prior to being placed in the cold. After 10 hr, the fetal retinal cells were harvested and cocultured with muscle cells. When assayed 1 day later, only 6% of the muscle cells that were innervated by fetal retinal neurons had evocable synaptic input. Thus, pretreatment with progesterone reduced the stress-mediated induction of evocable transmission by more than an 80%.

**Discussion**

The results indicate that a culture system can be used to identify and investigate possible etiologies of abnormal retinal development. A culture system was applied to study the pathophysiology of fetal retinal neurons in order to do assays that, at present, are virtually impossible to do in vivo. In the study presented here, the retina-muscle culture system was used to assay the ability of fetal retinal neurons to release acetylcholine at synapses in response to excitatory stimulation. This assay would be extremely difficult to do in vivo. However, with our culture system, the measurement of acetylcholine release at synapses is relatively simple. In fact, we demonstrated recently that it is possible, using this culture system, to monitor the release of neurotransmitter from a single, relatively isolated, visually identified, cholinergic retinal neuron.\textsuperscript{21,26} It appears that the use of the retina-muscle culture system provides a practical and potentially powerful method to help in the investigation of possible causes of abnormalities in the functional maturation of retinal neurons. However, even the most ardent supporter of in vitro experimentation must acknowledge that conclusions derived from studies of cultured neurons ultimately must be proved to be applicable in vivo.

In this study, glutamate was used as an excitatory stimulus. Glutamate is a putative excitatory retinal neurotransmitter\textsuperscript{27} that has been reported to increase sodium permeability of retinal neurons.\textsuperscript{28} Previously, it has been shown that glutamate-evoked transmission at retina-muscle synapses can be blocked by a glutamate-receptor antagonist and is dependent on extracellular calcium\textsuperscript{26} Importantly, earlier experiments demonstrated that the failure to evoke transmission at immature retina-muscle synapses is not specific to glutamate. Rather, since potassium also fails to evoke transmission,\textsuperscript{10,11} it appears that the immature cholinergic neurons studied in this system are unable to increase their output of acetylcholine in response to de-
polarizing stimuli in general. Glutamate, rather than potassium, was used in this study chiefly because the assays using glutamate are easier to do than those using potassium (for further discussion see reference 10). One of the goals of this study was to develop a relatively easy way to screen for possible causes of abnormal retinal development.

The major finding of this study was that, during a critical period in gestation, the functional development of cholinergic retinal neurons derived from the fetus could be altered if the mother rat had been injected with dexamethasone or stressed by cold exposure. Specifically, fetal retinal neurons could be induced to precociously acquire the capability of releasing acetylcholine at synapses in response to excitatory stimulation. My previous experiments using the retina-muscle culture system led to the hypothesis that exposure of retinal cells to glucocorticoid hormones in vitro could influence the development of mechanisms that couple neuronal depolarization with the release of acetylcholine. The results reported here strongly indicate that alterations in neuronal development detected with the use of the retina-muscle culture system are not restricted to manipulation to the culture conditions.

Based on the findings of this study, a number of hypotheses can be proposed concerning perturbations in the functional development of cholinergic retinal neurons. One idea is that the maturation of fetal retinal neurons can be influenced by factors affecting the mother, eg, dexamethasone injection or stress. Another notion is that timing of specific events in the developmental program for cholinergic retinal neurons may be most vulnerable to disruption during a particular period in gestation. For example, on embryonic days 19 and 20, the development of evachable acetylcholine release was found to be particularly sensitive to glucocorticoid exposure (see also reference 10). A third proposal is that one cause for abnormalities in retinal development may be the premature exposure to a normal developmental signal. This idea is based on the hypothesis that glucocorticoid hormones regulate the timing of certain aspects of neuronal maturation during normal retinal development in vivo. Support for this possibility is that a four-fold increase in the concentration of unbound glucocorticoids in the plasma of 18–21-day rat fetuses correlates temporally with the development of evachable synaptic activity reported here. Although this correlation is suggestive, further studies clearly are needed to establish that these hormones regulate the maturation of retinal synapses in vivo. Another question is whether glucocorticoids act directly on cholinergic retinal neurons or indirectly via other retinal cells such as the glia (see references 30 and 31). Also, the long-term effect of accelerating the functional maturation of cholinergic retinal neurons remains to be explored.

Finally, it would not be surprising to find that a variety of molecules, other than glucocorticoid hormones, also can influence developing retinal neurons. The cell culture system used here should prove useful in the subsequent identification and study of substances that may alter the functional maturation of the mammalian retina.

**Key words:** cell culture, cholinergic neurons, synaptic function, dexamethasone, stress

**References**


