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# Interocular effect of intravitreal injection of 6-hydroxydopamine and dopamine on spinule formation in teleost retina

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**Summary.** Terminal dendrites of cone horizontal cells (HCs) in teleost retinas show numerous spine-like protrusions named spinules, which are invaginated into the cone pedicles during light-adaptation, but retracted during dark-adaptation. Somata of HC show nematosomes whose size decreases as the number of spinules increases. Mechanisms regulating these changes in nematosomes and spinules are only partially understood, being an area of controversy in retinal cell biology. It has been suggested that efferent fibres from the brain to the retina might be involved in the control of spinule formation. Moreover, we have reported that actin depolymerization has an interocular effect on spinule formation, which could be mediated by these fibres. In the present report, we show an interocular effect on spinule dynamics: the monocular intravitreal injection of dopamine (DA) and 6-hydroxydopamine (6-OHDA), two drugs that affect the spinule formation, produces the same effects in the contralateral, untreated eye as in the injected eye. Our results reinforce the idea of an interocular central control of this phenomenon of synaptic plasticity. Dopamine-dependent events in the retina appear to be necessary to forge the afferent signals eliciting this interocular effect.

**Key words:** Spinules, Nematosomes, Dopamine, 6-hydroxidopamine (6-OHDA), Retinal efferent fibers

## Introduction

In teleosts, the synaptic connections between photoreceptors and cone horizontal cells (HCs) undergo a morphological re-arrangement, depending on the state of ambient illumination. During light-adaptation, the terminal dendrites of HCs extend neurites named spinules into the central cavity of the cone pedicle; these spinules disappear during dark-adaptation (Wagner, 1980). The spinules are typically 0.5 $\mu$m in length and about 0.1 $\mu$m in width and are characterized by electron-dense submembrane densities lining their tips. The formation and disappearance of spinules occurs within 45 min respectively. The feedback signal from HCs to cones varies with the ambient light conditions. Although the mechanism for these changes is poorly characterized, in the fish dark-adapted retina the HCs lose their colour opponency parallely to the spinule retraction. These data suggest that spinules may be the sites of feedback transmission from HCs to the cone pedicles (Raynauld et al., 1979; Weiler and Wagner, 1984; Djamgoz et al., 1985; Kirsch et al., 1990, 1991; Kamermans et al., 2001).

Related to spinules, the external horizontal cells have cytoplasmic inclusions termed nematosomes, whose number and size vary inversely with the number of spinules (De Juan et al., 1991). Specifically, nematosomes are larger and more numerous in dark-adapted retinas than in light-adapted ones. This suggests that electron-dense material observed in the spinules may originate from nematosomes and vice-versa (De Juan et al., 1991, 1996; De Juan and García, 1998, 2001).

Numerous neurotransmitters and neuromodulators have been implied in inducing formation or retraction of spinules (Douglas and Wagner, 1983; Weiler et al., 1988, 1990, 1991, 1998; Wagner et al., 1992; Weiler and Schultz, 1993; Behrens et al., 1993, 2000; Haamedi et al., 2001). For instance, the neuromodulator dopamine, released by the interplexiform cells, can induce spinule (Weiler et al., 1988; Khöler and Weiler, 1990; Kirsch et al., 1991; Wagner et al., 1992; Yazulla and Studholme, 1995; Yazulla et al., 1996). The role of dopamine in spinule formation has been studied with several experimental approaches. One approach was

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pharmacological using the application of dopamine and its agonist (Weiler et al., 1988; Khöler and Weiler, 1990; Kirsch et al., 1991). Another was neurotoxicological, using the depletion of retinal dopamine by selective destruction of interplexiform dopaminergic cells (DA-IPCs) by 6-hydroxydopamine (6-OHDA) (Khöler and Weiler, 1990; Kirsch et al., 1991; Wagner et al., 1992; Yazulla and Studholme, 1995; Yazulla et al., 1996).

