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Limits to the dependence of developing neurons on protein synthesis in their axonal target territory

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Summary. Our basic question was whether the survival of developing neurons is critically dependent on the level of protein synthesis in the axonal target region. The experiments were carried out on the projection from the isthmo-optic nucleus (ION) to the contralateral retina in chick embryos. The ION is known to undergo almost 60% neuronal death between embryonic days (E) 12 and E17 and to be critically dependent on the retina for trophic support throughout this period and shortly afterwards. Various concentrations of the protein synthesis inhibitor cycloheximide were infused into one eye from E15 to E19. Moderate inhibition (up to about 40%) of retinal protein synthesis, which did not lead to retinal degeneration, had no detectable effects on the number of neurons, nor on the general morphology, in the ION. Only when the inhibition was as high as 50%, leading to widespread degeneration in the retina, did massive degeneration occur also in the ION. It was also shown that a single intraocular injection of cycloheximide at E15 that inhibited retinal protein synthesis by as much as 70–90% during the subsequent 24 h had little effect on the ION in embryos fixed at E19. These results indicate that although the ION neurons are critically dependent on the retina, they can resist major reductions in the level of retinal protein synthesis, which argues against the widespread belief that neuronal survival during development is regulated by the limited production of trophic molecules in the axonal target area. The data are, however, compatible with alternative hypotheses. Most plausibly, survival may be regulated by limited access to a nonlimiting supply of trophic molecules.

Key words: Cell death – Chick embryo – Cycloheximide – Isthmo-optic nucleus – Neuronal death

Introduction

Neuronal death is a major event in neural development, the bulk of it occurring at approximately the time when the neurons are attempting to form synapses on their target cells (Clarke 1985a; Oppenheim 1991). Whether a neuron will live or die may be influenced by several factors (e.g. Clarke 1985a, b), but of these the best established, and probably the most important, is the receipt of a retrogradely transmitted signal from the axonal target zone. The signal is believed to be conveyed by the retrograde transport of a neurotrophic substance (or substances) which is in general different for different systems (e.g. Berg 1982; Davies 1986; Oppenheim et al. 1988). Although only one such neurotrophic substance has been unequivocally identified – nerve growth factor (NGF) – a few other candidates have been purified, and of these the most plausible are all proteins, as is NGF (Berg 1984; Barde 1988, 1989; Dreyer et al. 1989; Walicke 1989).

While it is widely accepted that failure to receive enough trophic substance is a major cause of neuronal death, it remains controversial why this failure should occur. The most straightforward and widely held hypothesis is that only limiting amounts of trophic substance are produced in the target zone, with the corollary that the dependent neurons must compete for the few available molecules (e.g. Davies 1986; Barde 1988). An alternative hypothesis is that it is not the quantity of the trophic factor produced that is limiting, but access to it (e.g. Clarke and Cowan 1976; Lamb 1984; Lamb et al. 1988). These two have recently been termed the *production hypothesis* and the *access hypothesis* (Oppenheim 1989), and we shall use these terms. For completeness, we shall briefly consider other hypotheses including even the possibility that neuronal death might in some cases not be regulated by trophic molecules at all.

Our chosen system for tackling this question is the isthmo-optic system of chick embryos. Virtually all the neurons of the isthmo-optic nucleus (ION) project to the contralateral retina where they form synapses mainly on amacrine cells and probably also on displaced ganglion cells (Maturana and Frenk 1965; Dowling and Cowan 1966; Cowan and Clarke 1976; Crossland and Hughes 1978). There is a transient *ipsilateral* isthmo-optic projection during embryogenesis (Clarke and Cowan 1976) but this is so small that it can be neglected in the present context. Some of the ION neurons may

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send collateral axons to various diencephalic structures (Galifret et al. 1971; Angaut and Repérant 1978), but these projections have not so far been established unequivocally (Crossland and Hughes 1978), and in any case do not provide the ION neurons with sufficient retrograde trophic support to survive in the absence of their target neurons in the retina (Clarke et al. 1976; Catsicas and Clarke 1987). Although there is abundant evidence for the trophic dependence of the ION on the retina (Cowan and Wenger 1968; Clarke et al. 1976; Clarke 1982a, 1984; O'Leary and Cowan 1984; Catsicas and Clarke 1987), the actual chemical nature of this support has not to our knowledge been investigated.

We were therefore obliged to devise an indirect approach that did not require knowledge of the trophic molecules. Our basic experiment has been to test the effect on the ION neurons of reducing protein synthesis in the retina, during the ION's period of natural neuronal death, by the intraocular injection of cycloheximide (CX). One of our reasons for selecting CX, rather than some other inhibitor of protein synthesis, was that CX is known not to act on mitochondrial ribosomes (Tedeschi 1976); it is therefore unlikely that the CX would directly affect the isthmo-optic axons. We would stress that our main question was not whether the ION neurons depend for survival on protein synthesis in the retina, but whether the extent of protein synthesis was the critical factor. To have posed the former question would have been fruitless for at least two reasons. First, such dependence is virtually certain on the basis of previous experiments (see above). Second, the reduction of protein synthesis will affect most or all aspects of retinal development and not just the production of the hypothetical trophic substance(s) for the ION neurons, so any resulting degeneration of the ION would be difficult to interpret.

But pilot experiments indicated that the ION neurons were surprisingly resistant to a substantial reduction in retinal protein synthesis. This resistance would be difficult to explain in terms of nonspecific mechanisms, so we were encouraged to pursue the project further. Some of the results have been published in abstracts (Blaser et al. 1988, 1989; Clarke et al. 1989).

Materials and methods

Fertile chicken eggs of the White Leghorn strain were incubated at 38° C and 60% relative humidity. At death, their developmental stages were estimated according to the criteria of Hamburger and Hamilton (1951).

Intraocular injections. A window was opened in the shell in the region of the air sac, the overlying membranes were removed, and the embryo's head was gently raised with an L-shaped plastic tool.

