

The autoregulation of retinal ganglion cell number

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SUMMARY

The development of the nervous system is dependent on a complex set of signals whose precise co-ordination ensures that the correct number of neurones are generated. This regulation is achieved through a variety of cues that influence both the generation and the maintenance of neurones during development. We show that in the chick embryo, stratified retinal ganglion cells (RGCs) are themselves responsible for providing the signals that control the number of RGCs that are generated, both by inhibiting the generation of new ganglion cells and by killing incoming migratory ganglion cells. Selective toxicological ablation of RGCs in the chick embryo resulted in the achronic generation of ganglion cells, which

eventually led to the repopulation of the ganglion cell layer and a large decrease in the physiological cell death affecting postmitotic migratory neurones. Interestingly, the application of exogenous NGF reversed the effects of ganglion cell ablation on ganglion cell death. Because the only source of NGF in the retina is that produced by the stratified ganglion cells, we infer that these differentiated neurones regulate their own cell number by secreting NGF, a neurotrophin that has previously been shown to be responsible for the death of migrating ganglion cells.

Key words: Chick embryo, Retina development, Neuron number, Neurogenesis, Apoptosis, Retinal ganglion cells, NGF

INTRODUCTION

Interactions between factors that influence the genesis and the death of cells inevitably influence the number of neurones that are formed during development and that are produced during regeneration. In the developing chick retina extracellular cues influence neuron birth (Turner and Cepko, 1987) and a number of growth factors have been shown to participate in this process (reviewed by Frade et al., 1999). However, whilst such factors exert a degree of control, during the normal development of the retina, an overproduction of retinal ganglion cells (RGCs) occurs and the excess RGCs are eliminated in two successive but distinct phases of cell death (Frade et al., 1997). The first of these phases peaks at embryonic day (E)6 and is associated with the differentiation of RGCs, whereas the second phase of cell death, coincident with arrival of RGC axons in the tectum (Rager et al., 1980), affects those RGCs unable to obtain sufficient neurotrophic input from the target area (reviewed by Lewin and Barde, 1996).

The initial phase of RGC death affects postmitotic cells during their migration from the ventricular to the vitreal surface of the retina and affects at least one third of the RGCs generated (Frade et al., 1997). This cell death has been shown to be induced by retina-derived NGF via p75^{NTR}, the common receptor for neurotrophins (Frade et al., 1996b) apparently as the result of an unsuccessful attempt of postmitotic cells to re-enter the cell cycle (Frade, 2000). Interestingly, the exogenous application of brain-derived neurotrophic factor (BDNF) inhibits the cell death that occurs during this phase of development (Frade et al., 1997). Hence, a balance exists

between the trophism of BDNF and the killing activity of NGF, probably linked to the relative levels of both TrkB (the high affinity receptor for BDNF; Klein et al., 1991; Soppet et al., 1991; Squinto et al., 1991;) and p75^{NTR} in individual cells, which determines the fate of migratory RGCs. Although these data together demonstrate which factors and receptors control cell death, nothing is known about the molecular mechanisms regulating their expression, nor about the cellular events that define why, how and which cells will die. We have addressed these questions by analysing the way in which RGC number is controlled in the chick. To this end, we selectively ablated stratified RGCs in the developing chick retina, a manipulation that resulted in the regeneration of the RGC layer by the inclusion of new neurones generated from the pool of neuroepithelial cells. In addition, since none or very few migrating RGCs undergo cell death during the initial phase of repopulation, we deduce that once layered at the vitreal surface, RGCs control their own number by employing two mechanisms: first, impeding the generation of new RGCs and, second, by killing incoming migratory RGCs.

MATERIALS AND METHODS

Intra-ocular injections

Fertilised eggs from White Leghorn hens were obtained from a local supplier. Eggs were incubated at 38.5°C at 70% humidity and embryos were staged as described by Hamburger and Hamilton (Hamburger and Hamilton, 1951). After 4 days incubation, a lateral window was made in the shell and 2 µg of saporin-conjugated anti-

chick Thy1 (2 µg/µl) was injected into the eye. The immunotoxin was synthesised by Advanced Targeting Systems, Carlsbad, Ca. In experiments where the immunotoxin was co-injected with 50 ng NGF at E4, a further injection of NGF alone was also administered at E5. To trace the birth date of RGCs, 50 µCi of [³H]thymidine was applied to E6.5 chick embryos, which were sacrificed at E8. Cryostat sections containing the central part of the retina of labelled embryos were treated for autoradiography (exposure time 21-28 days).

RT-PCR

The expression of different genes was determined by RT-PCR of mRNA extracted from dissected chicken neural retinae. Amplification of retinal TrkA mRNA was carried out as described by Frade et al. (1996a), except that poly(A)⁺ RNA was used for reverse transcription and the primers were annealed at 62°C. Other primers used for PCR amplifications were as follows: 5'-GTGTGTGACAGTGTGACAG-CATG-3' and 5'-ATTACAGGCTGAGGTAGGGG-3' (377 bp) for NGF (Ebendal et al., 1986); 5'-GAGCAACTTCATCGAGAACC-3' and 5'-GCTTCATGTACTCGAAGACC-3' (320 bp) for TrkA (Backstrom et al., 1996); 5'-AGCTGAGCCGTTGGTACGAA-3' (493 bp) for visinin (Yamagata et al., 1990); 5'-CGAGCGCTTAA-GGTGTTTGA-3' and 5'-AGCTGGGGGATGTTGTTCTC-3' (524 bp) for tyrosine hydroxylase (TH) (Ernsberger et al., 1995); 5'-CCTTAAACGTGTGACCATGG-3' and 5'-CCACAACAACATAC-AGGCA-3' (599 bp) for NSCL2 (accession no. AF 109013); and 5'-GGCTGCTAAGGCTGTGGGA-3' and 5'-TATCAGCCTCTCC-CACCTCC-3' (546 bp) for GAPDH (Panabieres et al., 1984). In order to compare expression between different samples, the exponential phase of amplification was determined for each gene and the amount of total mRNA in the amplification mixtures was equalized according to the GAPDH content.