In addition to these intrinsic retinal mechanisms, efferent fibers from CNS projecting to the retina might be involved in the control of the physiology of HCs and their spinules (Stell et al., 1984, 1987; Walker and Stell, 1986; Umino and Dowling, 1991). This idea of such central control is supported by five lines of evidence: 1) In teleost retinas, DA-IPCs, which make synapses on horizontal cells, receive inputs from centrifugal fibers originating in the olfactory bulb (Zucker and Dowling, 1987). 2) These fibers contain the neuropeptide GnRH, which mimics the effects of dopamine on horizontal cells, and FMRF-amide-like peptides that suppress the effects of GnRH on horizontal cells (Umino and Dowling, 1991). Moreover, it has been reported that GnRH induces light-adaptive spinule formation through the dopaminergic interplexiform cells (Behrens et al., 1993). 3) Spinule formation is subject to a circadian rhythm, which might be influenced by centrifugal fibers (Douglas and Wagner, 1983; Wagner et al., 1992). 4) Optic nerve integrity is necessary to yield spinule formation/disruption (De Juan et al., 1996). (A related aspect of the physiology of horizontal cells is that interruption of the optic nerve influences the density of gap junctions on these cells (Kurz-Isler, 1992)). 5) Finally, we have reported a direct interocular effect on spinule formation (De Juan and García, 1998). This effect is not entirely surprising, since the efferent fibers from the terminal nerve project bilaterally to both retinas (Stell et al., 1987; Repérant et al., 1989).

Here, we ask whether the afferent signals eliciting this interocular effect require dopamine-dependent events in the retina. The alternative is that non-dopaminergic processes are sufficient for the retinal signals of ambient illumination relevant for spinule modulation. In the present report, we discriminate between these alternatives by investigating a possible interocular effect on cone HC spinule formation and changes in nematosomes through monocular intravitreal injection of dopamine and depletion of dopamine using 6-OHDA.

## Materials and methods

The experiments were performed with the teleost black bass (*Micropterus salmoides*), with specimens a body length of 12-15 cm. The fish were kept in an aquarium under a 12h:12h light-dark cycle for at least 4 weeks prior to use. All experiments were carried out at noon to equalize circadian effects on spinule formation (Douglas and Wagner, 1983; Wagner et al., 1992). Two fish were light-adapted for 1 h and two other fish were dark-adapted for 1 h. They were used as controls of experimental animals to study both the normal spinule dynamics and changes in nematosome size. Three groups of experimental animals were used to test the effects of addition and depletion of dopamine as described next.

### Intraocular injections

The experimental drugs were injected intraocularly in a volume of 10 $\mu$l under anaesthesia with MS222 (Sigma, Saint Louis Missouri). Injection of drugs and sacrifice of dark-adapted animals was performed under dim red light. Three groups of animals were used for injection:

Group 1

To destroy dopamine-containing cells, 6-OHDA was injected intraocularly in one group of fish according to Dowling and Ehinger (1978). Six animals were injected on 2 consecutive days intravitreally in the right eye with 20 $\mu$g 6-OHDA (Sigma) and 10 $\mu$g pargyline (Sigma) dissolved in 10$\mu$l 0.9% NaCl solution containing 0.1 mM ascorbate. The left eye was injected with vehicle alone. Ten to fourteen days after the second injection, three fish were adapted to light and three fish were adapted to dark for 60 min and then sacrificed. These adaptations permited spinule formation or retraction respectively.

Group 2

To test the effect of exogenous dopamine, three fish were adapted to light and three fish adapted to dark for 2 h. After the adaptation, the fishes right eyes were given an intraocular injection of 10 $\mu$l dopamine 1mM (Sigma) dissolved in 10 $\mu$l 0.9% NaCl solution containing 0.1 mM ascorbate. Assuming a dilution of the injected dopamine by a factor of 20 in accordance with the size of the eyeball (Dearry and Burnside, 1986), the concentration at the retina was estimated as 50 $\mu$M. We used a concentration of 50 $\mu$M because it is in the range used by several authors in previous reports on spinule formation (Weiler et al., 1988; Khöler and Weiler, 1990). The left eye was injected with vehicle alone. After 60 min in the same light conditions to allow the possible effects on spinule dynamics induced by dopamine in the right eye, the fish were sacrificed.

Group 3

Finally, we had an additional control group. It addressed the possibility that the injection of vehicle alone could have caused the same effect as the drugs on spinule formation and changes on nematosome size. To address this possibility, six fish were treated as in Group 1 and six fish were treated as in Group 2. The difference was that the right eyes were injected intravitreally with

vehicle and the left eyes were not treated.

### Histological procedures

#### Preparation of retinas

After the application of drugs, the fish were killed by decapitation, and the eyes were enucleated and hemisected. The eyecups of fish from the control group and Group 1 were cut in half along the dorsal-ventral axis. One half was processed for electron microscopy, and the other for tyrosine-hydroxylase immunocytochemistry (TOH-IR) (described below). The retinas of fish from Groups 2 and 3 were only processed for electron microscopy.