The continuous intravitreal infusion of cycloheximide (CX) was achieved by means of Alzet mini-osmotic pumps, model 2001. These provided a continuous flow of 24 µl per day of CX solution (1 to 10 µg/day in 0.9% saline) that was fed through a polyvinyl catheter (20 cm long, 0.28 mm i.d., 0.64 mm o.d.) into the vitreous body of the right eye. The end of the catheter was preformed at right angles so as to leave a 7 mm distal part that was inserted through a small puncture in the sclera near the upper part of the corneal limbus. The catheter was fixed by a drop of cyanoacrylate glue at the puncture and by a stitch of fine thread through the upper beak. A loose coil of catheter was left within the egg so that it would not be wrenched from the eye when the embryo moved. The rate of flow was calculated from the speed with which the catheter was filled and its known cross-sectional area; the result was always 1.0 µl per hour, as expected. The mini-pumps were set in motion at E14.5 so that the catheters would be filled and ready to insert in the eye at the chosen moment (E15.0). The catheters were left in place until the time of sacrifice (E19.0).

Single intravitreal injections of CX (20 µg in 5 µl 0.9% saline) or [^{3}H]-L-leucine (see below) were made by means of a 10-µl Hamilton microsyringe, whose needle was inserted through the sclera into the centre of the vitreous body. For embryos older than E15 the sclera was anaesthetized with 2% xylocaine.

When the above operations were complete, the eggs were resealed with adhesive tape and returned to the incubator.

Tissue processing for paraffin sections. The embryos were heavily anaesthetized with chloral hydrate, after which the eyes and brains were removed and fixed by immersion in Carnoy's fixative for 2 h, dehydrated through a graded series of ethanols, oriented, and embedded in paraffin. Serial sections were cut at 12 µm, mounted on slides and counterstained with cresyl violet.

Quantitative analysis. In 13 brains, the neurons in both IONs were counted in the cresyl violet-stained paraffin sections. Counts were made in every fourth or fifth section, and numbers in the intermediate ones were derived by interpolation. A X40 objective was used, and the counting was facilitated by a grid of 5 squares × 5 squares inserted in an eyepiece. In the ION, the neurons can be distinguished by their large, round, pale-stained nuclei, which contrast with the small nuclei of the glia (Clarke 1982b; Catsicas and Clarke 1987). Neurons were counted only if a nucleolus was visible in the nucleus, and a correction factor (see appendix of Clarke 1985b) was applied, since most ION neurons have more than one nucleolus. Manifestly degenerate cells were not included in the counts.

In the retinas, cell densities and widths of layers were determined in the central region. The chosen sections were perpendicular to the plane of the retina, as could be seen by the fact that the receptors were parallel to the plane of section. Retinal cell densities were estimated by counting cell nuclei in standard strips of 30 µm in the inner nuclear layer and 60 µm in the ganglion cell layer using a X100 oil immersion objective. The widths of the inner nuclear and inner plexiform layers were determined using a calibrated scale in one eyepiece. To test whether the CX affected the tangential growth of the retina, equatorial sections of the retina were traced at about ×15 magnification, and their lengths measured by means of a ruler and a piece of cotton.

Leucine incorporation assay. Two hours after an intravitreal injection of [^{3}H]-L-leucine (2.5 µCi in 5 µl saline; specific activity 52 Ci per mmol; from Amersham International), the retinas were removed and homogenized in 2 mM potassium phosphate buffer, pH 6.8, at 4° C. The radioactivity in the TCA-precipitable fraction was estimated using the method of Mans and Novelli (1961) and was divided by that of the whole homogenate to eliminate the scatter that would otherwise have been introduced owing the rapid loss from the eye of a variable proportion (typically about 10%) of the injected [^{3}H]-leucine. From the ratio (R_{cx}) obtained in CX-treated retinas and that (R_u) obtained in CX-untreated retinas, the percentage inhibition of retinal leucine incorporation was derived: $(1-R_{cx}/R_u)100\%$.

Results

We chose to investigate the effects of continuous infusion of CX from E15 to E19, or of a single injection of CX on E15. Since neuronal death begins in the ION soon

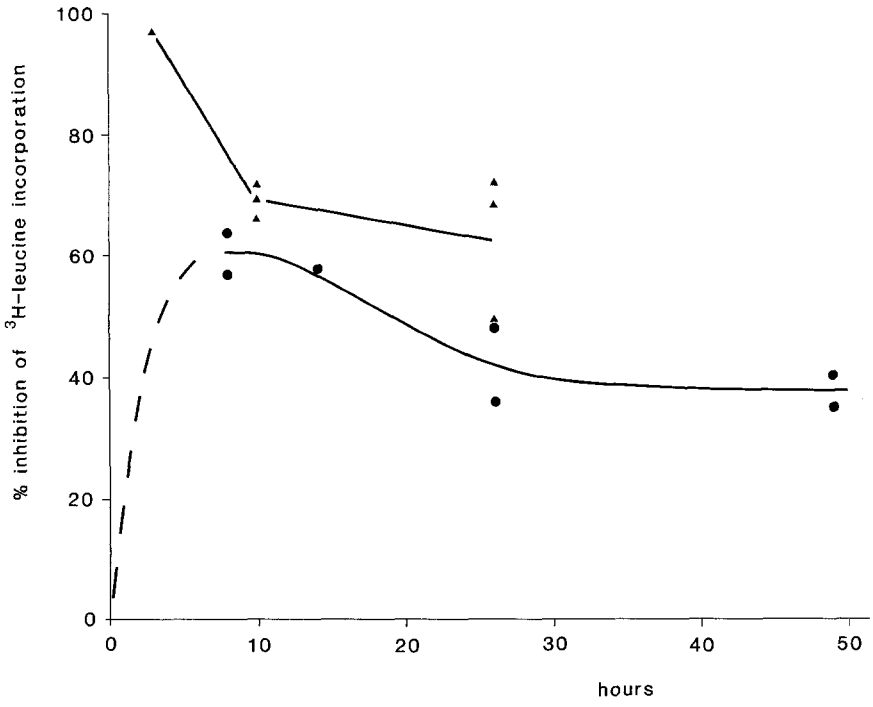

Fig. 1. Effects of continuous infusion of 6 µg/day CX (*circles*) or a single injection of 20 µg cycloheximide (CX) (*triangles*) into the eye at E15.0 on retinal [^{3}H]-leucine incorporation at various time intervals. Zero hours corresponds to E15.0. Each point represents a single embryo

