Photomechanical Movements of Cultured Embryonic Photoreceptors: Regulation by Exogenous Neuromodulators and by a Regulable Source of Endogenous Dopamine

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In the retina of nonmammalian vertebrates, light regulates photoreceptor morphology by causing rod photoreceptor elongation and cone photoreceptor contraction. The opposite photomechanical movements occur in the dark, and proceed with a circadian rhythm in many species *in vivo*. Using dissociated cultures of embryonic chick retina cells, we have recently demonstrated that photoreceptor cells that differentiate *in vitro* acquire the capacity of responding to light/dark cycles with photomechanical movements (Stenkamp and Adler, 1993). Here we report that the putative neuromodulators melatonin and dopamine can mimic the effects of darkness and light, respectively, on *in vitro* photomechanical movement. Pharmacological studies showed that dopamine appears to function by means of a D₂-type receptor negatively coupled to adenylate cyclase. The effects of light on the cultured photoreceptors were inhibited by dopamine D₂ receptor antagonists, and were attenuated by the dopaminergic neurotoxin 6-hydroxydopamine and by the dopamine synthesis inhibitor α -methyl-*p*-tyrosine. The possible existence of an endogenous source of dopamine in the cultures was also suggested by the presence of tyrosine hydroxylase-like immunoreactivity, and of an Na⁺-dependent mechanism for the accumulation of ³H-dopamine, which was predominantly associated with nonphotoreceptor cells. Additionally, ³H-dopamine release occurred *in vitro* through a Ca²⁺-dependent mechanism, as well as through reverse function of a nomifensine-sensitive dopamine transporter. Both of these putative release mechanisms appeared to be regulated by light and by melatonin, suggesting a mechanism whereby the putative dopaminergic cells may interact with other cells present in the cultures. These studies suggest that complex paracrine neuromodulatory mechanisms can differentiate in low-density embryonic cell culture, that dopaminergic activities exist *in vitro*, and that they are important for mediating photomechanical movements.

[Key words: photoreceptor, photomechanical movement, dopamine, melatonin, circadian rhythm, cell culture, dopamine transporter, D₂ receptor, retina]

The responses of vertebrate retinæ to light are not limited to visual transduction. For example, the rates of synthesis of opsin and several other visual proteins and mRNAs vary as a function of an external light cycle, with peak synthesis occurring at the time of light onset (Bowes et al., 1988; Korenbrot and Fernald, 1989). Similarly, a peak of rod photoreceptor outer segment disk shedding and phagocytosis occurs shortly following light onset (reviewed by Bok, 1985). In lower vertebrates, photoreceptor inner segment length is regulated by photomechanical movements, with light initiating rod elongation and cone contraction, and darkness inducing the opposite morphological changes (reviewed by Burnside and Dearth, 1986; Kunz, 1990). Some of these cyclic activities persist in the absence of external cues, and are therefore presumed to be circadian in nature (Dearth and Burnside, 1986).

Several neuromodulators are thought to participate in the regulation of retinal responses to light and dark and endogenous circadian clocks (Burnside and Dearth, 1986; Dowling, 1986; Besharse et al., 1988; Cahill and Besharse, 1991). The neuromodulators dopamine and melatonin act as reciprocal antagonists by mimicking the effects of light and darkness, respectively, for many of these rhythmic retinal changes. Their synthesis and release are in turn regulated by the light cycle and, in many cases, by each other (Kramer, 1971; Dubocovich, 1983; Iuvone, 1984; Iuvone et al., 1990). These neuromodulators presumably function in a nonsynaptic fashion, diffusing across the retina from the site of synthesis (Pierce and Besharse, 1985; Besharse et al., 1988; Dearth et al., 1990); these sites are photoreceptor cells for melatonin (Iuvone et al., 1990; Cahill and Besharse, 1993; Weichmann and Craft, 1993), and interplexiform cells or a subpopulation of amacrine cells for dopamine (Dowling and Ehinger, 1978; Dearth and Burnside, 1988).

We have begun to investigate cellular mechanisms for regulation and differentiation of cyclic retinal metabolism using low-density cell culture. Chick embryo neural retina precursor cells differentiate as either multipolar neurons or photoreceptors in low-density cultures, where they develop in the absence of retinal pigment epithelium (RPE) and Müller glia. These photoreceptors are anatomically and biochemically polarized, with Na⁺, K⁺-ATPase accumulating in the inner segment and visual pigment immunoreactivity strongest distal to it (Adler, 1986; Madreperla and Adler, 1989; Madreperla et al., 1989). Cultured

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retinal cells also contain serotonin *N*-acetyltransferase (NAT) activity, which appears to be localized to the photoreceptor cells (Iuvone et al., 1990). We recently showed that these isolated photoreceptors develop and maintain light cycle–dependent activities in the absence of detectable contact with other retinal cell types. When maintained on a 12 hr light/12 hr dark cycle, approximately half of the cultured photoreceptors elongate in response to light, and contract in response to darkness (L+), with a much smaller subpopulation showing the opposite responses (L–) (Stenkamp and Adler, 1993). The behavior of the more abundant (L+) subpopulation was chosen as assay to investigate the potential involvement of an intrinsic circadian clock and of various neuromodulator systems in these *in vitro* responses. The studies suggest that isolated cells establish *in vitro* a paracrine system of communication that participates in the control of light-regulated, rhythmic photoreceptor behaviors.

Materials and Methods

Cell culture. Methods for the preparation of the low-density neural retina cultures used in this study have been described (Adler, 1990). Briefly, embryonic day 8 (E8) chick embryo retinas were dissected free of pigment epithelium and other ocular tissues, trypsinized, and mechanically dissociated. The cells were seeded in medium 199 containing 10% fetal calf serum and 110 μ g/ml linoleic acid/serum albumin (GIBCO) on 35 mm polyornithine-coated dishes at an initial density of 8×10^5 per dish. Cultures were maintained at 37°C in a humidified atmosphere of 9% O₂, 5% CO₂, and 86% N₂.

Light/dark treatments. A timed cycle of 12 hr light and 12 hr darkness was in progress throughout the culture period in most experiments. A circular, cool white fluorescent tube, with a neutral density filter, was used to obtain 10 lux intensity at the level of the culture dishes. Constant darkness controls were either covered with aluminum foil, or grown in a separate, light-proof incubator.

Drug treatments. Dopamine, 6-hydroxydopamine (6-OHDA), α -methyl-*p*-tyrosine (AMPT), dibutyl cAMP (dbcAMP), serotonin, and melatonin were obtained from Sigma, and apomorphine, bromocriptine, SKF-38393, haloperidol, eticlopride, phenylephrine, fluperazine, nomifensine, and 2-iodomelatonin were purchased from Research Biochemicals, Inc. Stock solutions were prepared in Dulbecco's Modified Eagle's Medium (GIBCO) and/or ethanol, and were added to the cultures as indicated in Results, with the appropriate vehicle controls. A red safety light was used when cultures had to be treated during the dark phase of the light cycle.