#### Electron microscopy

The fish retinas were removed and immersed in the fixative (1% paraformaldehyde, 1,6% glutaraldehyde, 0.15 mM $CaCl_2$ in 0.1 M phosphate buffer, pH 7.4) for 2 h and then at 4 °C overnight. After fixation, the retinas were postfixed with 2% $OsO_4$ for 1 h, dehydrated in ethanol, and embedded in Epon-812 (Polysciences, Warrington, Pennsylvania). Thin vertical sections of the retinas were stained, examined, and photographed with a Zeiss EM10 (Zeiss, Oberkochen, Germany), and electron micrographs of cone pedicles and nematosomes were printed. Spinules were counted in fifty cone pedicles per retina and nematosome diameter was calculated for fifty nematosomes per retina. Statistical differences were checked by one-tailed Student t-tests. All quantitative data are expressed as means ± standard errors.

### Immunocytochemistry

Depletion of retinal dopaminergic cells was verified by demonstrating the loss of TOH-IR interplexiform cells. Three dark-adapted half retinas from different fish were processed. The retinas were fixed 1 h in 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at room temperature. Later they were postfixed overnight in 4% paraformaldehyde in 0.1 M sodium bicarbonate buffer (pH 10.4). After fixation, they were washed in 0.1 M sodium phosphate buffer, and then cryoprotected, and subjected to freezing and 1% borohydride treatment (Eldred et al., 1983). Next, the retinas were incubated in 10% normal goat serum (Vector, Burlingame, California) in 0.1 M sodium phosphate buffer containing 0.5% Triton X-100 for 1 hour at room temperature. This was followed by an incubation in the primary anti serum (rabbit-anti-TOH; Chemicon, Temecula, California) at dilution 1:1000 in 0.1M sodium phosphate buffer with 0.5% Triton X-100, for 4 days, at 4 °C. Subsequently, retinas were rinsed and incubated in the secondary antiserum, biotinylated goat anti-rabbit IgG (Vector), diluted 1:100 in sodium phosphate buffer for 2 days at 4 °C. The retinas were then rinsed in sodium phosphate buffer and transferred to a solution of ABC (Avidin Biotin Complex; Vector) in 0.1 M sodium phosphate buffer for 2 days at 4 °C. The antibody/antigen complex was visualized with the diamino-benzidine (DAB, Sigma) reaction. Internal control retinas were treated identically omitting the primary antiserum. Both control and experimental retinas were flat-mounted, coverslipped and observed in a Zeiss light microscope. Some wholemount retinas were dehydrated, embedded in Epon 812, and 10 $\mu$m sections, cut parallel to the cone´s long axis, were examined in a light microscope.

## Results

### Degeneration of interplexiform cells after treatment with 6-OHDA

The effectiveness of 6-OHDA lesioning was confirmed by the loss of TOH-IR in 6-OHDA-treated black-bass retinas. Whereas normal TOH-IR neurons were observed in control and untreated retinas (Fig. 1A) these neurons were absent from retinas of 6-OHDA-injected eyes (Fig. 1B) in agreement with previous reports (Yazulla and Studholme, 1995; Yazulla et al., 1996).

### Effects of light and dark adaptation

After 1 hour of light adaptation, control retinas were abundant with spinules per pedicle (Fig. 2A) and had small nematosomes (Fig. 3A). In contrast, dark-adapted retinas showed scanty spinules (Fig. 2B) and large nematosomes (Fig. 3B).

### Effects of 6-OHDA treatment

6-OHDA caused a significant reduction (t-test: $p<0.001$) in the density of spinules not only in light-adapted retinas (Figs. 2D, 4A) but also in contralateral, untreated retinas (Figs. 2C, 4A). However, 6-OHDA did not produce changes in nematosome size during light-adaptation, since nematosome size was similar in control and 6-OHDA-injected light-adapted retina (Fig. 4B). In dark-adapted retinas, 6-OHDA did not prevent either spinule retraction or increase in nematosome size. Under 6-OHDA, both the density of spinules and nematosome size were similar to those in control dark-adapted retinas. (Fig. 4A,B). The results of vehicle injection (Group 3) make it unlikely that damage to the eye or the injection alone caused the effects on spinule formation. This is because the lack of effects of vehicle injection was similar to that in control uninjected animals (Fig. 4A,B).

