This paper is here reprinted in its entirety because Fig. 2 in the original publication (pp. 2839–2843) and figure 5 in the preceding paper were interchanged during final assembly for printing.

D₁-type dopamine receptors inhibit growth cone motility in cultured retina neurons: Evidence that neurotransmitters act as morphogenic growth regulators in the developing central nervous system

(axon/dendrite/synapse)

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ABSTRACT Precedent exists for the early development and subsequent down-regulation of neurotransmitter receptor systems in the vertebrate central nervous system, but the function of such embryonic receptors has not been established. Here we show that stimulation of early-developing dopamine receptors in avian retina cells greatly inhibits the motility of neuronal growth cones. Neurons from embryonic chicken retinas were cultured in low-density monolayers, and their growth cones were observed with phase-contrast or video-enhanced-contrast-differential-interference-contrast (VEC-DIC) microscopy. Approximately 25% of the neurons responded to micromolar dopamine with a rapid reduction in filopodial activity followed by a flattening of growth cones and retraction of neurites. The response occurred at all ages examined (embryonic day-8 retinal neurons cultured on polylysine-coated coverslips for 1–7 days), although neurite retraction was greatest in younger cultures. Effects of dopamine on growth cone function could be reversed by haloperidol or (+)-SCH 23390, whereas forskolin elicited a response similar to dopamine; these data show the response was receptor-mediated, acting through a D₁-type system, and are consistent with the use of cAMP as a second messenger. The experiments provide strong support for the hypothesis that neurotransmitters, besides mediating transynaptic signaling in the adult, may have a role in neuronal differentiation as growth regulators.

Neuronal morphogenesis and the formation of appropriate synapses depend on the regulated growth of developing axons and dendrites. This growth is restricted to specialized regions, at the tips of neurites, called growth cones (1–3). In cell culture, neuronal growth cones are readily distinguished by their broad, fan-like appearance and their many motile filopodia (4–6). *In vivo*, they also exist as highly specialized structures (7, 8). Growth cones provide sites for new membrane addition (1, 9, 10) needed in neurite elongation, and they control the direction and extent of elongation (2, 3) through processes still poorly understood. Ultimately, growth cones become quiescent and disappear as final patterns of arborization are achieved.

Three types of extracellular cues have long been considered likely candidates for controlling neurite growth: (i) physical guidance (6, 11, 12), (ii) spatial/temporal gradients of diffusible (13–17) or bound (17–23) neurite growth-promoting molecules, and (iii) electric fields (24–27). The possibility has been raised that neurotransmitters also might play a role (28), as it has been reported that certain cultured

neurons isolated from the snail *Helisoma* stop neurite regeneration in response to serotonin or dopamine (29–31).

The current work addressed whether, in vertebrate central nervous systems, neurotransmitters might be used to control growth of axons and dendrites. This would be an important effect of molecules classically defined by their ability to mediate transynaptic signaling. For the snail experiments, detection of growth effects was aided by an ability to specifically identify individual cells, a distinct advantage of invertebrate systems. To approach the vertebrate central nervous system, we chose to examine the effects of dopamine on growth cones of cultured chicken retina neurons. Chicken retina neurons differentiate well in culture (32–34), and their growth cones have been studied in detail with respect to structure, behavior, and development (35–37). The cells express a robust dopamine-stimulated adenylate cyclase, which has been pharmacologically characterized. This activity differentiates *in ovo* several days before the appearance of synapses (38–40) and then shows extreme down-regulation as development proceeds (41). The transient nature of the response, which is mediated in part by an embryonic subtype of the D₁ receptor (40, 41), suggests the possibility of a developmental role.

The data presented here confirm and extend our preliminary observations that embryonic D₁ dopamine receptors mediate inhibition of growth cone motility and block neurite outgrowth in a subset of retina neurons (42). They strongly support the hypothesis that vertebrate central nervous system neurons use neurotransmitters as morphogenic signals.

METHODS

Measurement of Dopamine-Stimulated Adenylate Cyclase Activity. Freshly dissected retinas from White Leghorn chicken embryos and hatched chickens were preincubated for 10 min at 37°C in basal medium Eagle with 20 mM Hepes (pH 7.35) containing 0.1 mM ascorbate, 0.1 mM pargyline, and 0.5 mM 3-isobutyl-1-methylxanthine. Retinas were then incubated with dopamine (100 μ M), forskolin (40 μ M), or control medium. After 10 min, the reaction was stopped with trichloroacetic acid [final concentration 5% (wt/vol)], and materials were frozen and stored at –70°C for subsequent purification (43). cAMP levels were measured by the method of Gilman (44).