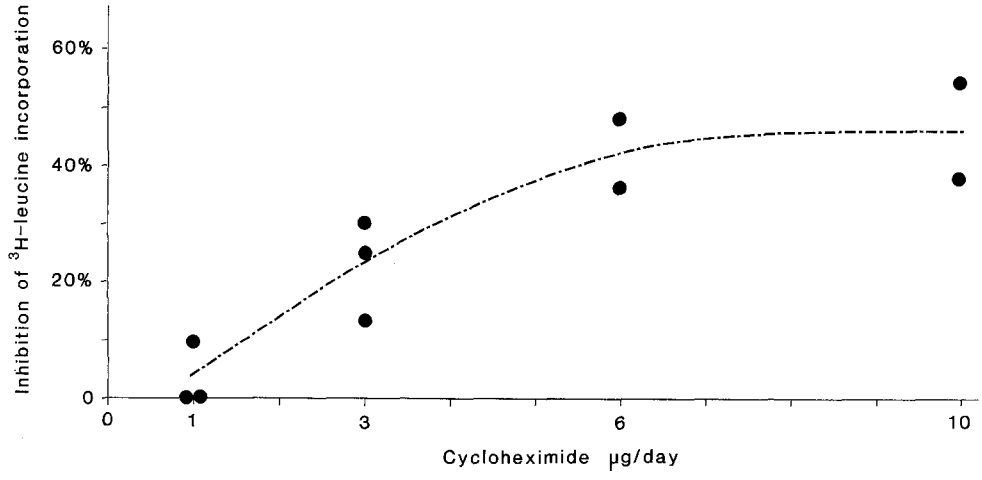

Fig. 2. Effects of continuous intraocular infusion of various doses of CX from E15.0 onwards on retinal [^{3}H]-incorporation 26 h later. Each point represents a single embryo

after E12 (Clarke et al. 1976) it would have been interesting to investigate the effects of CX before E15, but we declined to do so because the doses of CX required to inhibit protein synthesis substantially proved incompatible with the survival of the younger embryos. Moreover, we considered that the side-effects of CX on the retina would be less after E15.

Effects of CX on retinal [^{3}H]-leucine incorporation

Continuous CX-infusion (6 µg per day) into the eye from E15.0–E19.0 led to maximal inhibition of [^{3}H]-leucine incorporation within a few hours, after which the level remained relatively constant, as expected, although it declined somewhat during the latter part of the first day for unknown reasons (Fig. 1). We therefore took the level of inhibition reached after 26 h as representative of the whole period of infusion and evaluated the effect of varying the dose at this time point alone. The extent of inhibition increased with the dose level up to 6 µg CX per day, but began to saturate above this dose (Fig. 2).

In the single-injection experiments, a dose of 20 µg CX caused almost total inhibition 3 h later, and 60–70% inhibition for most of the first 26 h, after which the effects have not been investigated.

Effects of CX on retinal morphology

The retinal morphology was systematically studied in Nissl-stained sections. In most cases the main morphological features were preserved (Tables 1, 2, Fig. 3). The only exceptions were the two embryos (J3 and L6) which received a continuous infusion of 10 µg CX per day; in each of these, a large part of the retina of the infused eye had degenerated. In the non-degenerated parts of

Table 1. Cell densities in the inner nuclear layer and ganglion cell layer. Counts were made in regions of central retina that were oriented perpendicular to the plane of sectioning. Each figure in the table is the mean of three counts of the nuclei in a 30-μm (inner nuclear layer) or 60-μm (ganglion cell layer) strip through the thickness of the retina (± denotes SD). The percentages represent densities from CX-infused eyes as % of control

Embryo	μg CX per day	Inner nuclear layer		%	Ganglion cell layer		%
		CX-infused	Control		CX-infused	Control	
Continuous infusion							
K4	6	162± 7	164± 3	99%	26.7±3.8	30.0±1.0	89%
L1	6	125±13	167±12	75%	23.3±0.6	26.0±1.0	90%
L3	6	160±13	164± 6	98%	24.3±1.1	26.3±2.5	92%
J3[a, b]	10	144±12	141± 2	102%	23.0±1.7	20.7±0.6	111%
L6[a]	10	155±11	167± 8	93%	19.0±2.0	26.0±1.0	73%
Single injection (20 μg)							
013		147± 8	136± 9	108%	19.7±3.8	24.7±0.6	80%

[a] See footnote[b] of Table 3

[b] In these embryos, the ventral hemi-retina (J3) or nasal hemi-retina (L6) of the CX-infused eye had largely degenerated. Counts were made in regions of central retina that appeared intact

Table 2. Thickness of retinal layers. Measurements were made in regions of central retina that were perpendicular to the plane of sectioning. Each estimate is the mean of three measurements (± denotes SD). The percentages denote thicknesses from CX-infused eyes as % of control

Embryo	μg CX per day	Inner nuclear layer (μm)		%	Inner plexiform layer (μm)		%
		CX-infused	Control		CX-infused	Control	
Continuous infusion							
K4	3	75±4	65±1	115%	26±3	31±2	84%
L1	6	73±6	73±2	100%	24±2	33±1	73%
L3	6	65±2	77±2	84%	26±0	42±3	63%
J3[a, b]	10	51±1	53±3	96%	22±2	30±1	73%
L6[b]	10	71±1	64±2	111%	17±2	31±1	55%
Single injection							
O13		58±1	67±3	87%	25±1	37±4	68%

[a] See footnote[b] of Table 3

[b] In these embryos, part of the retina had largely degenerated (see footnote[b] of Table 1). The measurements were made in regions of the central retina that appeared intact

these retinas, and throughout the entirety of all others, the cell densities (per unit tangential area) in the inner nuclear and ganglion cell layers were approximately normal, as was the thickness of the inner nuclear layer. In contrast, the inner plexiform layer was narrower than normal in the CX-infused eyes. The injected and uninjected eyes did not differ significantly in size; nor, apparently, did the tangential extents of the two retinas, since the lengths of retina in equatorial sections were approximately equal. These results indicate that the CX did not affect retinal cell numbers (except in J3 and L6), but slowed the outgrowth of processes.