### Effects of injection of dopamine

As we expected, intraocular injection of dopamine into the right eye of light-adapted fish, did not modify

the normal effect of light on spinule formation. It was indistinguishable from spinule formation in control eyes adapted to light (Fig. 5A). In contrast, injection of dopamine into the right eye of dark-adapted fish caused an effect on spinule formation. Spinules now appeared in darkness, although their number was slightly lower than that induced by light (Fig. 2E, 5A). Furthermore, the contralateral untreated eye (vehicle-injected eye) showed a surprising increase in the density of spinules (Fig. 2F, 5A). This density was greater than in control dark-adapted retinas and the difference was statistically significant (t-test: $p<0.001$), although the density was lower than in the eye treated with dopamine (Fig. 5A). On the other hand, as we expected, nematosomes in the dopamine-injected eyes adapted to light remained as in control eyes. However, nematosomes in the dopamine-injected eyes adapted to darkness, showed a reduction in size not statistically significant, which became closer to the size in light-adapted eyes (Fig. 5B). The size of the nematosome in the contralateral untreated eye remained close to that in dark-adaptation and the difference was not statistically significant. Again, the results of vehicle injection (Group 3) were similar to those in the control animals (Fig. 5), showing that injection alone did not have an effect on spinule formation.

## Discussion

### Interocular effect in the spinule formation

The most interesting aspect of our results is that intraocular injections of 6-OHDA and dopamine into an eye produced similar densities of spinules and sizes of nematosomes in both the injected and the contralateral untreated eyes.

To explain this interocular phenomenon, we can consider three possibilities (De Juan and García, 1998, 2001): First, a nervous interaction between both eyes could be mediated by the centrifugal fibers. These fibers originate from cells in the olfactory bulb and comprise the terminal nerve. This nerve projects to the ipsilateral and contralateral retinas upon the dopaminergic cells (Stell et al., 1987; Zucker and Dowling, 1987; Réperant et al., 1989) and other targets in the retina (Stell et al., 1984, 1987). Second, as Negishi et al. (1991), have suggested, we cannot discard that the intraocular injection of these drugs evokes the synthesis or release of a humoral factor. This factor when released into the circulatory system, can perhaps produce the same effects in the untreated retina as in the treated one. For instance, the factor could activate superior visual pathways, which actuate through centrifugal fibers to the contralateral retina. Third, dopamine and 6-OHDA may be transported by the blood and cause, in a humoral way, the same effects on spinule formation in the contralateral retina as in the treated one. Our data suggest that this is unlikely. Whereas the 6-OHDA-treated eye shows a loss of TOH-IR, the contralateral untreated-eye is not directly affected by the drug, because TOH-IR is present in this eye.

In addition to an interocular effect in spinule formation, the influence of one eye on the contralateral eye has been shown in other physiological aspects of the retina. Owusu-Yaw et al. (1992) have reported that the removal of one eye or a monocular optic nerve lesion produces changes in rod precursor proliferation in the