Photomechanical movements assays. Two complementary assays were used throughout the study for evaluating photoreceptor responses to light (see Stenkamp and Adler, 1993). For both assays, cultures were fixed with 1% glutaraldehyde in phosphate-buffered saline (PBS), rinsed in buffer, and examined with a Nikon phase-contrast microscope. After quantitation of the total number of photoreceptors and nonphotoreceptor neurons present in the cultures (see Adler and Hatlee, 1989), the number of photoreceptors having an *elongated morphology* was determined, using as criterion the presence of a constricted inner segment myoid region (diameter of 1.5 μ m; see Fig. 1*B* and Stenkamp and Adler, 1993). This assay tends to *underestimate* the magnitude of the response to light: 50% of the photoreceptors actually increase in length in response to light, but only 20–30% show the above-mentioned constriction of the inner segment. However, previous studies have shown this characteristic to be a reliable index for assessing the morphological responses of a population of photoreceptors to a light cycle (Stenkamp and Adler, 1993). Ten microscopic fields were counted at 200 $\times$ magnification in each culture, representing approximately 1/1000 of the culture area. Counts were done in a “masked” fashion, by an investigator who was not aware of the identity of the cultures. Results are presented as means of triplicate cultures, $\pm$ standard deviation. Experiments were repeated a minimum of three times, with excellent reproducibility. Statistical significance was determined by a one-way analysis of variance, using either a SIDAK test or a Student-Newman-Keuls post hoc test ($\alpha = 0.05$).

A complementary assay involved measuring the length of individual photoreceptor cells between the base of the nuclear compartment (near

the origin of the neurite) and the center of the inner segment lipid droplet (Madreperla and Adler, 1989; Stenkamp and Adler, 1993). Fifteen randomly selected photoreceptors were measured in each of three replicate cultures. Results are shown as distribution of photoreceptor lengths for each condition. Although this assay was performed for all experimental conditions to verify the results obtained with the elongated photoreceptor assay, these data are shown only for selected experiments for the sake of brevity.

Western blot analysis of tyrosine hydroxylase-immunoreactive materials. After 6 d *in vitro*, (div) retinal cultures were washed once with Ca²⁺/Mg²⁺-free Hank's buffered saline (CMF-HBSS) and harvested in CMF-HBSS containing 0.2 mM EDTA (CMF-HBSS-EDTA). Ten 35 mm dishes were pooled for each experimental sample. Cell suspensions were centrifuged at 20,000 $\times$ g, and the resulting pellet was solubilized in Laemmli sample buffer (Laemmli, 1970). Freshly dissected chicken tissues were frozen in liquid nitrogen, thawed on ice, minced, and homogenized in a glass–glass homogenizer (Wheaton), suspended in CMF-HBSS-EDTA, centrifuged, and solubilized as for culture samples. For electrophoresis, 2 μ g of each sample was loaded per lane on a 10% polyacrylamide mini gel, electrophoresed, and transferred to a Nytran membrane (Schleicher and Schuell). The membranes were probed with a 1:500 dilution of a rabbit polyclonal antibody against bovine tyrosine hydroxylase (TH) (Eugentech), processed with an alkaline phosphatase detection kit (Vector), and visualized using the “X-Phos” and “NBT” reagents from a DIG riboprobe detection kit (Boehringer-Mannheim). A 1 mM concentration of levamisole (Sigma) was added during the color reaction to inhibit endogenous phosphatases.

³H-dopamine accumulation and release. Cultures were washed with 40 mM Tris-HEPES, 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 0.1 mM CaCl₂, pH 7.4 (THM), and incubated in THM containing ³H-dopamine (Dupont–New England Nuclear; specific activity, 33.25 Ci/mmol) at different concentrations (0.3 μ M for most experiments) for 15 min at 37°C. In some experiments, CoCl₂ was substituted for CaCl₂ (Ca²⁺-free THM), while choline chloride was substituted for NaCl (Na⁺-free THM) in others. The dopamine-transporter inhibitor nomifensine was present at 10 μ M in the incubation medium in several experiments. Following the incubation period, the cells were washed with THM and solubilized in Opti-Fluor O (Packard) fluid for determination of cellular radioactivity by liquid scintillation counting, or fixed with 1% glutaraldehyde in PBS for autoradiography (see below).

Autoradiography. Fixed cultures were washed with PBS, dehydrated with ethanol, and coated with a 50% dilution of NTB-2 emulsion (Kodak). Cultures were exposed in the dark for 7 d, developed with Amidol developer (Kodak), fixed in 5% sodium thiosulfate, and mounted with Polymount (Polysciences).

Results

Description of cultures

Freshly seeded, low-density chick neural retina cultures appear morphologically homogeneous in composition, but two morphologically identifiable cell types differentiate after 3–6 div (Fig. 1*A*). Multipolar neurons have large cell bodies and several long neurites. They have been previously characterized by their sensitivity to kainate toxicity, and by the presence of ChAT activity and high-affinity uptake mechanisms for GABA and glutamate (Adler, 1990). The photoreceptor cells differentiate somewhat later and can be identified by their polarized morphology, with a single short neurite, a cell body occupied exclusively by the nucleus, an inner segment with a conspicuous lipid droplet, and a distal cilium immunoreactive with visual pigment antibodies (Adler, 1986). These cells show light-sensitive photomechanical movements when maintained on a light cycle, with 50% of the photoreceptors elongating in response to light and contracting in darkness (L+ subpopulation; Stenkamp and Adler, 1993). A smaller subpopulation contracts in light and elongates in darkness (L–), while 30% of the cells show no changes (Lo cells). Figure 1 illustrates cultures fixed at the end of the dark period (Fig. 1*A*), when short photoreceptors predominate, and at the end of the light period (Fig. 1*B*), when elongated cells are most abundant. The figure also illustrates the

Figure 1. Embryonic chick neural retina cultures fixed at the same time of day (4:00 P.M.) at 6 div. *A*, Cultures incubated in constant darkness. *B*, Cultures incubated on a 12:12 hr light/dark cycle, fixed for photography during a light period. *Arrowheads*, photoreceptors; *arrows*, neurons. Photoreceptors indicated with *large arrowheads* in *B* have an "elongated photoreceptor" morphology; the narrow connection between the inner segment and the nuclear compartment is $<1.5\ \mu\text{m}$ in diameter (see Materials and Methods and Results). Scale bar, $10\ \mu\text{m}$.

constriction observed in the inner segment of many elongated photoreceptors, which was used throughout these studies as a practical criterion for quantitation of the frequency of elongated photoreceptors (see Materials and Methods).