Effects on the ION of continuous infusions of CX

Even in the saline control embryos, the neuronal numbers in both IONs were somewhat lower than normal, and particularly so in the left ION i.e. contralateral to the eye that received the infusion (Table 3). We attribute these reductions to the nonspecific effects of the surgery, and especially in the inevitable damage to the retina caused by the implantation of the cannula. In our attempt to allow for the bilateral nonspecific effects, we have expressed these neuronal counts as the percent

Table 3. Counts of neurons in the two IONs of chick embryos that received a constant infusion of CX from E15.0 to E19.0. All embryos were fixed at E19.0, when the ION would contain 9,000–9,500 neurons in unoperated chick embryos (Clarke et al. 1976)

Embryo	μg CX per day	Neurons in ION		$(I-N_L/N_R/100\%)$
		Left (N_L)	Right (N_R)	
W1	0[a]	4745	7325	35
W3	0[a]	7373	7974	8
K2	1.0	6115	6243	3
K3	1.0	6901	8165	15
K4	3.0	5668	7390	24
W4	3.0	5585	8615	35
W5	3.0	9291	9666	4
L1	6.0	5428	9281	44
L3	6.0	5874	8184	29
J3[b]	10.0	2241[c]	10280	79
L6	10.0	1118[c]	9864	89

[a] Embryos W1 and W3 received saline as a control

[b] In embryo J3 the minipump was stopped at E18.0 but fixation was at E19.0; this change in protocol presumably made no difference, since events in the retina take at least 24 h before affecting the ION

[c] The massive loss of ION neurons in embryos J3 and L6 may have been largely due to the occurrence of retinal degeneration

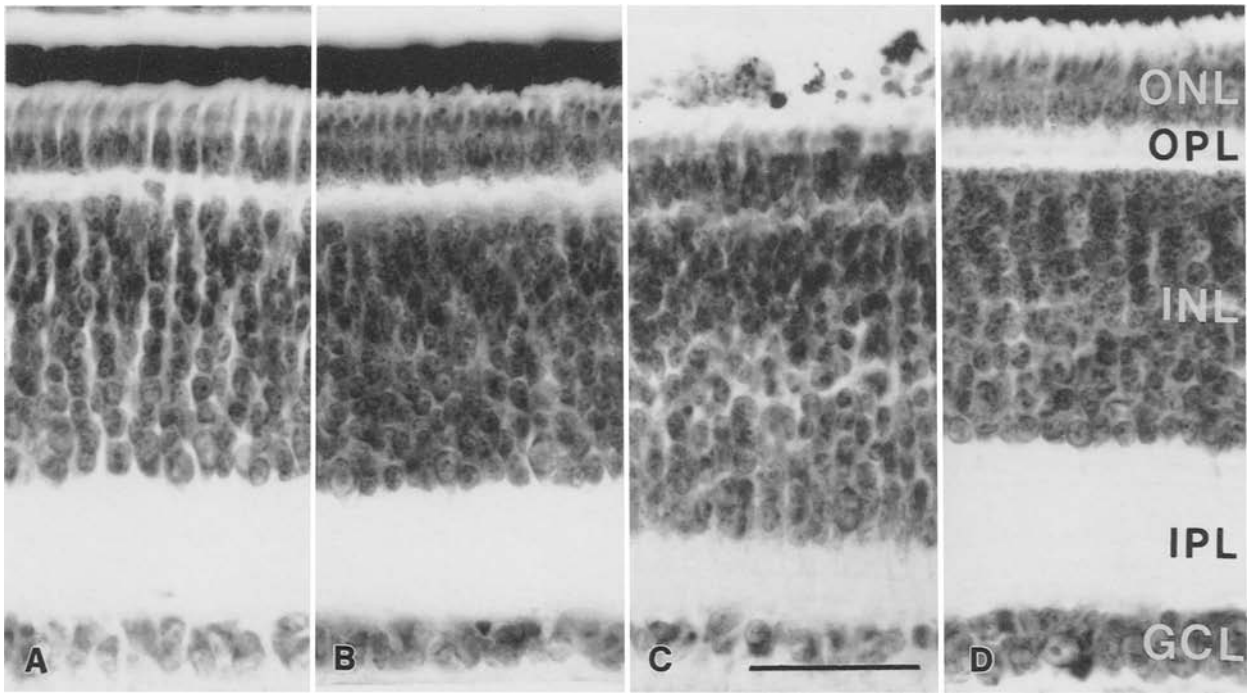

Fig. 3A–D. Retinas from embryos that were fixed at E19.0 after receiving intraocular infusions of CX continuously from E15.0. **A** 6 μg CX per day; **B** contralateral retina from same embryo. **C** 10 μg CX per day; **D** contralateral retina from same embryo. *ONL*, outer nuclear layer; *OPL*, outer plexiform layer; *INL*, inner nuclear layer; *IPL*, inner plexiform layer; *GCL*, ganglion cell layer. Bar 40 μm