In situ hybridisation

In situ hybridisation was performed on 20 µm cryostat sections as described by Schaeren-Wiemers and Gerfin-Moser (Schaeren-Wiemers and Gerfin-Moser, 1993). Digoxigenin-labelled sense and antisense probes were synthesised from the full coding sequence of chick NGF (Ebendal et al., 1986), bp1216-1814 of chick *nscl2* cDNA (accession no. AF 109013), bp1699-2281 of chick TrkA (Backstrom et al., 1996), bp 25-505 of chick *Bmn3a* (Lindeberg et al., 1997). For detection of the mRNAs, hybridisation was carried out at 67°C.

Histochemistry

Immunostaining of cryostat sections of E6 retinas with the RA4 mAb, specific for RGCs (Waid and McLoon, 1995), was performed as previously described (Frade et al., 1997). The TUNEL assay for the detection of apoptotic cells has also been described (Frade et al., 1997).

Cell counting

To quantify cell death, apoptotic nuclei were counted from three central sections per embryo, each section containing the entrance of the optic nerve. To quantify the [³H]thymidine-labelling in RGCs, about 7 central sections per embryo were examined and labelled cells along a 960 µm strip of the GCL dorsal to the optic nerve were counted. To quantify labelled amacrine neurons, about five central sections were examined and cells were counted within the inner nuclear layer, in a rectangle of 550 × 30 µm, the larger side located at the boundary of the inner nuclear layer with the inner plexiform layer. To quantify nascent RGCs, RA4-positive cells along a 514 µm strip of the central retina, located at the germinal, ventricular surface were counted.

RESULTS

Selective ablation of RGCs

In the dorsocentral part of the retina of E6 chick embryos, most

RGCs are postmitotic and have migrated to the inner vitreous surface, thereby forming the ganglion cell layer (GCL; Prada et al., 1981, Prada et al., 1991). Once layered, these RGCs express the Thy1 antigen (Sheppard et al., 1991), although lower levels of Thy1 expression can also be detected earlier in migratory RGCs (Bovolenta et al., 1996). To determine the role that RGCs play during retinal development, the anti-Thy1 mAb was chemically conjugated to the toxin saporin and intraocularly injected into E4 embryos to selectively ablate stratified RGCs. Antibody-based targeting of cell surface antigens has been widely used to kill malignant tumor cells, and in experimental neurobiology, to produce the toxicological ablation of neuronal phenotypes expressing defined membrane antigens (reviewed by Reiter and Pastan, 1998 and Wiley, 1996). Injected and mock injected embryos were sacrificed 12, 24, 36 and 48 hours after the injection and subjected to TUNEL histochemistry to determine the extent of the immunotoxin-induced cell death in the retina. A large number of dead cells were observed 12 hours after the injection, principally at the vitreal surface suggesting that they were most probably RGCs (Fig. 1A). Some apoptotic nuclei were also seen in other parts of the neuroepithelium where they probably corresponded to postmitotic migratory RGCs which have been previously shown to express low levels of Thy1 (Bovolenta et al., 1996). After 24 (not shown) and 36 hours, the number of dead cells decreased (Fig. 1B), although dead cells were still observed across the whole extent of GCL (Fig. 1C). Again, the location of the apoptotic nuclei strongly suggested that the cells affected by the treatment with the immunotoxin were either preferentially or exclusively RGCs. However, 48 hours after the injection, very few dead cells were detected in the retina (Fig. 1D). This reduction in cell death after 48 hours was probably due to the extensive clearance of RGCs although the inactivation or dilution of the immunotoxin may also contribute to this effect.

To further prove that the cells that were affected by the immunotoxin were indeed RGCs and that it is this cell type that was cleared from E6 retinas (as in Fig. 1D), we first analyzed the effects of immunotoxin injection on the mRNA localisation of genes that are specifically expressed in RGCs such as *brn3a* (Lindeberg et al., 1997; Fig. 2A,B) and *nscl2* (J. M. Frade, personal communication; Fig. 2C-E). Following immunotoxin treatment, no expression of either of these genes could be observed by in situ hybridisation in the retina (see Fig. 2). Taking into account the distribution of the apoptotic nuclei, these data indicated that most layered RGCs had vanished from immunotoxin-treated retinas 48 hr after immunotoxin injection. To confirm that the immunotoxin-induced ablation of RGCs was specific, we used PCR to determine the relative levels of expression of specific marker genes for distinct retinal cell types. As such, we compared the levels of expression of NGF and NSCL2 transcripts in RGCs, with that of visinin and tyrosine hydroxylase from cone photoreceptors and a subset of amacrine neurons (Yamagata et al., 1990; Ballesta et al., 1984). As shown in Fig. 2F, both NGF and NSCL2 mRNA specifically decreased following immunotoxin treatment, whereas the markers for amacrine and photoreceptor cells remained unchanged. These molecular data, together with the morphological studies following TUNEL immunohistochemistry demonstrated that saporin-conjugated anti-Thy1 specifically killed RGCs.

Layered RGCs control the generation of new RGCs

It has been shown that differentiated RGCs co-cultured with retinal progenitor cells, secrete a yet uncharacterised soluble factor that prevents the birth of new RGCs from cultured precursor cells (Waid and McLoon, 1998). Regardless of the nature of this inhibitor, we investigated whether stratified RGCs in vivo also negatively control the generation of new RGCs. Thus, in E6 control retinas, nascent RGCs are mostly found in the periphery, when visualised by immunohistochemistry using the RA4 mAb (Waid and McLoon, 1995; Fig. 3A), whereas in the dorsocentral retina the generation of these cells has almost terminated (Prada et al., 1991; Fig. 3B). In contrast, we encountered many new nascent RGCs in the central area of retinas where stratified RGCs had been eliminated by the prior injection of the Thy1 immunotoxin (Fig. 3C). Furthermore, the number of nascent RGCs in the central retina increased about fourfold with respect to controls (see Fig. 3D) and the injection of NGF did not modify the generation of RGCs (see below).