Morphological Responses. Embryonic day-8 retina neurons were cultured on poly(L-lysine)-coated glass coverslips for 1–7 days (E8C1–E8C7) (36). In the initial series of experiments, coverslips were inverted on top of a plexiglass and coverslip chamber, and cells were monitored with Zeiss phase optics. The image was converted to a video signal with

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FIG. 1. Dopamine receptors are strongly coupled to adenylate cyclase activity early in development, but the response decreases drastically after hatching. cAMP levels were assayed in freshly dissected retinas following a 10-min exposure to 1 μ M dopamine (●), 40 μ M forskolin (■), or control saline (▲) in the presence of 3-isobutyl-1-methylxanthine. Both dopamine- and forskolin-stimulated cAMP levels were significantly elevated above control levels throughout the embryonic period tested. Dopamine- and forskolin-stimulated adenylate cyclase activities doubled from embryonic day 8 to 13, plateaued for the next 5 days, and declined dramatically by 6 days after hatching. Control scale is on the left.

a Dage MTI (Michigan City, IN) video camera. In some of the later experiments, a Dvorak-Stoler chamber (Nicholson Precision Instruments, Gaithersburg, MD) was used; cells were monitored with a Nikon inverted rectified Nomarski microscope, and the video image was enhanced with a Quantex (Sunnyvale, CA) model DS-50 image processing system. Chamber temperature was maintained at a constant 37°C with a Sage Instruments (Boston) air-curtain incubator, and neurons were monitored for at least 15 min before testing to select for neurons with stable activity patterns.

Cells were photographed from videotaped images and presented as video micrographs or tracings.

Materials. SCH 23390 was obtained from Research Biochemicals (Wayland, MA). All other chemicals were obtained from Sigma. Solutions containing dopamine or apomorphine included ascorbate (1 μ M, final concentration) and were made fresh daily. Forskolin was dissolved in ethanol, and both forskolin and SCH 23390 stock solutions were stored at -70°C until the day of use.

RESULTS

Dopamine-Stimulated Adenylate Cyclase Activity. To determine the fraction of total adenylate cyclase activity that could be stimulated by dopamine in developing retina, we measured basal, forskolin-stimulated, and dopamine-stimulated cAMP levels in freshly dissected tissue (from embryonic day 8 to 6 days after hatching). Dopamine- and forskolin-stimulated adenylate cyclase activities doubled from embryonic day 8 to 13 and then plateaued (Fig. 1). At embryonic day 13, approximately the time of appearance of morphological synapses (45, 70), dopamine elicited a 10-fold increase in cAMP, equal to 13% of the total forskolin-stimulated activity. This fraction of total adenylate cyclase activity was sufficiently high to warrant screening individual cells for possible morphological responses to dopamine. Six days after hatching, there was a large decrease in total adenylate cyclase activity and in the adenylate cyclase that could be stimulated by dopamine.

Dopamine-Induced Morphological Responses. *Control behavior of growth cones.* All cells were examined in control medium before beginning experiments. Growth cones typically showed gradual changes in morphology, constant levels of filopodial activity, and steady rates of outgrowth. A small percentage (<5%) exhibited cyclic patterns of high and low activity and were not investigated further. Levels of activity varied slightly between neurons of the same age, but numbers of motile filopodia, total activity, and rates of neurite outgrowth all tended to decrease with increasing age. Exchange of control medium typically had no effect on the

FIG. 2. Phase-contrast micrographs and tracings of an E8C1 retina neuron showing neurite retraction response to 1 μ M dopamine. (A) Cell immediately before dopamine addition. (C) Same cell 10 min after dopamine addition. (B and D) Tracings of A and C.

FIG. 3. Video-enhanced-contrast-differential-interference-contrast micrographs of dopamine-induced neurite retraction and recovery. (Left) Cell immediately before dopamine addition. (Center) Cell 10 min after dopamine addition. (Right) Cell 10 min after return to control medium.

cells, although slight, transient (<1 min) increases in filopodial activity were occasionally noted.

Response to dopamine. A subset of neurons at each age tested (E8C1 to E8C7) showed an inhibition of filopodial activity, followed by a flattening and retraction of growth cones in response to dopamine. As illustrated with phase-contrast optics in Fig. 2, overall length could be significantly reduced within minutes after dopamine addition. Retraction was typically maximal by 10 min, with little change in length observed over the next 30 min. The rate and extent of retraction decreased with increasing culture age. At E8C1, neurite lengths (cell body to front of growth cone) often were reduced by $<50\%$; at E8C7, only slight reductions in neurite length were observed, although filopodial activity was inhibited.