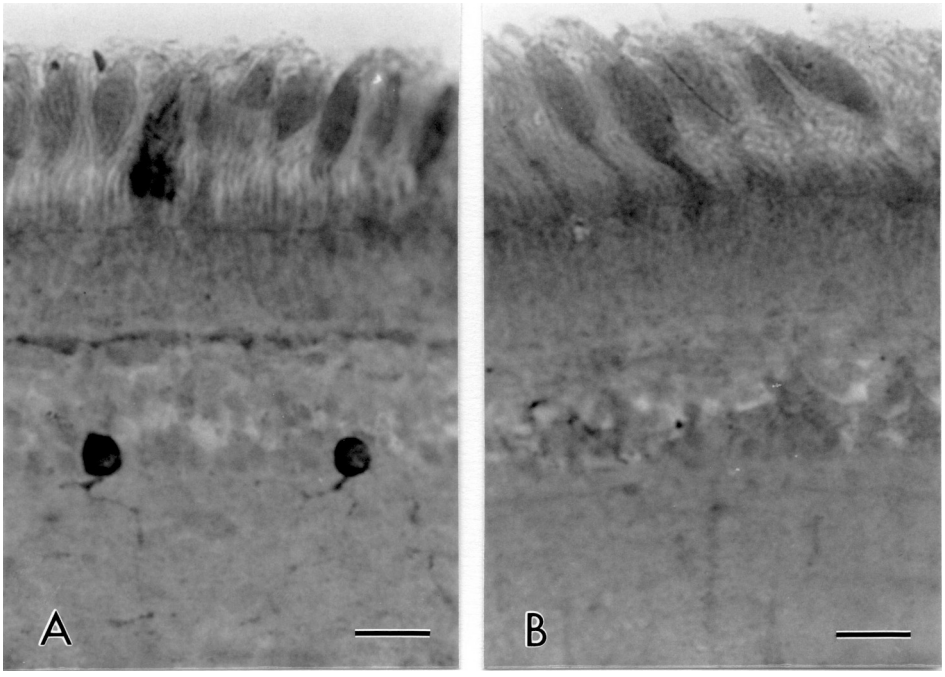


**Fig. 1.** Light-micrographs of black-bass retinas processed for TOH-IR. **A.** Control and untreated retinas show the somas of DA-IPCs and their processes in both plexiform layers. **B.** In retinas injected with 6-OHDA 10-14 days prior to the histological procedures, the immunoreactivity is absent. Bar: 20 $\mu$m.