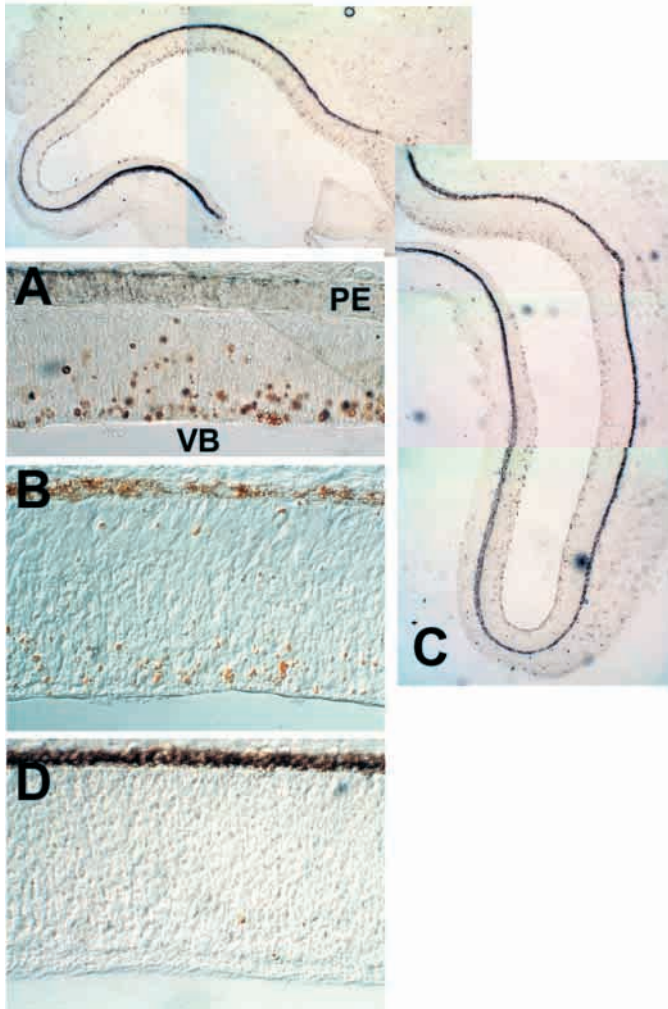


Fig. 1. Selective destruction of RGCs by injecting sporran-conjugated anti-Thy1 into the eyes of E4 chick embryos. TUNEL histochemistry on central sections of the retina (A) 12 hours, (B) 36 hours and (D) 48 hours, after the injection. (C) General view of the retina. Observe that cell death mainly affects the vitreal area of the retina. PE, pigment epithelium; VB, vitreous body.

To further examine the origin of these apparently late born RGCs, we carried out a number of [3 H]thymidine labelling experiments. In the central retina of E8 control embryos, which had been labelled at E6.5, very few [3 H]thymidine-positive cells were observed in the GCL (Fig. 4A), indicating that most of the cells were postmitotic at the time of labelling. In contrast, following immunotoxin treatment, at E8 the number of [3 H]thymidine-positive cells in the GCL increased 7.4 fold with respect to the control values (Fig. 4B,C). Since immunotoxin-treated embryos contained, in general, fewer [3 H]thymidine-positive cells, this increase in the de novo generation of neurones appeared to specifically affect RGCs. Indeed, when we counted labelled amacrine neurones (those cells located at the innermost area of the inner nuclear layer

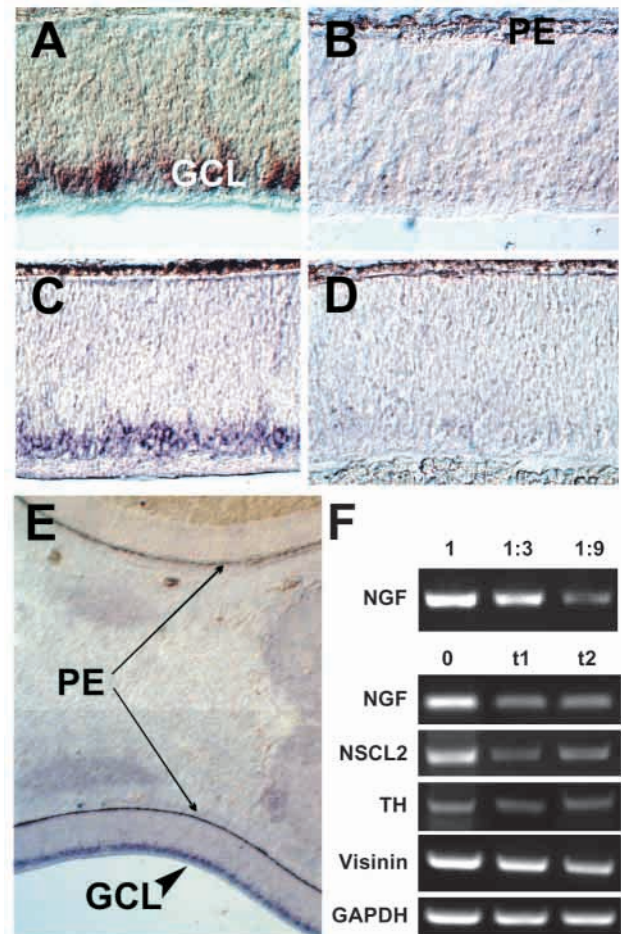


Fig. 2. Intra-ocular injections of anti-Thy1-saporin destroy RGCs as assessed by the disappearance RGC-specific markers. The ablation of RGCs was assessed by localising the mRNA of *bm3a* (A,B), and *nsc2* (C,D) by in situ hybridisation of control (A,C) and injected (B,D) E6 retinas. E is a low magnification micrograph shown expression of *nsc2* in the non-injected eye (lower), and lack of expression in the injected eye (upper). (F) To further assess the selective nature of the ablation, relative levels of the RGC markers NGF and NSCL2 mRNA, were compared with that of visinin mRNA, a marker for cone receptors, and tyrosine hydroxylase (TH), a marker for a subset of amercing neurones. In retinas of two immunotoxin-treated embryos (t1 and t2), only levels of RGC markers were clearly decreased with respect to the control (0) GCL, ganglion cell layer; PE, pigment epithelium; VB, vitreous body.