In vitro photomechanical movements can be regulated by melatonin and dopamine

General design of the experiments. The experimental paradigms used to test the effects of pharmacological agents related to dopamine and melatonin are shown in Figure 2. To test for inhibition of the effects of light, compounds were added in the dark, prior to light onset, and cultures were fixed 6 hr later, during the subsequent light period (Fig. 2*A*). To investigate possible inhibition of the effects of darkness, compounds were added prior to light offset, and cultures were fixed 6 hr later, during the subsequent dark period (Fig. 2*B*). The experiments described in Figure 2*C–D* were possible because rhythmic photomechanical responses were found *not* to persist in the absence of a light/dark cycle (data not shown; Stenkamp and Adler, 1993), allowing the testing of compounds for their ability to *mimic* the effects of light [by acting on cells kept in the dark during a light phase of the cycle ("expected light/actual darkness"); Fig. 2*C*], or to *mimic* darkness [by acting on cells kept in the light during an expected dark period ("expected darkness/actual light"); Fig. 2*D*]. For experiments illustrated in Figure 2, *C* and *D*, controls included cultures that were actually exposed to the appropriate "expected" condition, as well as cultures exposed to the "actual" illumination condition.

Effects of melatonin and melatonin agonists. As shown in Figure 3*A*, treatment of the cultures during the light cycle with $1\ \mu\text{M}$ melatonin (a dark-adaptive neuromodulator; reviewed by Dubocovich, 1988) or 2-iodomelatonin (a melatonin receptor agonist; Dubocovich and Takehashi, 1987) resulted in a signif-

icant decrease in the frequency of elongated photoreceptors as compared to controls. The same reagents also showed the capacity to mimic darkness (Fig. 3*B*); serotonin was ineffective at a similar concentration ($1\ \mu\text{M}$). Photoreceptor length distributions in expected light/actual light and expected darkness/actual light cultures treated with melatonin or 2-iodomelatonin were strikingly similar to those in dark control cultures (Table 1).

Figure 2. Experimental paradigms used to investigate effects of various drugs on photomechanical movements *in vitro*. *Solid bars* indicate a 12 hr dark period; *open bars*, a light period. Each design asks a specific experimental question, as indicated to the *right*.

Table 1. Effects of melatonin and melatonin agonists on photoreceptor length distributions *in vitro*

Exptl. paradigm ^a	Expected condition	Actual condition	Melatonin agonist	Photoreceptor length distribution (%)				
				10–14 μm	15–19 μm	20–24 μm	25–29 μm	>30 μm
A	Dark ^b	Dark ^b	—	40.0	46.7	11.1	2.2	0
	Light	Light	—	4.4	48.9	31.1	11.1	4.4
	Light	Light	Melatonin ^c	20.0	37.8	35.6	6.7	0
	Light	Light	Melatonin ^d	44.4	46.7	6.7	2.2	0
	Light	Light	I-melatonin ^d	33.3	37.8	28.9	0	0
D	Light ^b	Light ^b	—	17.8	35.6	37.8	6.7	2.2
	Dark	Dark	—	35.6	44.4	13.3	4.4	2.2
	Dark	Light	—	20.0	40.0	28.9	11.1	0
	Dark	Light	Serotonin ^d	26.7	40.0	17.8	13.3	2.2
	Dark	Light	Melatonin ^d	46.7	33.3	13.3	6.7	0
	Dark	Light	I-melatonin ^d	31.1	44.4	15.6	6.7	2.2

Neural retina cultures were examined after a 6 hr treatment with melatonin or melatonin receptor agonist. Forty-five photoreceptors were measured for each condition (15 in each of three 35 mm dishes) and were assigned to 5 μm bins based on length. The table shows distributions of these lengths according to illumination condition and drug treatment.

^a Experimental paradigms as described in Figure 2.

^b Assayed prior to addition of drug/vehicle.

^c Drug added at 100 nM.

^d Drug added at 1 μM .

Effects of dopamine and dopamine agonists. Treatment of cultures with dopamine resulted in robust, dose-dependent increases in the frequency of elongated photoreceptors in cultures in expected light/actual darkness (Fig. 4), with 10 μM dopamine mimicking and 100 μM surpassing the light-stimulated percentages of elongated photoreceptors. Analysis of photoreceptor length distributions corroborated that dopamine exerts light-adaptive effects during expected light (Table 2).

Results with various dopamine agonists using two different

assays are summarized in Tables 2 and 3. When added at a concentration of 10 μM , the D_2 -like receptor agonist bromocriptine was more effective than the nonselective dopamine receptor agonist apomorphine or the D_1 -selective agonist SKF-38393. This is consistent with a putative D_2 receptor pharmacology (Cohen et al., 1992).

In contrast to its effect in expected light/actual darkness, dopamine at 10 μM failed to increase the number of elongated photoreceptors when tested in expected darkness/actual dark-

Figure 3. Effects of melatonin and melatonin receptor agonists on photomechanical responses *in vitro*. Cultures were treated with 1 μM melatonin (MT), 1 μM 2-iodomelatonin (I-MT), or 1 μM serotonin (ST) for 6 hr and analyzed for the presence of elongated photoreceptors. *A*, Effects during expected light/actual light (experimental paradigm in Fig. 2*A*). *B*, Effects during expected darkness/actual light (experimental paradigm in Fig. 2*D*). The following differences were significant ($\alpha = 0.05$; see Materials and Methods): *A*, dark versus light, light versus MT, light versus I-MT; *B*, dark versus light, light versus MT, light versus I-MT.

Table 2. Effects of dopamine (DA) agonists and antagonists on the frequency of elongated photoreceptors *in vitro*

Exptl. para-digm ^a	Expected condition	Actual condition	DA agonist	DA antagonist	% Elongated photo-receptors (±SD)
A	Dark ^b	Dark ^b			6.1 (2.2)
	Light	Light			24.9 (2.5)
	Light	Light		Fluperazine ^c	11.4 (1.2)
	Light	Light		Spiperone ^c	13.4 (2.6)
	Light	Light		Sulpiride ^c	14.5 (1.0)
	Light	Light		Eticlopride ^c	15.9 (3.2)
	Light	Light		Haloperidol ^c	29.2 (3.7)
C	Light	Dark			5.6 (1.4)
	Light	Dark	Dopamine ^c		26.6 (6.0)
	Light	Dark	Dopamine ^c	Sulpiride ^d	9.4 (1.8)
	Light	Dark	Dopamine ^c	Fluperazine ^d	11.7 (1.6)
	Light	Dark	Dopamine ^c	Spiperone ^d	17.4 (3.1)
	Light	Dark	Dopamine ^c	Eticlopride ^d	18.0 (5.0)
	Light	Dark	Dopamine ^c	Haloperidol ^d	23.0 (4.1)
	Light	Dark	Bromocriptine ^c		17.6 (5.1)
	Light	Dark	Apomorphine ^c		12.6 (4.2)
	Light	Dark	SKF38393 ^c		11.7 (2.2)
	Light	Dark	Phenylephrine ^c		3.7 (0.9)
	Light ^b	Light ^b			20.4 (3.2)
B	Dark	Dark			9.4 (1.9)
	Dark	Dark	Dopamine ^c		9.1 (2.8)
	Dark	Dark	Bromocriptine ^c		17.8 (1.5)
	Dark	Dark			
D	Dark	Light			20.8 (1.3)
	Dark	Light	Spiperone ^c		4.0 (1.4)