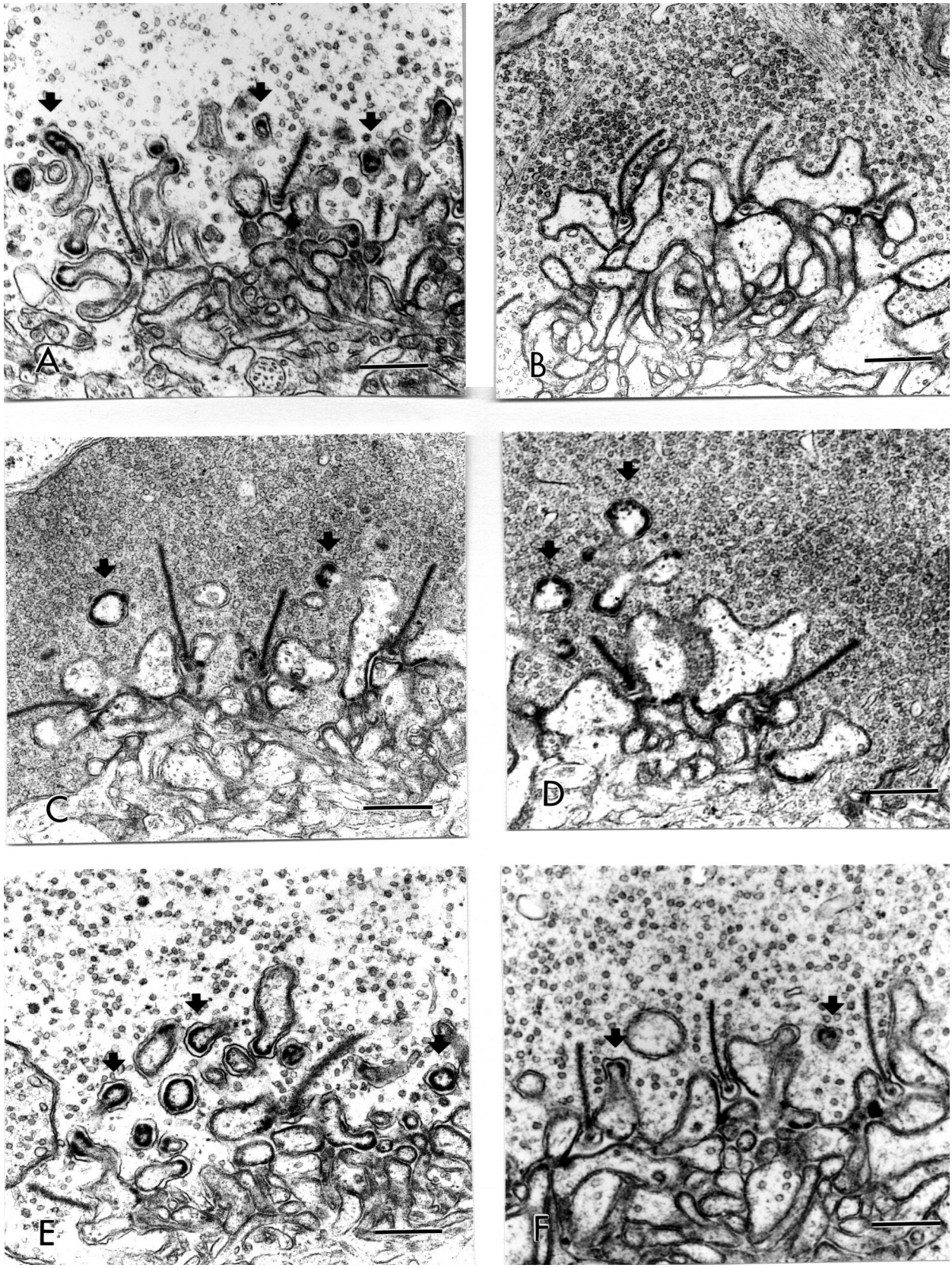


**Fig. 2.** Electron micrographs from vertical sections of cone pedicles in control **(A, B)**, 6-OHDA-treated **(C, D)**, and dopamine-treated **(E, F)** fish. **A.** Light-adapted retinas **(A)** show numerous spinules (arrows). In dark-adapted retinas **(B)**, no spinules are present. 6-OHDA-untreated light-adapted retinas **(C)** have a reduced number of spinules (arrows), similar to the contralateral 6-OHDA-treated ones **(D)**. In dopamine-treated dark-adapted retinas **(E)**, numerous spinules are formed (arrows). In untreated contralateral dark-adapted retina **(F)**, a few spinules are present. Bar: 0.5 $\mu$m.

remaining eye. Similarly, Negishi et al. (1991), have shown that the treatment of one retina with 6-OHDA or tunicamycin produces a slow increase of rod precursor proliferation in both retinas. Bodeustch et al. (1999) have reported that the unilateral injury to the adult rat optic nerve causes multiple cellular responses in the contralateral site. In addition, a variety of consensual retinal effects have been described in humans. In particular, changes in sensitivity of the dark-adapted retina occur during light-adaptation of the other eye (Denny et al., 1991; Auerbach et al., 1992), and the unilateral ocular vascular stress produces a supranormal oscillatory potential index in the contralateral eye (Kergoat and Lovasik, 1994). As has been proposed (Schütte, 1995), these interocular effects may be essential for matching the inputs from both eyes to higher visual centers. We propose a similar explanation for the interocular effect in spinule formation. Such interocular effects are a reminder that for many experimental manipulations of an eye, the fellow eye is not a control.

### Dopamine as co-regulator of spinule formation

If retinal dopamine is necessary for binocular spinule formation, then one must address the literature's controversy on the role of dopamine (Wagner and Djamgoz, 1993, 1994; Baldridge and Ball, 1994; Weiler, 1994; De Juan et al., 1996). It has been reported that spinule formation through light adaptation is mimicked by exogenously-applied dopamine (Weiler et al., 1988; Kohler and Weiler, 1990), partially inhibited in 6-OHDA-lesioned retinas (Kohler and Weiler, 1990; Kirsch et al., 1991; Wagner et al., 1992), and inhibited by dopamine D1 antagonists (Kirsch et al., 1991). These data suggest that dopamine released by interplexiform cells in teleost fish induces light-dependent formation of spinules (Weiler et al., 1988; Kohler and Weiler, 1990; Kirsch et al., 1991; Wagner et al., 1992). In this paper, we have confirmed that dopamine injected intravitreally into an eye in the dark induces the formation of spinules. These results suggest an essential role for dopamine in the induction of spinules. However, in agreement with results reported earlier (Kohler and Weiler, 1990; Kirsch et al., 1991; Wagner et al., 1992; Yazulla and Studholme, 1995; Yazulla et al., 1996), depletion of retinal dopamine with intraocular injections of 6-OHDA produced only a partial (albeit significant) reduction of spinule formation. These results suggest that dopamine is not the only signal for the appearance of spinules in the light (Kohler and Weiler, 1990; De Juan et al., 1990, 1996; Yazulla and Studholme, 1995). In support, dopamine injected into an eye previously depleted of dopaminergic cells could not form spinules (Kohler and Weiler, 1990). Hence, an additional regulator probably exists, which together with dopamine induces spinule formation. Is there a negative correlation between the nematosome´s size and the formation of spinules?