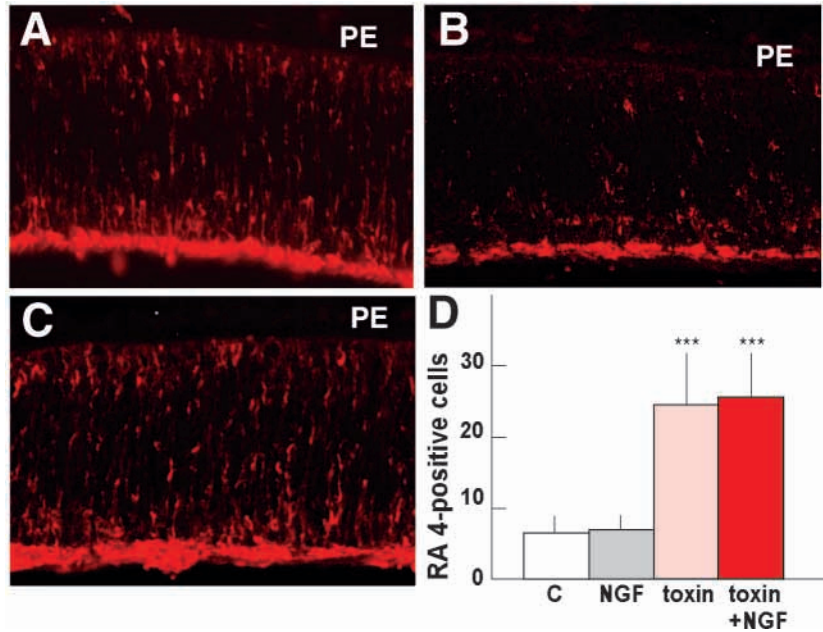


Fig. 3. Ablation of RGCs induces the achronic, late generation of new RGCs. In the peripheral area of an E6 chick control retina RGCs are being born as revealed by RA4 mAb immunohistochemistry (A), whereas in the central retina RGC genesis has mainly terminated (B). In contrast, in the central retina of immunotoxin-treated eyes, extensive birth of RGCs is observed (C). Nascent RGCs were quantified in D in the retinas of control (C), NGF-treated (NGF), immunotoxin-treated (toxin), and immunotoxin+NGF-treated (toxin+NGF) embryos. Observe that the ablation of RGCs was followed by a 3.5-fold increase of nascent RGCs, whereas NGF had no effect on the birth of these cells. PE, pigment epithelium.

that expressed tyrosine hydroxylase (Ballesta et al., 1984) and that were postmitotic at E8), in the retinas of treated embryos there were 40% fewer labelled amacrine cells than control retinas (Fig. 4C). This further suggests that the generation of RGCs in treated embryos occurred at the expense and to the detriment of the generation of other neurons such as amacrine cells. Taken together, these data confirm that on the one hand, the ablation of the normally born RGCs was followed by a generation of new RGCs to compensate for the loss of the former, and on the other hand, they imply that stratified RGCs negatively control the birth of additional RGCs *in vivo*.

Layered RGCs influence the death of incoming RGCs

A second mechanism by which RGCs may influence their own cell number resides in the control of cell death that specifically affects cells of this phenotype. Thus, shortly after their birth and during their migration to the vitreal zone of the retina, many RGCs undergo programmed cell death. Intuitively, one might consider that RGC number would be more precisely controlled when two opposite mechanisms influenced this process. However, it appears that RGC number is instead controlled by NGF, acting via its p75^{NTR} receptor, which kills a proportion of these cells, and is

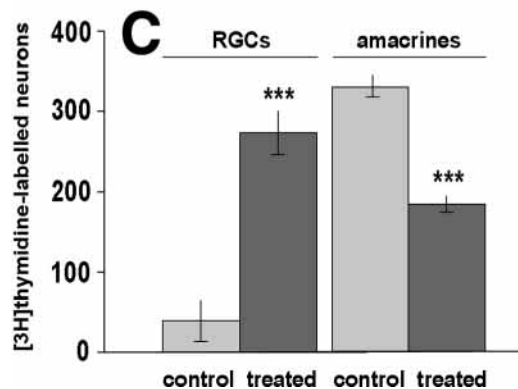
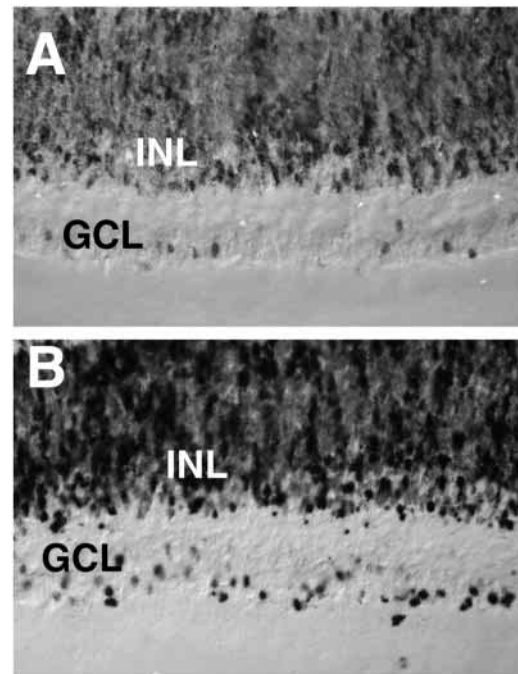


Fig. 4. Newly born RGCs can restore the GCL. After ablation of the stratified RGCs, the newly generated RGCs can reconstruct a GCL as seen in E8 retinas. Application of [³H]thymidine to E6.5 control embryos revealed that few RGCs were labelled in the central GCL of E8 embryos (A). In contrast, many RGCs of immunotoxin-treated embryos were labelled (B), indicating a later generation of RGCs, which form the ganglion cell layer. (C) Labelled amacrine cells at the innermost area of the inner nuclear layer were also counted and, unlike the RGCs, their number was lower in treated embryos. (***) $P < 0.0008$; INL, inner nuclear layer; GCL, ganglion cell layer.