Neural retina cultures were examined after 6 hr treatment with dopamine or dopamine agonist by counting the number of photoreceptors having an "elongated morphology" in triplicate 35 mm dishes. The table quantifies these elongated photoreceptors as a percentage of total photoreceptors. The following were significantly different ($\alpha = 0.05$; see Materials and Methods): all dark versus light control comparisons, dark versus dopamine (paradigm C), dark versus bromocriptine (paradigms C and B), light versus all antagonists except haloperidol (paradigm A), dopamine versus dopamine plus all antagonists except haloperidol (paradigm C), and light versus spiperone (paradigm D).

^a Experimental paradigm as described in Figure 2.

^b Assayed prior to addition of drug/vehicle.

^c Drug added at 10 μ M.

^d Drug added at 100 μ M.

ness (Table 3), and elicited only a limited degree of elongation at 100 μ M under these conditions (data not shown). Bromocriptine (10 μ M) was equally effective in expected light/actual darkness and in expected darkness/actual darkness (Table 3). The difference between the effects of dopamine in the two experimental paradigms may imply that the relative susceptibility of photomechanical response to dopamine varies in a circadian manner.

Effects of dopamine antagonists and dbcAMP. Various dopamine antagonists were tested for their ability to inhibit the effects of dopamine, using two complementary assays (Table 2, and data not shown). At a concentration of 10 μ M, the D₂-selective antagonist sulpiride was more effective than the less selective antagonist haloperidol.

Since D₂- and D₄-type dopamine receptors appear to be negatively coupled to adenylate cyclase in the retina (Cohen et al., 1992), cAMP analogs should counteract the effects of dopamine. Our results were consistent with this prediction, since 50 μ M

dbcAMP prevented dopamine from eliciting light-adaptive photomechanical movements in expected light/actual darkness (Fig. 5A, Table 3).

Effects of K⁺-evoked depolarization. Depolarization by high extracellular [K⁺] was tested for effects on photomechanical responses by adding 20 mM KCl to culture medium before light onset. KCl significantly inhibited the response to light but responses could be restored by the addition of 10 μ M dopamine (Fig. 5B, Table 4). KCl was also effective during expected darkness/actual light, but the addition of 20 mM NaCl had no effect in either condition (not shown).

Analysis of endogenous dopaminergic activities

Effects of dopamine antagonists upon photoreceptor responses to light. Dopamine receptor antagonists were also effective at inhibiting light-adaptive photomechanical movements (Table 2), with the selective D₂ antagonists sulpiride and spiperone being more effective at 10 μ M concentrations than the nonse-

Figure 4. Effects of dopamine (DA) on photomechanical responses *in vitro*. Cultures were treated for 6 hr and analyzed for the presence of elongated photoreceptors. Experimental paradigm in Figure 2C is used (expected light/actual darkness). The following differences were significant ($\alpha = 0.05$; see Materials and Methods): light versus dark, dark versus 10 μ M DA, dark versus 100 μ M DA.

lective antagonist haloperidol. dbcAMP was also found to significantly attenuate the response of the cultures to light (Fig. 5A, Table 3). These results suggested that the signaling pathways for light and dopamine may converge at or before this second messenger regulatory system, and that dopamine receptor activation may be involved in light-adaptive photomechanical responses *in vitro*. This possibility raised questions about the existence of endogenous sources of dopamine in the cultures.

TH immunoreactivity. The potential existence of dopaminergic activities in the cultures was approached by investigating the presence of TH, the rate-limiting enzyme for catecholamine synthesis (Levitt et al., 1965). Western blots of cultured retinal cell extracts, reacted with a polyclonal antibody against bovine TH, showed two bands with relative molecular sizes of approximately 65 and 123 kDa, consistent with the expected behavior of TH monomers and dimers, respectively (Oka et al., 1983). Similar results were obtained with control samples of

Figure 5. Effects of 50 μ M dibutyryl cyclic AMP (dbcAMP) and K⁺-evoked depolarization on photomechanical responses *in vitro*. Cultures were treated for 6 hr and then analyzed for the presence of elongated photoreceptors. **A**, Effects of dbcAMP on responses to light (experimental paradigm in Fig. 2A), and effects of dbcAMP on photoreceptor responses to 10 μ M dopamine (DA) during expected light/actual darkness (experimental paradigm in Fig. 2C). **B**, Effects of 20 mM KCl on photoreceptor responses to light, and their inhibition by 10 μ M dopamine (DA) (experimental paradigm in Fig. 2A). The following differences were significant ($\alpha = 0.05$; see Materials and Methods): **A**, light versus dbcAMP, light versus dark, dark versus DA, DA versus DA-dbcAMP; **B**, dark versus light, light versus KCl, KCl versus KCl-DA.

Table 3. Effects of dbcAMP on light- and dopamine-stimulated photomechanical responses *in vitro*

Exptl. paradigm ^a	Expected condition	Actual condition	Dopa-mine ^b	dbc-AMP ^c	Photoreceptor length distribution (%)				
					10–14 μ m	15–19 μ m	20–24 μ m	25–29 μ m	>30 μ m
A	Dark ^d	Dark ^d			31.1	55.6	11.1	2.2	0
	Light	Light			6.7	46.7	31.1	11.1	4.4
	Light	Light		X	28.9	40.0	31.1	0	0
C	Light	Dark			46.7	33.3	17.8	2.2	0
	Light	Dark	X		13.3	48.9	20.0	17.8	0
	Light	Dark	X	X	33.3	51.1	6.7	8.9	0

Neural retina cultures were examined after a 6 hr treatment with dopamine and/or dbcAMP. Forty-five photoreceptors were measured for each condition (15 in each of three 35 mm dishes), and were assigned to 5 μ m bins based on length. The table shows distributions of these lengths according to illumination condition and drug treatment.

^a Experimental paradigm as described in Figure 2.

^b Drug added at 10 μ M.

^c Drug added at 50 μ M.

^d Assayed prior to addition of drug/vehicle.