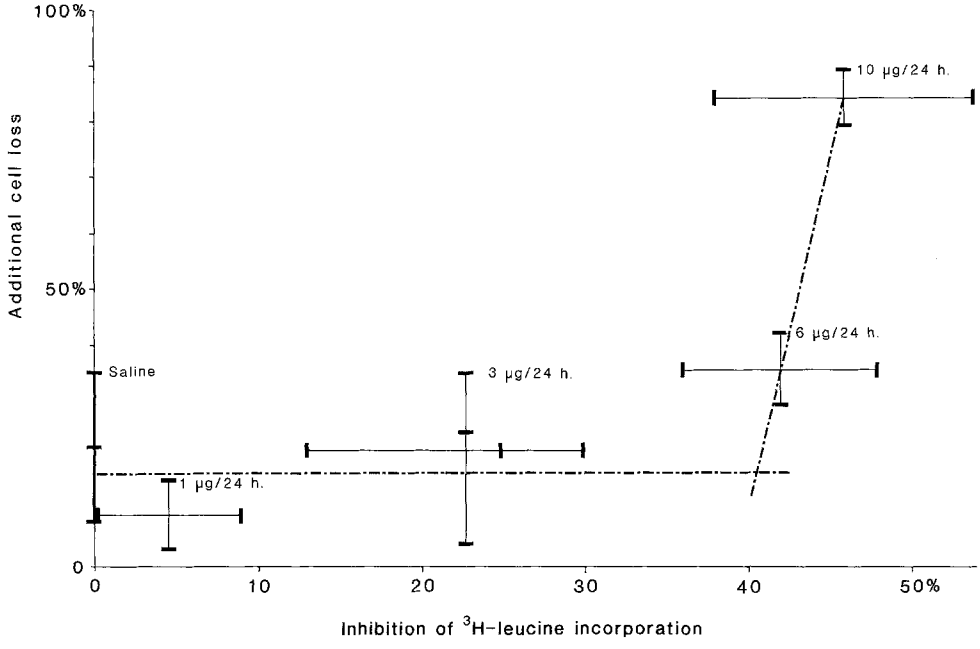

Fig. 4. The effect of inhibiting protein synthesis (inhibition of [^{3}H]-leucine incorporation measured at E16.0) by continuous intraocular infusion of CX (from E15.0) on the survival of ION neurons (counted at E19.0). CX was infused from E15.0 to E19.0. The experimental design necessitated using different embryos for the counts and for the measurements of [^{3}H]-leucine incorporation. Thus, the thick horizontal bars show individual counts at the mean inhibition for that particular infusion rate of CX; and vice versa for the thick vertical bars. See Table 3

additional cell loss $=(1-N_L/N_R)100\%$, where N_L and N_R are the numbers of healthy-looking neurons in the left and right IONs (Table 3, Fig. 4).

The main conclusion was that only the highest dose of CX (10 μg per day) caused massive degeneration in the ION (Figs. 4, 5); it may be significant that much of the retina degenerated at this dose, as is described above. With 6 μg per day, there was a small patch of frank degeneration in the lateral part of the ION in one of the two embryos (Fig. 5), and the percent additional cell loss was slightly greater than with saline, but this difference was scarcely significant (Table 3, Fig. 4). Thus the ION can tolerate a reduction of up to about 40% in retinal protein synthesis between E15 and E19 without there being a major increase in the ION neuronal death.

We have not carried out a detailed morphometric analysis of the IONs, but our impression is that when