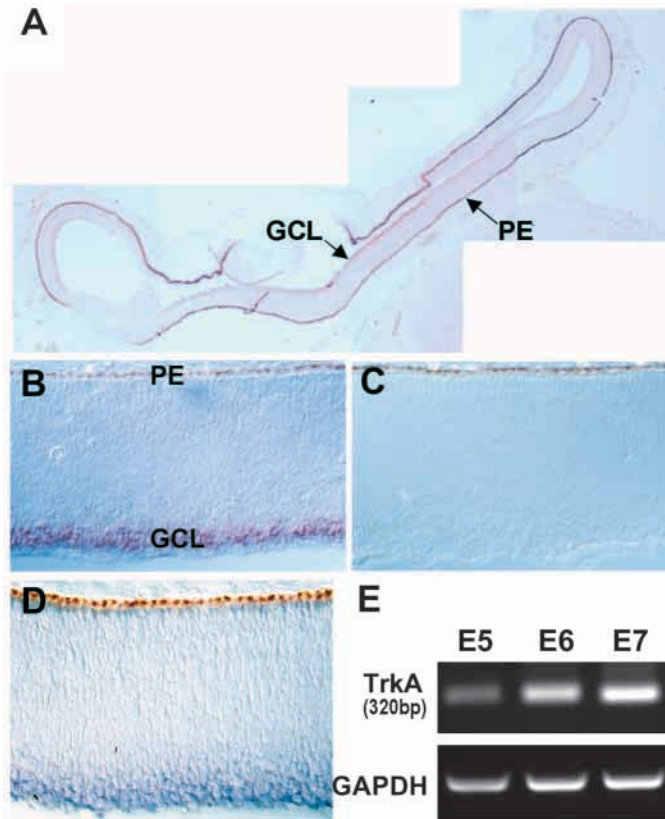


Fig. 5. NGF and TrkA mRNA are produced by the stratified RGCs. (A,B) NGF mRNA was detected by in situ hybridisation in layered RGCs. (C) A parallel in situ hybridisation experiment with the corresponding sense probe did not give any signal. (D) TrkA mRNA was also detected in the ganglion cell layer by in situ hybridisation and (E) by RT-PCR in E5-7 neural retina. GCL, ganglion cell layer; PE, pigment epithelium.

modulated by BDNF, which is needed for their survival (Frade et al., 1997). It still remains unclear how NGF induces the death of migratory RGCs, where NGF is produced at this precise developmental stage, and the physiological meaning of this control in neuronal development.

In the developing retina of E5-7 chick embryos, both NGF mRNA and mRNA for its TrkA receptor were detected in the layered stratified RGCs by in situ hybridisation and by RT-PCR in the case of TrkA (Fig. 5). Attempts to detect NGF mRNA in retinas prior to these ages were unsuccessful (not shown). In view of these results we postulated that RGC-derived NGF could be responsible for the apoptotic cell death found within the retinal neuroepithelium from E6 embryos (Frade et al., 1996b; Frade et al., 1997). If this were to be the case, the disappearance of RGCs as a result of the saporin-conjugated anti-chick Thy1 injections would remove the source of NGF and should lead to a reduction in cell death. We therefore analysed the physiological cell death in the retina by TUNEL histochemistry at E6, 48 hours after immunotoxin injection, when cell death has ended (Fig. 1D). In injected retinas, physiological cell death was reduced to 15% of the control levels, indicating that the absence of layered RGCs, and therefore that of NGF, prevented the death of incoming newly

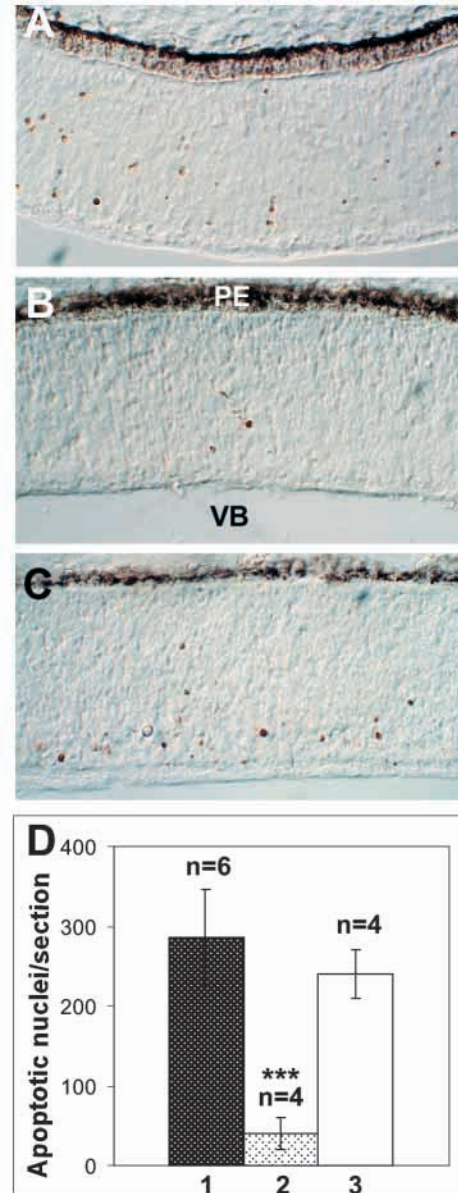


Fig. 6. Ablation of RGCs results in a large decrease in the physiological cell death in the E6 chick retina. Neuronal differentiation-associated death has been described previously and is observed at E6 (A). After ablation of the RGCs with the immunotoxin, the physiological cell death was reduced to 15% of control values (B). However, simultaneous injection of NGF restored cell death to normal values (C). (D) Cell death was assayed by TUNEL and was quantified in central sections of retinas from several embryos (*n*). (***) $P < 1.95 \times 10^{-9}$. PE, pigment epithelium; VB, vitreous body.

born RGCs that were generated in the retina (see Fig. 6). Furthermore, to prove that the depletion of NGF was responsible for the reduction in physiological cell death, this neurotrophin was co-injected in immunotoxin-treated embryos resulting in an increase in retinal cell death to levels similar to those of controls (Fig. 6C,D). Together these results demonstrate that, by producing NGF, layered RGCs induce the death of incoming RGCs.

DISCUSSION

The data presented here shed light upon two important issues in the development of the vertebrate retina. Firstly, the birth of neuronal cells, or at least of the RGCs, is not restricted to a specific temporal programme. If favourable local cues are available and/or inhibitory ones are absent, neuron generation may occur extemporaneously, as long as progenitor cells exist. This also indicates that, as previously demonstrated, neural progenitors are multipotent and may generate any type of neuron (Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988; Turner et al., 1990) while precursor cells are still available. Secondly, one cell type such as the RGCs, may regulate its own cell numbers.