Figure 6. TH antiserum immunoblot. Lanes 1 and 2, chick neural retina cultures harvested during dark and light periods, respectively, on div 6; lane 3, cultures treated with 6-OHDA at 4 div, harvested during a light period on div 6; lane 4, E16 chick retina; lane 5, postnatal day 5 (P5) chick retina; lane 6, P5 chick heart. Positions of molecular size standards are indicated to the right.

tissues known to contain TH, such as posthatch chicken heart and retina, while blots processed with control, nonimmune serum failed to show detectable bands (not shown).

The bands of TH-immunoreactive materials were reduced in Western blots of cultures treated at 3 div with 7 μ M 6-OHDA, which is toxic for dopaminergic neurons (Johnsson et al., 1975; Braisted and Raymond, 1992) and attenuates *in vitro* photomechanical movement (see below). Higher 6-OHDA concentrations could not be studied due to nonspecific cytotoxic effects. We observed no apparent differences in the amount of TH-immunoreactive material in cultures harvested during the dark as compared to the light phases of the cycle (Fig. 6).

Inhibition of photomechanical movement by drugs that attenuate dopamine synthesis. AMPT, a TH inhibitor, eliminated

almost completely the light-induced appearance of elongated photoreceptors in 5–6 div cultures exposed to the drug since *in vitro* day 4 (Fig. 7A). The occurrence of photoreceptor elongation when exogenous dopamine was added at 6 div to some of these AMPT-treated cultures indicated that AMPT did not have deleterious effects upon the photoreceptors themselves. In a second group of experiments, complete attenuation of photomechanical responses was observed at 5–6 div in cultures treated at 3 div with 7 μ M 6-OHDA (Fig. 7B). Responsiveness to exogenous dopamine could not be studied in this case due to toxic effects apparently resulting from the exposure to both 6-OHDA and dopamine (data not shown). However, photoreceptor elongation could be elicited in the 6-OHDA-treated cultures at 6 div by cytochalasin D, which has been shown to trigger photoreceptor elongation by inhibiting actin-dependent contractile mechanisms (Madreperla and Adler, 1989; D. L. Stenkamp and R. Adler, unpublished observations).

Na⁺-dependent ³H-dopamine accumulation. The presence of Na⁺-dependent uptake mechanisms for dopamine is characteristic of dopaminergic neurons. Preliminary experiments indicated that ³H-dopamine accumulation was more readily measured when Co²⁺ was substituted for Ca²⁺ in the incubation medium, probably due to inhibition of Ca²⁺-dependent, exocytotic release mechanisms (Paes de Carvalho et al., 1990; Efthimiopoulos et al., 1991).

³H-dopamine accumulation in Ca²⁺-free, Co²⁺-supplemented buffers (Fig. 8A) was concentration dependent, and Eadie-Hofstee analysis revealed a low-affinity, high-capacity transport component and a high-affinity, low-capacity transport component. This resembles findings in rat cerebellum, striatum, and frontal cortex (Efthimiopoulos et al., 1991). Cellular accumulation was reduced by roughly 75% under Na⁺-free conditions (as compared to Ca²⁺-free conditions). Autoradiographic analysis (Fig. 8B) showed heavy labeling of multipolar neurons and of round, morphologically undifferentiated cells in the presence, but not in the absence, of Na⁺ ions (not shown). Scattered silver grains were associated with photoreceptors under both Na⁺-containing and Na⁺-free conditions, suggesting that they may

Figure 7. Effects of dopamine synthesis inhibitors on the frequency of elongated photoreceptors in culture (expressed as a percentage of total photoreceptors). Solid and open bars at the bottom indicate dark and light periods, respectively (see Materials and Methods). **A**, Circles, control (vehicle added at 4 div); squares, 50 μ M α -methyl-*p*-tyrosine (AMPT) added at 4 div; diamond, AMPT at 4 div and 10 μ M dopamine (DA) at 6 div. **B**, Circles, control (vehicle added at 4 div); squares, 7 μ M 6-hydroxydopamine (6-OHDA) added at 4 div; diamond, 6-OHDA at 4 div and 2 μ M cytochalasin D (CCD) at 6 div. In addition to all light versus dark comparisons for controls, the following differences were significant ($\alpha = 0.05$; see Materials and Methods): **A**, light control versus light AMPT, light AMPT versus light AMPT-DA; **B**, light control versus light 6-OHDA, light 6-OHDA versus light 6-OHDA-CCD.

Figure 9. *A*, Effects of Ca²⁺ on intracellular accumulation of ³H-dopamine in the constant presence of nomifensine under various conditions. Experimental design in Figure 2*D* is used, showing increased accumulation in the absence of Ca²⁺, which is inhibited by 1 μ M melatonin (MT) during expected darkness/actual light. *B*, Effects of nomifensine on intracellular accumulation of ³H-dopamine in the constant absence of Ca²⁺, under various conditions. Experimental design in Figure 2*D* is used, showing increased accumulation in the presence of nomifensine, which is inhibited by expected darkness/actual darkness, and by 1 μ M melatonin (MT) during expected darkness/actual light. The following differences were significant ($\alpha = 0.05$; see Materials and Methods): *A*, for all conditions, Ca²⁺-containing versus Ca²⁺-free; *B*, for all conditions except MT, -NOMI versus +NOMI.

represent background labeling. We have not determined whether the nonphotoreceptor neurons that accumulate ³H-dopamine are also those that take up glutamate, GABA, or taurine under similar conditions (Adler, 1983; Pessin and Adler, 1985; Politi and Adler, 1987). It must be noted, however, that the coexistence of uptake mechanisms for as many as three different neurotransmitters has been demonstrated in these cultured cells (Pessin and Adler, 1985).

A nomifensine-sensitive dopamine transporter has been described that can transport dopamine both into (Efthimiopoulos et al., 1991) and out of cells (Lonart and Zigmond, 1990; Jacocks and Cox, 1992). In our cultures, nomifensine stimulated very effectively the accumulation of ³H-dopamine in the cultures in a dose-dependent manner (Fig. 8*C*). By analogy with results in other tissues (Lonart and Zigmond, 1990; Jacocks and Cox, 1992), therefore, it appears that the nomifensine-sensitive dopamine transporter may be functioning as a nonexocytotic dopamine release mechanism in cultured retinal cells. This possibility is further explored in the Discussion.