Video-enhanced-contrast-differential-interference-contrast microscopy markedly improved visualization of morphological details (Fig. 3). Dopamine-sensitive neurons had small cell bodies and were monopolar or bipolar. Their neurites were mostly unbranched with narrow growth cones and short thin filopodia. No responses were seen in multipolar neurons with highly branched processes or in neurons with long neurites and large well-defined growth cones (Fig. 4). Approximately 25% of the neurons in a randomly selected population retracted neurites in response to dopamine.

Spontaneous recovery of growth was not observed in cells monitored for up to 1 hr in the continuous presence of dopamine. Removal of dopamine permitted recovery (Fig. 3), but not if exposure to dopamine had exceeded 15 min. Complete recovery from a 10-min dopamine exposure typically required 30 min. Neurite retraction and recovery could be induced at least three times in the same cell, but the response decreased with each trial.

Inhibition of dopamine effects by antagonists. Two antagonists were used to verify that the morphological responses to dopamine were receptor mediated and to characterize the receptor subtype involved: haloperidol, which blocks both D_1 and D_2 receptors, and (+)-SCH 23390, which blocks only D_1 receptors (46–48). Both $1 \mu\text{M}$ haloperidol and 25 nM (+)-SCH 23390 blocked the effects of dopamine on cells that subsequently showed a neurite retraction response to dopamine alone. Furthermore, reversal of neurite retraction and inhibition of motility could be observed 10 min after adding either haloperidol or (+)-SCH 23390 in the continuous presence of dopamine (Figs. 5 and 6). Recovery induced by either substance decreased with increasing dopamine exposure times. (–)-SCH 23390, an inactive stereoisomer of the D_1 antagonist, had no effect. These results establish that the morphological response to dopamine was mediated by D_1 -type receptors.

Mimicking of dopamine effects by forskolin. D_1 -type dopamine receptors are known to activate adenylate cyclase,

suggesting a possible role for cAMP in mediating the morphological response. This possibility was further supported by the morphological response to forskolin, which stimulates adenylate cyclase directly. As with dopamine, forskolin elicited an inhibition of filopodial activity and a flattening and retraction of neurites in sensitive neurons (Fig. 6). The rate and extent of neurite retraction, qualitatively dose dependent with $5\text{--}40 \mu\text{M}$ forskolin, appeared somewhat reduced compared to the dopamine response. Recovery from forskolin was typically more rapid than recovery from dopamine and could be induced after longer stimulations (20 min).

Although forskolin should stimulate adenylate cyclase activity in all cells, only 25% of the cells responded to it. To test for a possible correlation of dopamine and forskolin sensitivities, we tested 21 E8C1–E8C7 neurons with both dopamine and forskolin. Of these, 8 cells exhibited a neurite-retraction response to both, 13 cells responded to neither, and no cells responded to only one substance. Forskolin-induced neurite retraction thus occurred only in cells that also were sensitive to dopamine.

DISCUSSION

Data presented here show that stimulation of D_1 dopamine receptors inhibits growth cone motility in a subset of chicken retina neurons, blocking both filopodial activity and neurite outgrowth. The results strongly support the hypothesis that neurotransmitters act as growth regulators in the developing vertebrate central nervous system.

FIG. 4. Video-enhanced-contrast-differential-interference-contrast micrograph of dopamine nonresponsive neuron. (Left) Cell immediately before dopamine addition. (Right) Cell 10 min after dopamine addition.

FIG. 5. Forskolin mimics the effects of dopamine. Tracing of an E8C1 neuron exposed to forskolin (Forsk) and dopamine (DA). Vertical columns illustrate the same cell at successive time points ≈ 1 min apart. Numbers indicate the time in minutes after the beginning of the experiment. Asterisks indicate that conditions were changed immediately after this time point. (A) Neuron in control medium with ethanol vehicle. (B) First 3 min after the addition of $40 \mu\text{M}$ forskolin. (C) After forskolin stimulation. (D) After return to ethanol control medium. (E) In plain control medium. (F) First 3 min after the addition of $1 \mu\text{M}$ dopamine. (G) After dopamine stimulation. (H) After the addition of dopamine plus haloperidol (H).

Previous reports have established that functional D_1 dopamine receptor systems develop early in the chicken retina. Dopamine stimulation of adenylate cyclase activity can be detected as early as embryonic day 7 (nearly 1 week before the appearance of morphological synapses) and maximal dopamine stimulated activation of adenylate cyclase is observed by embryonic day 14 (Fig. 1 and ref. 38). Dopamine itself can be detected in amacrine cells by immunofluorescence by embryonic day 14 (49). Furthermore, a spontaneous rise in basal cAMP levels on embryonic day 15, which can be blocked by dopamine antagonists, indicates that significant quantities of dopamine are released by this age *in ovo* (50). Effects of dopamine on growth cone motility were observed throughout this early developmental period.