The control of RGC birth

The RGCs are the first neurons to differentiate in the vertebrate retina (Kahn, 1973; Prada et al., 1981; Prada et al., 1991). They can be readily identified during or immediately after the last mitotic cycle of their precursor cells, at the ventricular (germinal) surface of the retina (MacLoon and Barnes, 1989; Waid and McLoon, 1995), suggesting that their phenotype is established concomitant with their genesis. The acquisition of the ganglion cell phenotype has been considered by some authors to be a default pathway for all neural progenitor cells, i.e. most retinal progenitors isolated in culture, and free of inhibitory forces, differentiate into RGCs (Waid and McLoon, 1998; Reh, 1992; Austin et al., 1995). However, studies in our laboratory have shown that progenitors require IGF-I, NT-3 and laminin-1 to differentiate into RGCs, not only in vitro (de la Rosa et al., 1993) but also in vivo (Frade et al., 1996a; Bovolenta et al., 1996). Recent studies from our laboratory indicate that p75^{NTR} is expressed by RGC progenitors before the M phase of the last mitotic cycle and that by binding NT-3, at a time when TrkC is not yet expressed, the genesis of RGCs is positively controlled in vivo (M. L. Cotrina, R. Mates, J. M. Frade and A. R.-T., unpublished observations). These data, combined with those revealing that p75^{NTR} mediates NGF-induced cell death in the retina (Frade et al., 1996b; see below), highlight the astounding fact that the same neurotrophin receptor, by binding two distinct neurotrophins, participates in both the birth and death of retinal neurons. In addition, the facts that p75^{NTR}, in association with the Trks, also mediates neurotrophin-induced trophism (Lewin and Barde, 1996), and that it plays a role in neurite outgrowth (Yamashita et al., 1999) makes this receptor extremely versatile and polyvalent. These data stress the importance of elucidating the different transduction pathways that this receptor uses depending on the cell type and developmental status (Cassademunt et al., 1999; Yamashita et al., 1999).

The birth of RGCs is subject to positive control by neurotrophic factors originating in the pigment epithelium as well as being influenced by other trophic factors (reviewed by Frade et al., 1999). However, the birth of these neurons is eventually constrained in development and its production stops while other neuronal phenotypes are still being generated (Prada et al., 1991). Such a constraint on RGC differentiation may be due either to the disappearance of inductive signals, the change or loss of response to such signals (e.g. by changing or down-regulating the expression of receptors to specific growth

factors (Lillien, 1995)), or to the expression of factors that inhibit the further production of RGCs.

As in other tissues, differential activation of Notch receptors serves to control the production of neurons and, ultimately, the generation of neuronal diversity in the retina (reviewed by Perron and Harris, 2000). Specifically, it has already been shown that RGC differentiation is negatively regulated by mechanisms associated to Notch signalling (Austin et al., 1995; Ahmad et al., 1997; Henrique et al., 1997). In addition, there are indications that unidentified factor(s) secreted by differentiated RGCs prevent(s) the differentiation of progenitor cells into RGCs in vitro (Waid and McLoon, 1998). These data, together with those showing that differentiated amacrine neurons may also limit their generation in re-aggregate cultures (Belliveau and Cepko, 1999) have generated new insights into how neuron number is self-limited.

Here, we present evidence in vivo that the elimination of differentiated RGCs releases a constraint that deters newborn neurons from forming RGCs. Thus, as demonstrated by both RA4 immunohistochemistry and [³H]thymidine labelling experiments, shortly after the toxicological ablation of the RGCs, new RGCs are generated and they safely migrate to the vitreal surface of the retina to reconstruct a new ganglion cell layer. The increase in neuron generation upon immunotoxin treatment seemed to affect RGCs only. We also counted newly generated amacrine neurons and their number, unlike RGCs, was significantly decreased. It is, therefore, possible that the extra generation of RGC drains progenitor cells, partially inhibiting the production of other neuron phenotypes. Thus, our data show that once that RGCs accumulate in sufficient number to constitute the ganglion cell layer, they obstruct the birth of new RGCs, probably by producing an inhibitory factor(s). Conversely, the ablation of the stratified RGCs removes this constraint and new RGCs are born.

The control of RGC death

The fact that differentiated RGCs die in two successive phases (Frade et al., 1997; Rager, 1980), clearly suggests that an excess (of about 2.5 fold) of these cells are generated and, therefore, the control mechanisms described above are insufficient to determine the final number of RGCs. Thus, once RGCs have been born and populated their layer, a second mechanism must be put into effect to limit the number of migratory cells that are capable of introducing themselves into the ganglion cell layer. Indeed, a substantial amount of RGCs die on their way to the vitreal surface and the factor responsible for inducing this death is NGF via an interaction with its p75 receptor (Frade et al., 1996b). In E4 embryos and earlier, the source of retinal NGF has been reported to be invasive macrophages (Frade and Barde, 1998). In addition, cell death increased in the retina when vitreous body-derived macrophages were added to cultures of E3 eye balls (Frade and Barde, 1998). However, we have failed to detect cells producing NGF mRNA outside the ganglion cell layer at E6, the stage at which most of our experiments were performed. Indeed, NGF message was consistently found associated with stratified RGCs, indicating that NGF and its associated killing activity is solely derived from these cells. Furthermore, we present additional evidence that RGC-derived NGF is responsible for the physiological cell death observed in the retina. Toxicological ablation of RGCs resulted in a robust