Effect of light and melatonin on dopamine release. The findings reported in the preceding section provided indirect evidence for the existence of two putative dopamine release mechanisms in the cultures: one appeared to be Ca²⁺ dependent and Co²⁺ sensitive, whereas the second appeared to involve the nomifensine-sensitive dopamine transporter. Direct investigation of their relative contributions was somewhat hampered by the susceptibility of low-density retinal cultures to damage by protocols

involving repeated and quick washes. As an alternative, we estimated Ca²⁺-dependent release by measuring intracellular accumulation of ³H radioactivity after incubating cultures with ³H-dopamine in the presence of 10 μ M nomifensine (to prevent transporter-dependent release), and in the presence or absence of Ca²⁺. As shown in Figure 9*A*, intracellular accumulation of ³H-dopamine was higher under Ca²⁺-free conditions in expected light/actual light, expected darkness/actual darkness, and expected darkness/actual light conditions. However, the effects of Ca²⁺-free medium were abolished when expected darkness/actual light cultures were simultaneously treated with 1 μ M melatonin.

The reciprocal experiment involved incubating cultures with ³H-dopamine in the absence of Ca²⁺ (to block Ca²⁺-dependent release), and in the presence or absence of nomifensine. As shown in Figure 9*B*, intracellular accumulation of ³H-dopamine was increased approximately threefold in the presence of nomifensine in expected light/actual light, and over twofold in expected darkness/actual light conditions. Smaller differences were observed in expected darkness/actual darkness cultures. Melatonin at 1 μ M caused reductions in dopamine accumulation in expected darkness/actual light conditions, which were particularly evident in the presence of nomifensine.

Nomifensine reversibly inhibits light-dependent photomechanical responses. Since nomifensine appears to block dopamine release *in vitro*, it should be expected to attenuate light-induced photoreceptor elongation if, as postulated, endogenous sources

Figure 8. Cellular accumulation of ³H-dopamine. *A*, Dose-dependent accumulation under Ca²⁺-free conditions. Open symbols indicate accumulation in the presence of Ca²⁺ ($\diamond$) and in Na²⁺-free conditions ($\circ$) at a concentration of 0.3 μ M ³H-dopamine in the medium. Inset, Eadie-Hofstee analysis of ³H-dopamine uptake, showing a low-affinity, high-capacity uptake component and a high-affinity, low-capacity uptake component. *B*, Autoradiography of cultures incubated with ³H-dopamine in the absence of Ca²⁺. Panels show the same photographic field examined under bright-field and phase-contrast optics showing silver grains concentrated over morphologically undifferentiated cells (open arrows) and neurons (solid arrows), but not over photoreceptors (arrowheads). Scale bar, 10 μ M. *C*, Effect of nomifensine (NOMI) on accumulation of cellular ³H-dopamine. The following were significantly different ($\alpha = 0.05$; see Materials and Methods): *A*, for 0.3 μ M, Ca²⁺-free versus Ca²⁺-containing, Na²⁺-free versus Ca²⁺-free; *C*, control versus 10 μ M NOMI, control versus 100 μ M NOMI.

Table 4. Effects of KCl on light-stimulated photomechanical responses *in vitro*

Exptl. paradigm ^a	Expected condition	Actual condition	KCl ^b	Dopamine ^c	Photoreceptor length distribution (%)				
					10–14 μ m	15–19 μ m	20–24 μ m	25–29 μ m	>30 μ m
A	Dark	Dark ^d			35.6	48.9	24.4	0	0
	Dark	Light			8.9	31.1	31.1	17.8	11.1
	Dark	Light	X		51.1	28.9	15.6	4.4	0
	Dark	Light	X	X	6.7	24.4	35.6	11.1	22.2

Neural retina cultures were examined after a 6 hr treatment with KCl and/or dopamine by measuring 45 photoreceptors for each condition (15 in each 35 mm dish); photoreceptors were assigned to 5 μ m bins based on length. The table shows distributions of these lengths according to illumination conditions and drug treatment.

^a Experimental paradigm as described in Figure 2.

^b Drug added at 20 μ M.

^c Drug added at 10 μ M.

^d Assayed prior to addition of drug/vehicle.

of dopamine mediate these effects of light. As shown in Figure 10, 10 μ M nomifensine did indeed reduce significantly, by approximately 50%, the frequency of elongated photoreceptors in light-exposed cultures. Moreover, concomitant treatment of the cultures with nomifensine and 10 μ M dopamine restored photoreceptor elongation, indicating that nomifensine did not interfere directly with photoreceptor responsiveness to dopamine. Nomifensine effects upon photoreceptor elongation were similar in expected light/actual light and expected darkness/actual light conditions; no effects were detected upon dark-adaptive responses (not shown). Theoretically, elimination of Ca^{2+} ions from the culture medium should also inhibit light-dependent photomechanical responses by inhibiting Ca^{2+} -dependent dopamine release. This hypothetical mechanism could not be verified experimentally because of the deleterious effects of Ca^{2+} -free, EDTA-containing medium upon the cultured cells during the 6 hr assay period necessary for these studies.

Discussion

Effects of neuromodulators *in vitro*

The experiments aimed at analyzing the susceptibility of photomechanical responses to regulation by neuromodulators can be summarized as follows: (1) light-dependent, rhythmic photomechanical responses of cultured embryonic photoreceptors can be regulated by exogenous neuromodulatory agonists and antagonists; (2) melatonin and its receptor agonist inhibit the effects of light and mimic the effects of darkness; (3) dopamine and its receptor agonists mimic the effects of light with a D_2 -type pharmacology; (4) dopamine receptor antagonists inhibit the effects of dopamine as well as those of light, with a D_2 -type pharmacology; (5) the cyclic nucleotide analog dbcAMP has similar inhibitory effects upon dopamine- and light-induced photoreceptor elongation; and (6) K^+ -evoked depolarization has dark-adaptive effects upon photomechanical responses.

Taken together, these experiments suggest that the cultured cells have appropriate receptors and second-messenger signal systems that mediate the effects of exogenous neuromodulators. Although the cellular distribution of these receptors remains undetermined, possible involvement of RPE and Müller cells can be excluded, since they are not present in the cultures. We hypothesize that photoreceptors are likely sites for dopamine D_2 receptors for reasons of parsimony; this would also be consistent with the tentative localization to cultured chick photoreceptors of D_2 receptor-regulated *N*-acetyltransferase activity

(Iuvone et al., 1990), and with studies of melatonin release from isolated *Xenopus* photoreceptors (Cahill and Besharse, 1993). In the retina, *in vivo*, D_2 -like receptors have been identified with radioligand binding in the outer nuclear layer (Zarbin et al., 1986), as well as in rod outer segments (Brann and Jelsena, 1985). Melatonin receptors have been detected primarily in the inner plexiform layer of the retina *in vivo*, suggesting a distribution that includes amacrine or interplexiform cells (Dubocovich, 1988). Their *in vitro* distribution remains unknown. Activation of melatonin receptors has been shown to occur at nanomolar or even subnanomolar concentrations. However, a 100 nM concentration was needed to elicit a dark-adaptive response in chick retinal cultures, possibly due to the susceptibility of melatonin to oxidation. This is consistent with the observation that higher melatonin concentrations were needed to activate photoreceptor disk shedding in the absence than in the presence of the antioxidant ascorbic acid (Besharse and Dunis, 1983; Besharse et al., 1984).