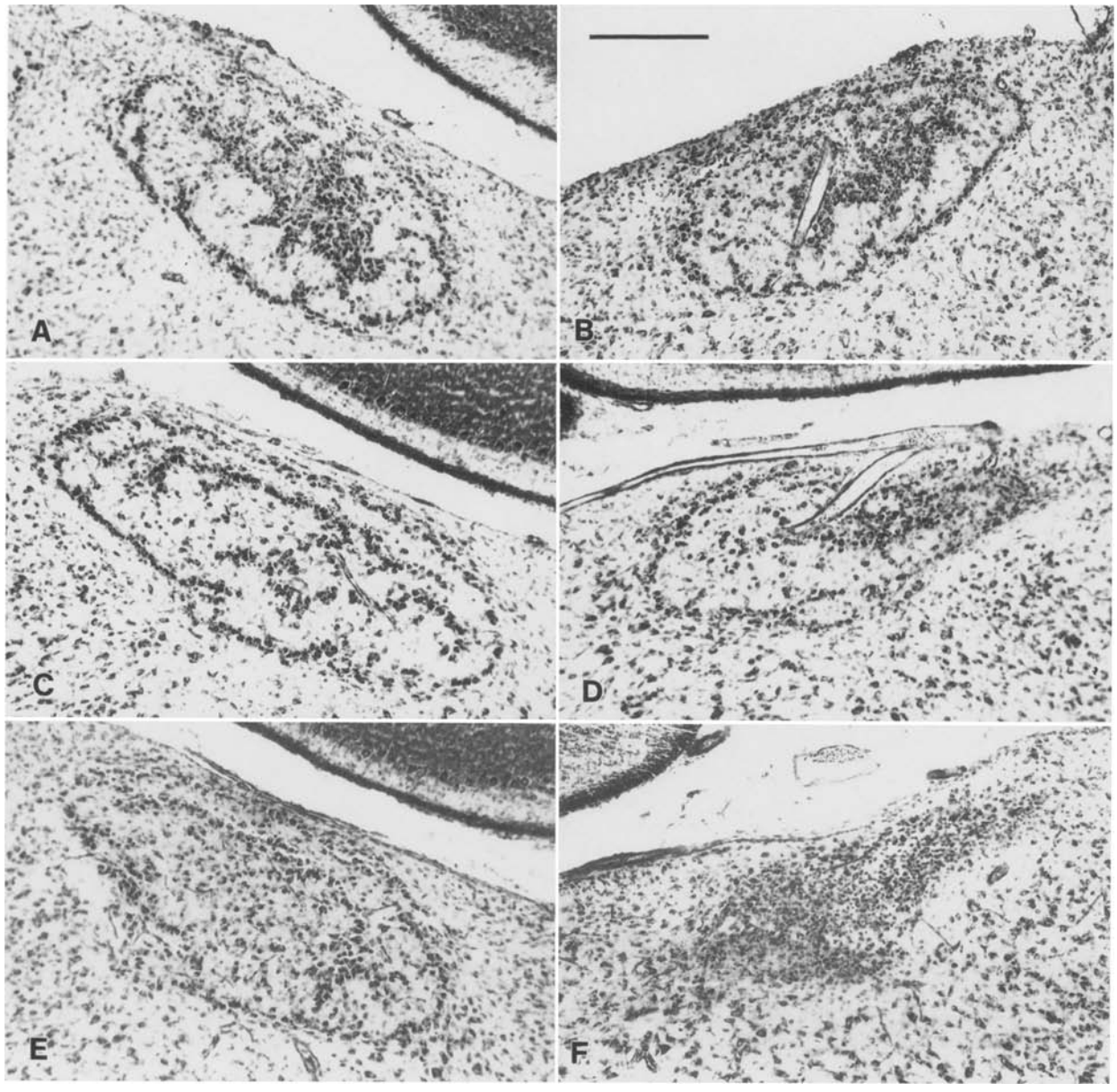

Fig. 5A–F. Isthmo-optic nuclei from embryos that were fixed at E19.0 after receiving intraocular infusions of CX continuously from E15.0: 1 μg per day (**A**, **B**), 6 μg per day (**C**, **D**) or 10 μg per day (**E**, **F**). The IONs contralateral to, and therefore liable to be affected by, the CX-infusions are shown right. The other IONs (left) serve as controls. Dorsal is up, medial is inwards. Bar 200 μm

cell death was not enhanced other characteristics – lamination, nuclear size, perikaryal size, intensity of Nissl-staining – were normal.

Effects on the ION of single injections of CX

The first series of experiments suggested that trophic material is produced in excess in the retina, raising the possibility that it might accumulate to form a trophic reserve (see Discussion). According to this latter hypothesis, the ION neurons should be able to tolerate a still greater reduction in retinal protein synthesis than in the infusion experiments if its duration was relatively brief, since there could be no effect on the ION until the reserve had been depleted. We have tested this possibility by making a single intraocular injection of 20 μg CX at E15.0, which caused a substantially greater inhibition of leucine incorporation over the first 26 h than occurred with even the highest dose (10 μg/day) in the continuous infusion experiments. Despite almost 100% inhibition initially, followed by almost 70% inhibition for more than a day (Fig. 1), there was almost no detectable effect on the histology of the retina (Fig. 6, Table 1), and there was little effect on the ION (Fig. 7). Thus in an embryo that received such an injection at E15 and was allowed to survive until E19, there were 6,566 neurons in the contralateral ION and 6,174 in the ipsilateral ION. In a second such embryo that survived until E20, there were 7,046 neurons in the contralateral ION and 6,061 in the ipsilateral. These figures are admittedly lower than normal (about 9,500 – Clarke et al. 1976), but since the reduction was no greater (and in fact slightly smaller)

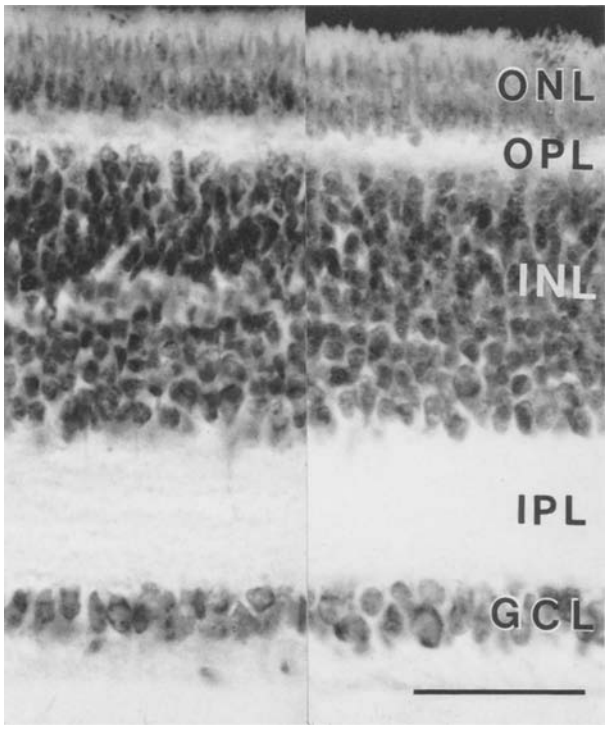

Fig. 6. Retias from an embryo that received a single intraocular injection of 20 μg CX at E15.0, and was fixed at E19.0. *Left*, retina from injected eye; *right*, contralateral retina. For layering, see Fig. 3. Bar 40 μm

contralateral to the injection than ipsilaterally, it may most plausibly be ascribed to nonspecific effects of the operation.

Discussion

The main result of this study was that the neurons of the isthmo-optic nucleus were able to survive a considerable reduction (up to about 40% for several days or 70–90% for a day) of protein synthesis in their axonal target territory, the retina, at a time when they are known to be very dependent on the retina for survival (Catsicas and Clarke 1987). Although a still larger reduction in retinal protein synthesis led to increased isthmo-optic neuronal death, the latter may most plausibly be ascribed to the accompanying widespread retinal degeneration. These results indicate that the isthmo-optic neurons are not critically dependent on the precise amount of trophic factor produced, which argues against the production hypothesis.

Ambiguities and possible artifacts

First, we would emphasize that the CX-induced reduction in the supply of the hypothetical trophic factor(s) to the isthmo-optic fibres cannot be assumed to be proportional to the overall reduction in retinal protein synthesis, for reasons explained in Fig. 8. This difficulty is dealt with at various points in the following discussion.