In previous reports, we showed an inverse relationship between the incidence of electron-dense material in spinules and the size of nematosomes (De Juan et al., 1991, 1996, 1998). We suggested that the electron-dense material lining the spinules originates in nematosomes. The results obtained here with exogenous dopamine are in agreement with this spinule-nematosome hypothesis. Injection of dopamine in the dark produces an increase in the density of spinules and a reduction in the nematosome diameters. However, our results also showed that although the retinas treated with 6-OHDA present a reduction in the density of spinules,

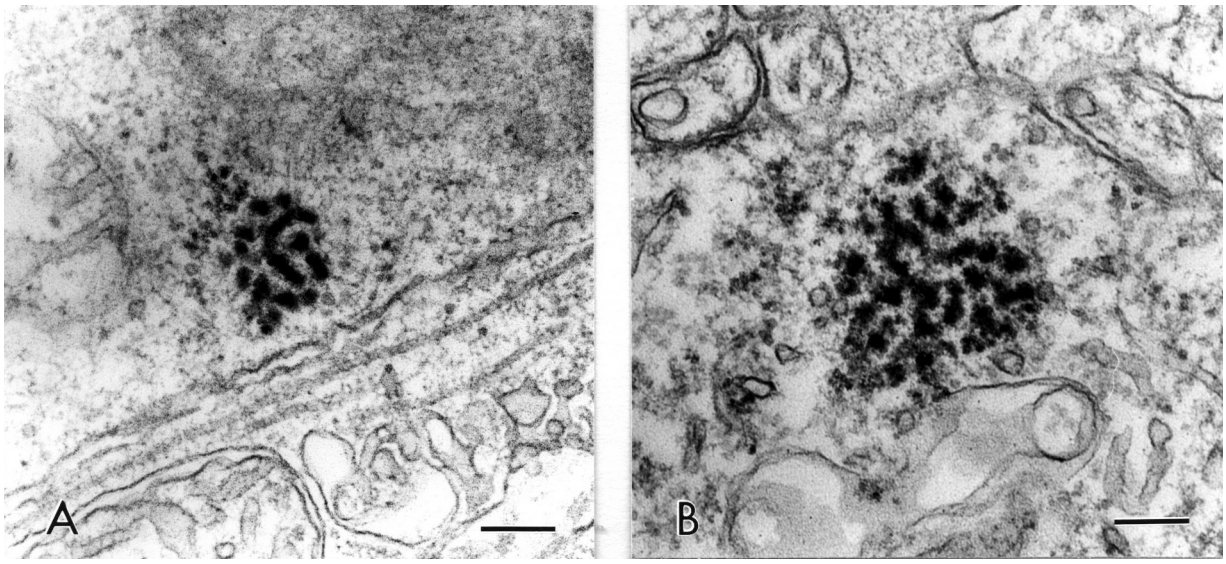


**Fig. 3.** Electron-micrographs of nematosomes in horizontal cells. Nematosomes in the light-adapted retina **(A)** are smaller than those in the dark-adapted retina **(B)**. Bar: 0.2 $\mu$m.