Although rhythmic photomechanical responses did not ap-

Figure 10. Effects of 10 μ M nomifensine (NOMI) on photomechanical movement *in vitro*. Cultures were treated for 6 hr and examined for the presence of elongated photoreceptors. Experimental paradigm in Figure 2A is used, showing effects during expected light/actual light. DA, 10 μ M dopamine added. The following differences were significant ($\alpha = 0.05$; see Materials and Methods): dark versus light, light versus NOMI, NOMI versus NOMI-DA.

Figure 11. Model for regulation of *in vitro* photomechanical responses to dark (A) and light (B). D₂, D₂-type dopamine receptor; cAMP, cyclic AMP; NAT, serotonin *N*-acetyltransferase; CSK, cytoskeleton. See Discussion for explanation.

pear circadian, it is noteworthy that dopamine was more effective in mimicking the effects of light during expected light/actual darkness conditions than during expected darkness/actual darkness. The relative lack of effects during expected darkness/actual darkness could imply that some cell properties, such as sensitivity to dopamine, change in a circadian manner. Two possible mechanisms would be downregulation of dopamine receptors and/or upregulation of dopamine catabolism during expected darkness. Relevant to this discussion is the observation that bromocriptine, a D₂ agonist, was effective at eliciting photoreceptor elongation during expected darkness. Furthermore, dopamine receptor antagonists inhibited the effects of light in both expected light and expected darkness. At present, the data do not allow us to discriminate between the two postulated mechanisms.

The dopamine receptors involved in the regulation of photomechanical responses *in vitro* appear to be of the D₂ or D₄ type, as suggested by the relative effects of both agonists and antagonists. However, the concentrations of dopamine needed for an effect greatly exceeded those usually needed to activate D₂-type receptors. As in the case of melatonin (see above), this discrepancy may be explained by the susceptibility of dopamine to oxidation in solution. Previous studies showing effects of dopamine at lower concentrations (Pierce and Besharse, 1985; Dearth and Burnside, 1986) were carried out in the presence of ascorbic acid, an antioxidant that could not be used in our culture medium due to deleterious effects upon the cells. It is also noteworthy that Pierce and Besharse (1985) observed that high concentrations of dopamine (50 μ M) were necessary to elicit maximal responses in *Xenopus* eyecups. Additional evidence for the participation of D₂-type receptors in the chick retinal cultures is provided by the observation that dbcAMP counteracts the effects of both light and dopamine; the latter decreases cAMP accumulation when acting on D₂- or D₄-type receptors (Iuvone, 1986; Cohen et al., 1992). While this interpretation assumes that cyclic nucleotides act only as intracellular second messengers, it is noteworthy that a recent report suggested that cAMP may act also as an *intercellular* first messenger in the retina (Burnside and Garcia, 1992).

A "paracrine network" *in vitro*

The finding that dopamine antagonists and dbcAMP inhibited both the effects of dopamine and those of light is consistent with a convergence of dopamine and light at the level of the D₂

dopamine receptor or its signal transduction pathway. This hypothetical mechanism would require the presence of endogenous sources of dopamine in the cultures. Our investigation of this hypothetical mechanism can be summarized as follows: (1) dopamine D₂ receptor antagonists inhibit photoreceptor responses to light; (2) Western blot analysis shows the presence of TH-like immunoreactivity in extracts of the cultured cells; (3) TH-like immunoreactivity is less abundant in cultures treated with 6-OHDA, a neurotoxin specific for dopaminergic cells; (4) photomechanical responses to light are attenuated after treatment of the cultures with 6-OHDA or with AMPT, an inhibitor of TH activity; (5) nonphotoreceptor cell types (e.g., multipolar neurons and morphologically undifferentiated cells) accumulate ³H-dopamine under Ca²⁺-free conditions, in a Na⁺-dependent manner; (6) nomifensine, an inhibitor of the dopamine transporter, affects both the intracellular accumulation of dopamine and photoreceptor elongation in response to light; and (7) intracellular accumulation of ³H-dopamine can be regulated by light and by melatonin, at least in part through a reduction in dopamine release.

Taken together, these findings are consistent both with the presence of dopaminergic cells in the cultures, and with their involvement in the regulation of photomechanical responses *in vitro*. Moreover, the data suggest the development of a "paracrine network" in the cultures, whereby specific cell types communicate information regarding light conditions in the external environment, resulting in the coordination of complex responses such as photomechanical movements. A hypothetical model, consistent with results from the literature and our own data, is summarized in Figure 11. This working model proposes that light and melatonin have opposite effects upon the efflux of dopamine from nonphotoreceptor cells into the extracellular environment and that light, initially detected by the photoreceptor cell, also causes a decrease in melatonin synthesis and release. In the following paragraphs we will discuss the premises on which this model is based, including (1) that photoreceptors are the light-sensitive cells in the cultures; (2) that cultured photoreceptors are a source of melatonin, which can be regulated by dopamine and/or by light; (3) that cultured nonphotoreceptor neurons (and/or morphologically undifferentiated cells) are a source of dopamine, which can be regulated indirectly by light and/or directly by melatonin; and (4) that these cell types communicate with each other *in vitro* by regulating levels of melatonin and dopamine in their environment.

Photoreceptors as the light-sensitive cell in vitro. Cultured photoreceptors resemble their *in vivo* counterparts in many respects, including structural and molecular polarity, the accumulation of Na^+ , K^+ -ATPase in the inner segment plasma membrane, and the presence of an inner segment lipid droplet (a characteristic of cone photoreceptors and some embryonic photoreceptors). Of particular relevance to this discussion is the expression of opsin-immunoreactive materials (Adler, 1986; Madreperla and Adler, 1989; Madreperla et al., 1989; reviewed in Adler, 1993) which, however, would not be sufficient for phototransduction in the absence of the visual pigment chromophore 11-*cis* retinaldehyde (Hubbard and Wald, 1952). While retinyl acetate and retinol are present in the culture medium used for these experiments, 11-*cis* retinaldehyde is not (Stenkamp and Adler, unpublished observations). This raises questions regarding the source of chromophore in the cultures, which are grown in the absence of RPE. The RPE is considered the primary site for reisomerization of chromophore in adult bovine retina (Bernstein et al., 1987), but it is not clear whether this is also true for other species. Separate studies in our laboratory have shown that cultured neural retina cells can produce retinaldehyde isomers from retinol and retinyl acetate present in their culture medium (Stenkamp et al., 1993; Stenkamp and Adler, 1994). However, we have not been able to demonstrate the presence of 11-*cis* retinaldehyde among these isomers, perhaps due to the limits of sensitivity of the HPLC method used. Thus, the existence of light-sensitive photopigment in the cultures is likely, but has not yet been conclusively demonstrated. It is worth mentioning that cultured cells that clearly lack photoreceptor-type visual pigments have been shown to be sensitive to light *in vitro*, through still undetermined mechanisms (Giebul-towicz et al., 1989; Albrecht-Buehler, 1991).