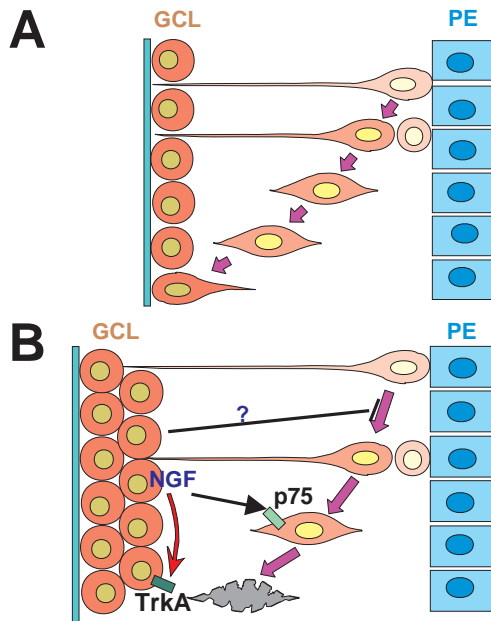


Fig. 7. Schematic representation of the control of RGC numbers. At an early stage of development (A), the generation of RGCs is unrestrained and depends on positive signals. As development proceeds (B), more RGCs accumulate in the ganglion cell layer (GCL), thereby producing increasing amounts of both a yet unidentified factor (?), which prevents the generation of new RGCs, and NGF, which by diffusion kills the incoming migratory RGCs via the p75^{NTR} receptor expressed on their surface. Layered RGCs can survive the apoptotic effect of NGF by expressing the high affinity TrkA receptor, which switches the pro-apoptotic signaling of p75^{NTR} into a neurotrophic one. PE, pigment epithelium; GCL, ganglion cell layer.

decrease of apoptotic cell death within the E6 retina. Consistent with this finding, exogenously applied NGF restored the levels of cell death to physiological values. Together these data demonstrate that by producing and secreting NGF, RGCs impede the arrival of new RGCs, thus negatively controlling their number. Application of exogenous NGF did not however influence the birth of new RGCs, thus implying that the action of RGC-derived NGF is restricted to the induction of cell death.

Conclusion

The main findings of this work are summarised in Fig. 7. Thus, the ingenious mechanism by which RGCs control their own number, implies that the local concentration of NGF will increase as more RGCs are added to the growing GCL. Once threshold levels of NGF are reached, this neurotrophin may diffuse into the retina, killing migrant RGCs by interacting with p75^{NTR} that is actually expressed in these cells (unpublished results). In contrast, TrkA (Fig. 5D,E), TrkB and TrkC, the high-affinity specific receptors for NGF, BDNF and neurotrophin 3, respectively, are highly expressed later in the layered RGCs (this study; Das et al., 1997), where they probably couple to p75^{NTR}, thereby switching the apoptotic activity of the latter into a neurotrophic one as has been observed in some in vitro systems (Yoon et al., 1998), thus preventing the stratified RGCs from committing suicide.

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REFERENCES

- Ahmad, I., Dooley, C. M. and Polk, D. L. (1997). Delta-1 is a regulator of neurogenesis in the vertebrate retina. *Dev. Biol.* **185**, 92-103.
- Austin, C. P., Feldman, D. E., Ida, J. A. and Cepko, C. L. (1995). Vertebrate retinal ganglion cells are selected from competent progenitors by the action of Notch. *Development* **121**, 3637-3650.
- Backstrom, A., Soederstrom, S., Kylberg, A. and Ebendal, T. (1996). Molecular cloning of the chicken trkA and its expression in early peripheral ganglia. *J. Neurosci. Res.* **46**, 67-81.
- Ballesta, J., Terenghi, G., Thibault, J. and Polak, J. M. (1984). Putative dopamine-containing cells in the retina of seven species demonstrated by tyrosine hydroxylase immunocytochemistry. *Neuroscience* **12**, 1147-1156.
- Belliveau, M. J. and Cepko, C. L. (1999). Extrinsic and intrinsic factors control the genesis of amacrine and cone cells in the rat retina. *Development* **126**, 555-566.
- Bovolenta, P., Frade, J. M., Marti, E., Rodríguez-Peña, M. A., Barde, Y. A. and Rodríguez-Tébar, A. (1996). Neurotrophin-3 antibodies disrupt the normal development of the chick retina. *J. Neurosci.* **16**, 4402-4410.
- Casademunt, E., Carter, B. D., Benzel, I., Frade, J. M., Dechant, G. and Barde, Y.-A. (1999). The zinc finger protein NRIF interacts with the neurotrophin receptor p75(NTR) and participates in programmed cell death. *EMBO J.* **18**, 6050-6061.
- Das, I., Hempstead, B. L., MacLeish, P. R. and Sparrow, J. R. (1997). Immunohistochemical analysis of the neurotrophins BDNF and NT-3 and their receptors trkB, trkC, and p75 in the developing chick retina. *Vis. Neurosci.* **14**, 835-842.
- de la Rosa, E. J., Arribas, A., Frade, J. M. and Rodríguez-Tébar, A. (1994). Role of neurotrophins in the control of neural development: neurotrophin-3 promotes both neuron differentiation and survival of cultured chick retinal cells. *Neuroscience* **58**, 347-352.
- Ebendal, T., Larhammar, D. and Persson, H. (1986). Structure and expression of the chicken beta nerve growth factor gene. *EMBO J.* **5**, 1483-1487.
- Ernsberger, U., Patzke, H., Tissier-Seta, J. P., Reh, T., Goridis, C. and Rohrer, H. (1995). The expression of tyrosine hydroxylase and the transcription factors cPhox-2 and Cash-1: evidence for distinct inductive steps in the differentiation of chick sympathetic precursor cells. *Mech. Dev.* **52**, 125-136.
- Frade, J. M. (2000). Unscheduled cell cycle re-entry induced by NGF precedes cell death in nascent retinal neurones. *J. Cell Sci.* **113**, 1139-1148.
- Frade, J. M. and Barde, Y. A. (1998). Microglia-derived nerve growth factor causes cell death in the developing retina. *Neuron* **20**, 35-41.
- Frade, J. M., Bovolenta, P. and Rodríguez-Tébar, A. (1999). Neurotrophins and other growth factors in the generation of retinal neurons. *Microsc. Res. Tech.* **45**, 243-251.
- Frade, J. M., Bovolenta, P., Martínez-Morales, J. R., Arribas, A., Barbas, J. A. and Rodríguez-Tébar, A. (1997). Control of early cell death by BDNF in the chick retina. *Development* **124**, 3313-3320.
- Frade, J. M., Martí, E., Bovolenta, P., Rodríguez-Peña, M. A., Pérez-García, D., Rohrer, H., Edgar, D. and Rodríguez-Tébar, A. (1996a). Insulin-like growth factor-I stimulates neurogenesis in chick retina by regulating expression of the alpha 6 integrin subunit. *Development* **122**, 2497-2506.
- Frade, J. M., Rodríguez-Tébar, A. and Barde, Y.-A. (1996b). Induction of cell death by endogenous nerve growth factor through its p75 receptor. *Nature* **383**, 166-168.
- Hamburger, V. and Hamilton, H. (1951). A series of normal stages in the development of chick embryo. *J. Morphol.* **88**, 8487-8491.
- Henrique, D., Hirsinger, E., Adam, J., Le Roux, I., Pourquie, O., Ish-Horowicz, D. and Lewis, J. (1997). Maintenance of neuroepithelial progenitor cells by Delta-Notch signalling in the embryonic chick retina. *Curr. Biol.* **7**, 661-670.
- Holt, C. E., Bertsch, T. W., Ellis, H. M. and Harris, W. A. (1988). Cellular determination in the Xenopus retina is independent of lineage and birth date. *Neuron* **1**, 15-26.