An almost trivial explanation of the present results would be that the lack of sensitivity to intraocularly injected CX was due to its leaking into the general circulation and thence reaching the cell bodies of the isthmo-optic neurons in sufficient amounts to block the intracellular mechanisms required for their death. Indeed, it has long been known that naturally occurring cell death is in many cases prevented by blockade of transcription or translation (Tata 1966; Lockshin and Zakeri-Milovanovic 1984), and this has recently been shown for chick embryo motoneurons (Oppenheim et al. 1990). However, recent experiments on E15 chick embryos (M. Catsicas and Clarke 1990) indicate that although systemically administered CX does prevent the death of isthmo-optic neurons deprived of retrograde maintenance, the minimal quantity of CX required to produce such an effect at E15 exceeds that which would have been available systemically in any of the present embryos. A further control is provided by experiments (Blaser et al., in preparation) in which the injection of 20 μg CX into

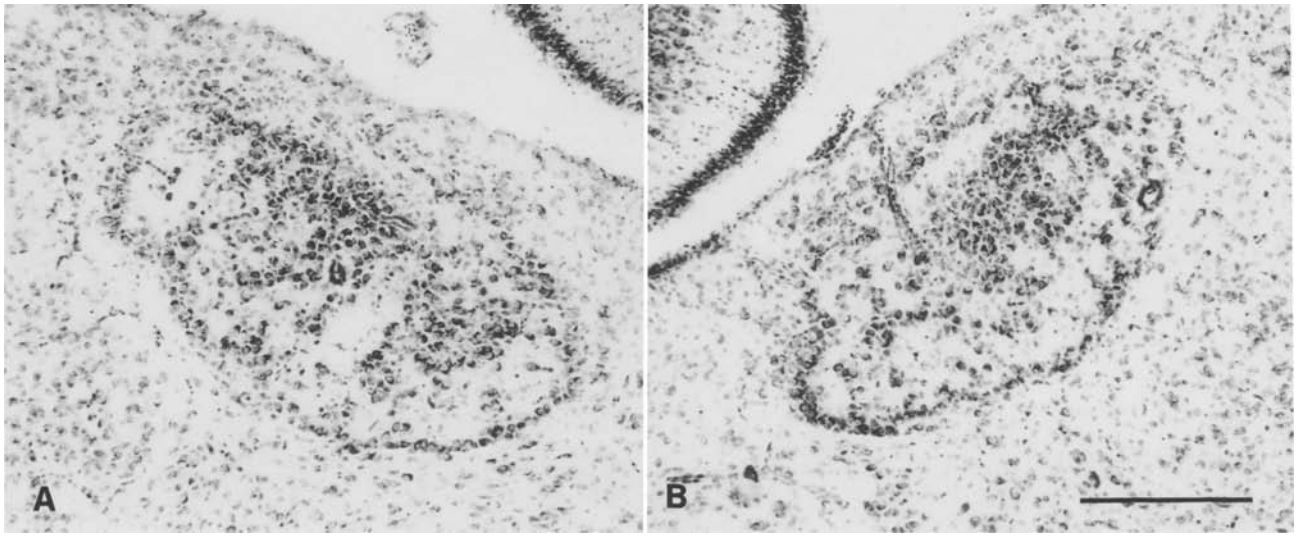

Fig. 7A, B. Isthmo-optic nuclei from an embryo that received a single intra-ocular injection of 20 μg CX at E15.0 and was fixed at E19.0. **A** ION contralateral to the injection; **B** control ION. Dorsal is up, medial is inwards. Bar 200 μm

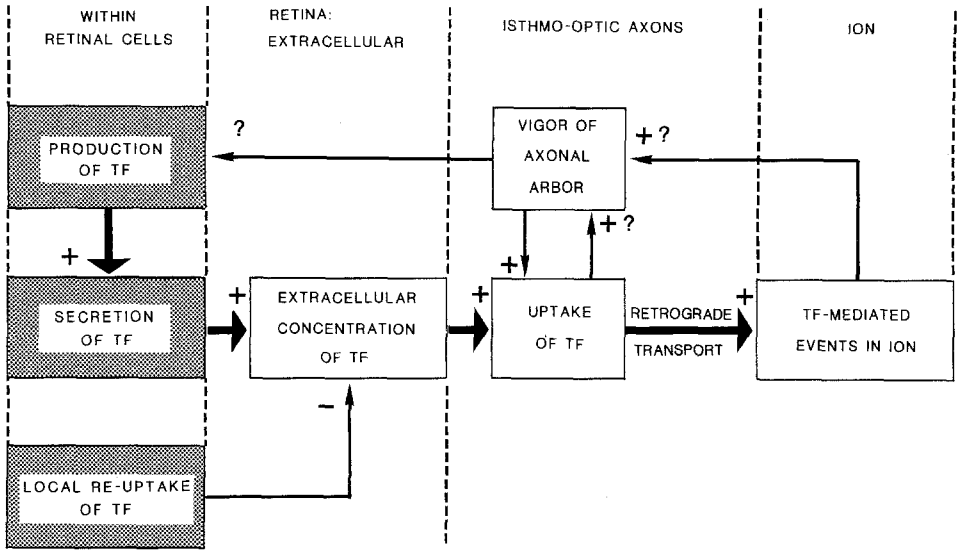

Fig. 8. Diagram showing that the supply of trophic factor (TF) to the ION will depend not only on the synthesis of TF but also on other events that will have been modified as a result of the CX, including the synthesis of other retinal proteins (e.g. those involved in the mechanisms of secretion and re-uptake). Only the shaded boxes (all in the retinal cellular compartment) will be directly affected by the CX, but the resulting changes in TF-uptake will affect various feedback loops (*thin arrows*) that will further complicate the interpretation. The direct pathway from production of CF to its effect on the ION is shown with *thick arrows*. This diagram is by no means exhaustive; e.g. it ignores the possibility of post-translatory modification of TF, presumably by enzymes whose concentration would be reduced following a reduction in their synthesis. The + and − signs indicate the probable normal interactions (*not* the CX-induced changes)

one eye at E15 (as in the present single-injection embryos) was combined with the injection of colchicine into the other eye at E15 or E16 (or both, in some cases) thereby blocking axoplasmic transport. The extent of the resulting ION neuronal death in nine such embryos was compared with that in ten controls that had received colchicine alone, and it was found to be virtually unchanged, impliying that leaked CX was insufficient to protect target-deprived ION neurons. It is noteworthy, however, that in the embryos subjected to a continuous infusion of CX, the number of neurons in the *ipsilateral* eye tended to be slightly higher for the larger doses (Table 3), which does suggest that there may have been a *slight* reduction of naturally occurring neuronal death in the ION because of leaked CX in the embryos which received 6 μg/day or 10 μg/day. But if such a reduction occurred, it was certainly small.