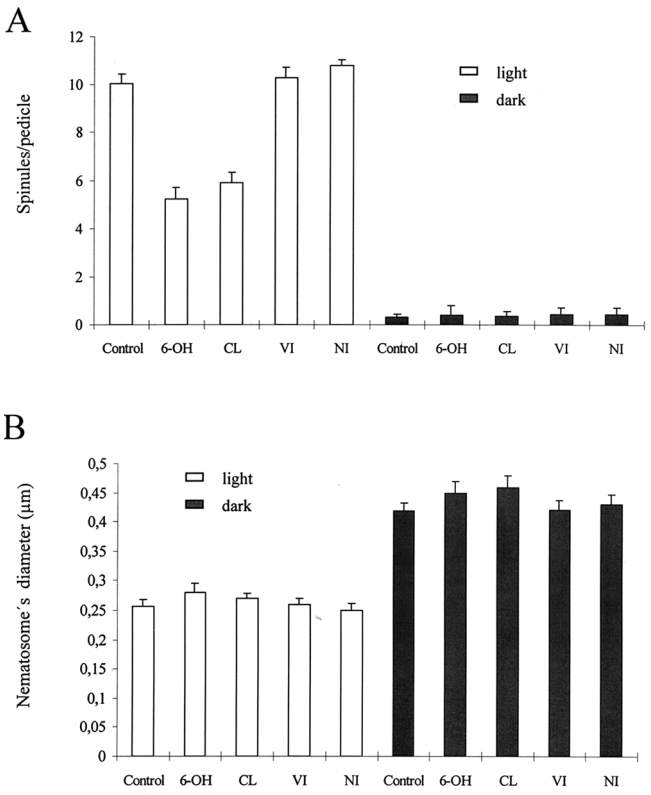


**Fig. 4.A.** Histograms summarizing the effects of monocular intravitreal injection of 6-OHDA on the average number of spinules per cone pedicle during light and dark-adaptation. Control: untreated eyes from animals light- or dark-adapted for one hour to study normal spinule dynamics. Following the injection of 6-OHDA into the right eye, the number of spinules per pedicle is reduced in both the injected eye (6-OH) and the contralateral eye (CL) during light-adaptation. However, 6-OHDA injection does not prevent the retraction of spinules during dark adaptation. The results for vehicle-injected (VI) eyes and non-injected (NI) fellow eyes are similar to those for the control animals (see Methods: Group 3 in Intraocular injections). **A.** Same as **B** but for nematosome diameters, 6-OHDA does not produce significant effects in any group of animals. Asterisks indicate values that differ statistically from control values ($p<0.001$).

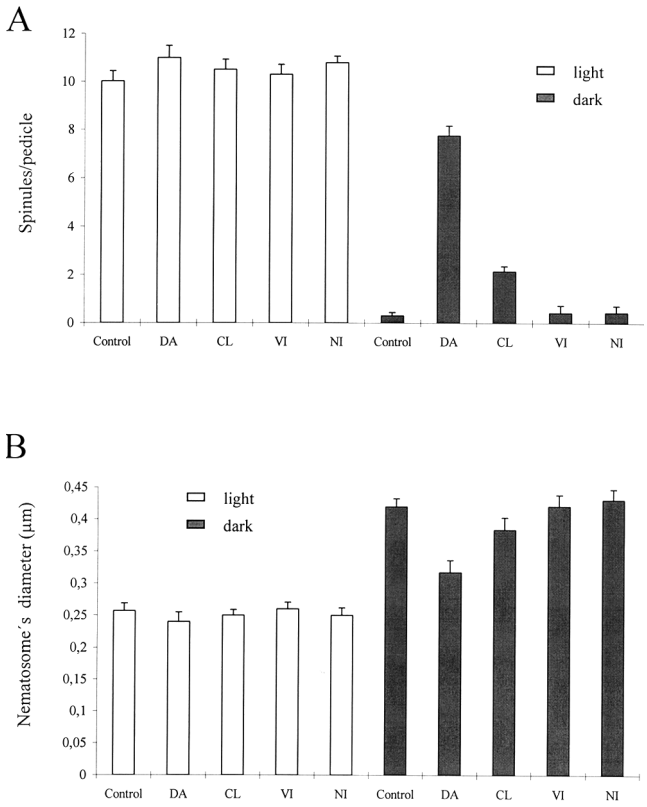


**Fig. 5.** Histograms as in Figure 4 but for monocular intravitreal injection of DA. DA injection into the right eye (DA) produces a great increase in the number of spinules per pedicle in dark-adapted fish. Their contralateral eye (CL) has a low but statistically significant increase in the density of spinules. In contrast, dopamine-injection in light has no effect on spinules. The results for vehicle-injected (VI) and non-injected (NI) fellow eyes are similar to those for control animals. Dopamine reduces the diameter of nematosomes in dopamine-injected dark-adapted eyes, to a size intermediate between those obtained in light-adapted control and dark-adapted control. The results for vehicle-injected eyes and non-injected fellow eyes are similar to those for dark-adapted control animals.

this drug has no effect on the size of nematosomes in light-adapted retinas. More research will be necessary to clarify the significance of nematosomes.

This report confirms that 6-OHDA partially inhibits the spinule formation during light adaptation, and that dopamine "in vivo" in the dark is capable of inducing spinule formation. However, our main conclusion is that intraocular injection of these drugs into an eye produces similar effects in the contralateral eye on spinule formation. Our results reinforce the idea of an interocular central control of this phenomenon of synaptic plasticity. Dopamine-dependent events in the retina appear to be necessary to forge the afferent signals eliciting this interocular effect. Moreover, in agreement with other reports (Owusu et al, 1992; De Juan and García, 1998), our data must serve as a cautionary reminder that chemically manipulating one eye while using the other as a control may not produce meaningful results.

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