Although results reported here show that dopamine can inhibit growth cone motility and data from the literature indicate that both dopamine and dopamine receptors are present early in development, whether and how this response might be used *in vivo* remains to be determined. Several developmental functions could be subserved. First, a signal that inhibits growth cone motility could facilitate synapse formation by stabilizing cell-cell contacts. We have proposed (37) that adhesive contacts between filopodia may constitute an early event in the formation of synapses, and quieting

filopodial activity may be important in stabilizing such nascent junctions. Dopamine inhibition of neurite outgrowth also could be used to restrict neurite outgrowth, either limiting total arborization or preventing outgrowth into specific inappropriate regions. This possibility may be testable in the future by exposing embryos to dopamine antagonists and examining the arborization pattern of neurons labeled with antibodies to DARPP-32, a specific marker for neurons with dopamine receptors (51, 52).

The present work was done with embryonic cells to assess possible developmental roles for the early-maturing dopamine receptors. If the mechanisms that couple receptors to morphological changes in growth cones are preserved as the central nervous system matures, they also could function as mediators of structural plasticity in the adult. We have not yet tested whether neurons from the adult avian central nervous system respond morphologically to neurotransmitters. However, reports from two laboratories have shown that neurotransmitters can alter the morphology of adult neurons in other systems. Dopamine and serotonin inhibit neurite elongation of snail neurons regenerating in culture (29–31), and dopamine, acting through a D_2 receptor, stimulates retraction of photoreceptors in teleost fish (53).

The elevation of cAMP levels by dopamine and the ability

FIG. 6. Effects of dopamine are reversed by haloperidol or (+)-SCH 23390. Tracings of E8C1 neurons showing dopamine-induced neurite retraction, inhibition of filopodial activity, and recovery in the presence of antagonists. Vertical columns illustrate the same cell at successive time points 30 sec apart. Numbers indicate time in minutes after the beginning of the experiment. Asterisks indicate that conditions were changed immediately after this time point. Three cells in close contact, with cell bodies indicated by white regions. Neurites shorten and cell bodies move closer together when antagonist is removed. From left to right: cells in control medium with $1 \mu\text{M}$ dopamine (DA) and $1 \mu\text{M}$ haloperidol (H) (A), after the addition of dopamine alone (B), and after return to dopamine plus haloperidol medium (C). (D) Neuron in control medium with ascorbate. (E) After the addition of $1 \mu\text{M}$ dopamine plus 25 nM of the inactive (-)-SCH 23390 [(-)SCH]. (F) After the addition of dopamine plus 25 nM (+)-SCH 23390 [(+)SCH].

of the adenylate cyclase activator forskolin to mimic the effects of dopamine on growth motility and neurite outgrowth strongly imply that elevation of cAMP is involved in this response. Since forskolin maximally elevates adenylate cyclase in all cells (54), the observation that only dopamine-responsive neurons responded morphologically to forskolin suggests that cAMP acts through specific target molecules that are present only in neurons with dopamine receptors, such as DARPP-32, a substrate for cAMP-dependent protein kinase A (51, 52). A likely mechanism for control of morphogenesis may be modification of assembly regulating components of the cytoskeleton. Both globular actin (55) and microtubule-associated protein (55–57) are known to be phosphorylated by a cAMP-dependent protein kinase in a manner that inhibits polymerization of actin fibers and microtubules, respectively. Actin-myosin interactions are thought to form the basis of filopodial movements (58), and microtubule assembly is necessary for elongation and stabilization of neurites (59). Another possible mode of action might be control of calcium levels. cAMP influences Ca^{2+} channels (60) and Ca^{2+} mobilization (61, 62), and Ca^{2+} has been implicated as an important regulator of neurite outgrowth (63–68). Regulation of contractile events in cardiac muscle by cAMP-dependent phosphorylation of phospholamban, and subsequent increases in calcium transport (69) may serve as a model for events mediating neurite retraction.

Although only a subset of neurons in the retina responded to dopamine, others may respond to different neurotransmitters. Preliminary work indicates that nicotinic receptors may mediate a response similar to dopamine, but in a different morphological class of retina neurons (M. I. Fonseca and W.L.K., unpublished data). The current data thus strongly support the hypothesis that neurotransmitters act as regulators of neuronal morphogenesis in the vertebrate central nervous system. Molecules, classically characterized as mediators of synaptic transmission, thus may play an additional role in regulating critical stages of neural differentiation.

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