A related possibility is that, by preventing naturally occurring cell death within the retina of the CX-injected eye, the CX might have increased the number of available target cells sufficiently to counteract the effects of the reduced availability of trophic factor. This is not very plausible, however, because cell death in the inner nuclear layer of chick embryos is believed to end at E15 (Hughes and LaVelle 1975), and moreover our present observations indicate that the numbers of cells in the inner nuclear layer (and ganglion cell layer) of the CX-injected eyes were approximately normal.

Another possibility is that if there is normally competition between isthmo-optic fibres and intraretinal axons, a CX-induced reduction in the competitive strength of the latter might compensate for the CX-induced reduction in production of trophic substance (Fig. 8). We cannot exclude this, but there is at present no evidence for such competition, and even if it occurred it would surely be a surprising coincidence that the CX-induced reduction in competition should be of exactly the magnitude required to counteract the reduction in trophic factor reduction.

The question arises also whether the CX may have blocked the synthesis of different proteins to different extents, with relative sparing of the hypothetical trophic molecule(s). CX is believed to block the synthesis of all proteins nonspecifically by inhibiting the initiation and extension of peptide chains (Beard et al. 1969; Obrig et al. 1971), but experiments in vitro indicate that CX treatment can in specially contrived situations cause a selective accumulation of a few particular mRNAs (Garfield and Moscona 1974; Subramaniam and Shanmugam 1988). Whether that could be the case in our experiments in unclear; even if it could, we do not think that such an effect could be great enough to explain our results – especially in the case of the single injections of CX, since the inhibition of protein synthesis then attained almost 100%, albeit briefly.

Finally, it might be suggested that the trophic substance(s) were formed by post-translational modification of a much more abundant inactive precursor molecule, and that modest reductions in the pool of precursor did not affect the production of trophic factor. We cannot rule out this possibility, but the post-translational modification would presumably be performed by enzymes whose synthesis was reduced by the CX, so it is difficult even on this scenario to see how the amount of trophic factor could be totally unaffected.

The production hypothesis and its alternatives

The present experiments were intended as a test of the *production hypothesis*. By their very design they cannot distinguish between the alternatives, which are several but include the *access hypothesis* as well as the possibility that the ION might not be dependent on trophic molecules at all.

Production hypothesis. The best evidence for this stems from quantitative assay of the concentration of nerve growth factor (NGF) and of its mRNA in the peripheral nervous system. It has been shown that NGF is not produced in the sympathetic and sensory ganglion cells, but only in their target organs, where the levels of NGF and its mRNA are roughly proportional to the density of sympathetic innervation (Korsching and Thoenen 1983; Shelton and Reichardt 1984). Moreover, the quantity of NGF is not only small, but is sufficiently small to be largely removed by the innervating axons, by which most of the NGF produced is transported to the parent ganglia (Heumann et al. 1984; Davies et al. 1987). As might be expected on the basis of this latter result, there is competition between axons for NGF, as is shown by the fact that elimination of the sympathetic innervation of a field can lead to an almost threefold increase in the level of NGF in the sensory neurons innervating the same field (Korsching and Thoenen 1985).

But while all of this tends to support the production hypothesis, it is not decisive, for two main reasons. First, the amount of trophic factor required for the survival of a given neuron is unknown; without this information it remains possible that the small quantities of NGF available may nevertheless be substantially above the threshold necessary to maintain all the dependent neurons. Second, although inter-axonal competition for NGF has been demonstrated (see above), it is possible that this operated at least in part via competition for access to localized sources of NGF. In addition, since all the quantitative measurements of NGF levels were carried out in the peripheral nervous system, the target territories being mostly skin and/or muscle, one should be cautious in extrapolating to the central nervous system.

A number of experiments do in fact tell against the production hypothesis. The report that immature retinal ganglion cells could survive *partial* blockade of axoplasmic transport in their axons (Matthews et al. 1982) is difficult to reconcile with it, as is the observation in *Xenopus* tadpoles that when both sides of the spinal cord were induced to innervate a single hindlimb bud, an almost normal population of motoneurons survived on each side (Lamb et al. 1988).

The access hypothesis is grounded to a great extent on evidence against the production hypothesis, in situations where there is experimental support for trophic molecules. Thus, Oppenheim (1989) emphasizes that the production hypothesis cannot account for the reduction in motoneuron death that occurs when neuromuscular synapses are blocked by curare, since there is then no enhancement of the survival-promoting capacity of muscle-extract when tested on motoneurons in vitro (Tanaka 1987) or in vivo (Houenou et al. 1989).

The access hypothesis also accommodates most readily the observation that many of the neurons which die in development are those whose axons grow to targets that are inappropriate by adult standards (Clarke 1981; O'Leary et al. 1986), as has been shown in at least one system where there is direct evidence for trophic molecules – the neuromuscular system (Lamb 1984; Lamb et al. 1988) – and, of particular relevance to this study, in the isthmo-optic projection in chick embryos (Clarke and Cowan 1975, 1976; O'Leary and Cowan 1982; Catsicas et al. 1987). It would seem plausible that the "aberrant" axons might have subnormal access to trophic molecules.

Other alternatives. Logically, several possibilities remain. For example, ION neurons which normally survive might inherently require many times less trophic material than those that normally die. Or the maintenance of the ION by the retina might not be due to trophic molecules at all, which seems unlikely in the light of current knowledge, but cannot be entirely ruled out[1]. Combinations of the different possibilities are also conceivable. For example, limited production of trophic substance might exacerbate problems of access to it.

The present results clearly militate against the production hypothesis. The fact that almost 50% inhibition of protein synthesis for several days was required before the isthmo-optic neuronal death was increased shows that during the period investigated (E15 onwards) the overall level of trophic factor production was not a critical factor in the regulation of the extent of neuronal death. Of the alternatives, the access hypothesis is the most likely on the basis of current theory.

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[1] To cite just one of several possibilities, direct interactions between the pre- and post-synaptic membranes might induce second messengers presynaptically.

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