Photoreceptors as a regulable source of melatonin in vitro. The photoreceptor cell type is a likely source of melatonin *in vivo* (reviewed by Dubocovich, 1988; Cahill and Besharse, 1991). The chicken retina has been shown to contain melatonin-synthesizing activities (Iuvone, 1990; Thomas and Iuvone, 1991) that can be regulated by light and cyclic nucleotides. The mRNA for hydroxyindole-*O*-methyltransferase, the terminal enzyme of the melatonin biosynthetic pathway, has been localized to photoreceptors in chicken retina (Wiechmann and Craft, 1993). The key regulatory enzyme NAT is already present in chick retinas at E6, and begins to be regulated by light by E20 (Iuvone, 1990). NAT activity is present in low-density chick retina cultures similar to the ones used here, is markedly higher in cultures enriched for photoreceptors, is downregulated by dopamine, with a D_2 -type receptor pharmacology, and is increased by treatments that cause increases in cAMP concentrations (Iuvone et al., 1990). K^+ -evoked depolarization, a treatment that we have now found to elicit dark-adaptive photomechanical movements *in vitro*, is likely to act directly on the photoreceptors since it is known to increase cAMP (Iuvone et al., 1991) as well as NAT activity in photoreceptor-enriched cultures (Avendano et al., 1990); the increase in melatonin release that could be presumed to result from these changes could be expected to inhibit any depolarization-induced release of dopamine. While these results are consistent with cultured photoreceptors being a regulable source of melatonin in our experiments, we have not been able to measure reliably possible changes in NAT activity in response to light in our cultures.

Nonphotoreceptor neurons as a regulable source of dopamine. Dopamine synthesis and release has been documented in the

retinae of several species (Kramer, 1971; Iuvone et al., 1978). The dopamine synthetic enzyme TH has been localized to subtypes of amacrine cells (Brecha et al., 1984) and/or to interplexiform cells (Savy et al., 1989). In developing chick retina, TH-immunoreactive cells are first seen on E11, are initially morphologically undifferentiated (Araki et al., 1982; Kagami et al., 1991), and assume their adult positions and morphology by E20. Evidence for the presence of dopaminergic cells in retinal cultures is compelling, but fragmentary. Positive evidence includes the presence of TH-like immunoreactivity in Western blots, its decrease in cultures treated with 6-OHDA, and the intracellular accumulation of ^3H -dopamine in an Na^+ -dependent, nomifensine-sensitive manner. Moreover, autoradiographic evidence suggests further that dopamine uptake is predominantly, if not exclusively, associated with nonphotoreceptor neurons and morphologically undifferentiated, process-free round cells.

Accumulation of dopamine into the cultured cells has been demonstrated not only with ^3H -dopamine, but also by HPLC analysis of cells incubated in the presence of "cold" dopamine (not shown). The radioactive experiments suggest the presence of an uptake system consisting of a high-affinity, low-capacity component and a low-affinity, high-capacity component, similar to those described in other neurons (Efthimiopoulos et al., 1991). Accumulation of ^3H -dopamine, on the other hand, was not easily measurable unless the incubations were carried out in Ca^{2+} -free buffers, suggesting the presence of a Ca^{2+} -dependent dopamine release mechanism similar to that present in the retina (Dubocovich, 1983; Boatright et al., 1989). The difference between intracellular radioactivity in the absence versus the presence of Ca^{2+} ions was therefore likely to reflect differences in Ca^{2+} -dependent release; that difference was increased by light (suggesting increased release), and significantly decreased by melatonin (suggesting decreased release). Although these are only indirect estimates of release (which, as explained in Results, was not examined with more direct methods due to the susceptibility of the cells to repeated washing and the low levels of radioactivity involved in the measurements), our results are consistent with known effects of light and melatonin on dopamine release *in vivo* (Kramer, 1971; Dubocovich, 1983; Boatright et al., 1989; Nowak et al., 1989). To our knowledge, however, a release mechanism via the dopamine transporter has not been investigated in the retina. This mechanism also appears to be regulated by light and melatonin in a manner similar to that observed with the Ca^{2+} -dependent releasable dopamine pool. It is unclear whether both mechanisms coexist in individual cells or are present in different cell types. The presence of distinct and separate releasable pools is suggested by the inability of the Ca^{2+} -dependent mechanism to compensate for the reduced release when the transporter mechanism is blocked by nomifensine.

While the above-mentioned results are consistent with the presence of cells with dopaminergic features, we were frustrated in our attempts to demonstrate the presence of TH-immunopositive cells in the cultures by immunocytochemistry; it is uncertain whether this is due to low antigen concentration in individual cells, and/or to technical problems. Reliable measurements of cellular dopamine or TH activity in the cultures were similarly difficult.

Intercellular communication regulates the response to light in vitro. Pharmacological evidence shows that perturbing the dopaminergic systems *in vitro* disrupts photomechanical responses to light/dark cycles, suggesting an important role for paracrine

communication in the regulation and/or establishment of these responses. To our knowledge, *in vitro* paracrine neuromodulatory systems of this nature have not been reported, although trophic interactions between two cell types in culture have been described (see Watanabe and Raff, 1992), indicating that communication "across the dish" in the absence of visible cell contacts is possible.

A currently active area of research is the investigation of the respective contributions of external cues and internal modulators in the regulation of rhythmic behaviors such as photomechanical movements (cf. Cahill and Besharse, 1993; Pierce et al., 1993). In the studies described here there is no apparent circadian regulation (but see above), and neuromodulators appear to be secondary to light as regulatory mechanisms. However, since disruption of at least one of these neuromodulatory systems leads to an absence of observed photomechanical responses, we can conclude that in this system, dopaminergic input may be necessary for the regulation and/or establishment of photomechanical responses.

In conclusion, these findings are consistent with the establishment of paracrine communication among cultured cells that are isolated from the retina prior to differentiation. The expression of complex activities such as photomechanical movement and neuromodulator uptake and release, which may be regulated in a coordinated manner, suggests that the differentiation programs for neural retina cells can be expressed in the absence of RPE and glia, in the absence of intraretinal signals, and in the absence of significant cell-cell contact. These differentiation "master programs," once set into action by undetermined mechanisms, may therefore proceed as cell-autonomous programs, with only the necessity for "permissive" cues such as dopamine or light for L+ photomechanical elongation.

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