- Kahn, A. J.** (1973). Ganglion cell formation in the chick neural retina. *Brain Res.* **63**, 285-290.
- Klein, R., Nanduri, V., Jing, S. A., Lamballe, F., Tapley, P., Bryant, S., Cordon-Cardo, C., Jones, K. R., Reichardt, L. F. and Barbacid, M.** (1991). The trkB tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. *Cell* **66**, 395-403.
- Lewin, G. and Barde, Y. A.** (1996). Physiology of the neurotrophins. *Annu. Rev. Neurosci.* **19**, 289-317.
- Lillien, L.** (1995). Changes in retinal cell fate induced by overexpression of EGF receptor. *Nature* **377**, 158-162.
- Lindeberg, J., Klint, P., Williams, R. and Ebendal, T.** (1997). Identification of a chicken homologue in the Brn-3 subfamily of POU-transcription factors. *Dev. Brain Res.* **100**, 169-182.
- McLoon, S. C. and Barnes R. B.** (1989). Early differentiation of retinal ganglion cells: an axonal protein expressed by premigratory and migrating retinal ganglion cells. *J. Neurosci.* **9**, 1424-1432.
- Panabieres, F., Piechaczyk, M., Rainer, B., Dani, C., Fort, P., Riaad, S., Marty, L., Imbach, J. L., Jeanteur, P. and Blanchard, J. M.** (1984). Complete nucleotide sequence of the messenger RNA coding for chicken muscle glyceraldehyde-3-phosphate dehydrogenase. *Biochem. Biophys. Res. Commun.* **118**, 767-773.
- Perron, M. and Harris, W. A.** (2000). Determination of vertebrate retinal progenitor cell fate by the Notch pathway and basic helix-loop-helix transcription factors. *Cell. Mol. Life Sci.* **57**, 215-223.
- Prada, C., Puelles, L. and Genis-Gálvez, J. M.** (1981). A golgi study on the early sequence of differentiation of ganglion cells in the chick embryo retina. *Anat. Embryol.* **161**, 305-317.
- Prada, C., Puga, J., Pérez-Méndez, L., López, R. and Ramírez, G.** (1991). Spatial and temporal patterns of neurogenesis in the chicken retina. *Eur. J. Neurosci.* **3**, 559-569.
- Rager, G. H.** (1980). Development of the retinotectal projection in the chicken. *Adv. Anat. Embryol. Cell Biol.* **63**, 1-90.
- Reh, T. A.** (1992). Cellular interactions determine neuronal phenotypes in rodent retinal cultures. *J. Neurobiol.* **23**, 1067-1083.
- Reiter, Y. and Pastan, I.** (1998). Recombinant Fv immunotoxins and Fv fragments as novel agents for cancer therapy and diagnosis. *Trends Biotechnol.* **16**, 513-520.
- Schaeren-Wiemers, N. and Gerfin-Moser, A.** (1993). A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridisation using digoxigenin-labelled cRNA probes. *Histochemistry* **100**, 431-440.
- Sheppard, A. M., Konopka, M., Robinson, S. R., Morgan, I. G. and Jeffrey, P. L.** (1991). Thy1 antigen is specific to ganglion cells in chicks. *Neurosci. Lett.* **123**, 87-90.
- Soppet, D., Escandon, E., Maragos, J., Middlemas, D. S., Reid, S. W., Blair, J., Burton, L. E., Stanton, B. R., Kaplan, D. R. and Hunter, T.** (1991). The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the trkB tyrosine kinase receptor. *Cell* **65**, 895-903.
- Squinto, S. P., Stitt, T. N., Aldrich, T. H., Davis, S., Bianco, S. M., Radziejewski, C., Glass, D. J., Masiakowski, P., Furth, M. E. and Valenzuela, D. M.** (1991). TrkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. *Cell* **65**, 885-893.
- Turner, D. L. and Cepko, C. L.** (1987). A common progenitor for neurons and glia persists in rat retina late in development. *Nature* **328**, 131-136.
- Turner, D. L., Snyder, E. Y. and Cepko, C. L.** (1990). Lineage-independent determination of cell type in the embryonic mouse retina. *Neuron* **4**, 833-845.
- Waid, D. K. and McLoon, S. C.** (1995). Immediate differentiation of ganglion cells following mitosis in the developing retina. *Neuron* **14**, 117-124.
- Waid, D. K. and McLoon, S. C.** (1998). Ganglion cells influence the fate of dividing retinal cells in culture. *Development* **125**, 1059-1066.
- Wetts, R. and Fraser, S. E.** (1988). Multipotent precursors can give rise to all major cell types of the frog retina. *Science* **239**, 1142-1145.
- Wiley, R. G.** (1996). Targeting toxins to neural antigens and receptors. *Semin. Cancer Biol.* **7**, 71-77.
- Yamagata, K., Goto, K., Kuo, C. H., Kondo, H. and Miki, N.** (1990). Visinin: a novel calcium binding protein expressed in retinal cone cells. *Neuron* **4**, 469-476.
- Yamashita, T., Tucker, K. L. and Barde, Y. -A.** (1999). Neurotrophin binding to the p75 receptor modulates Rho activity and axonal outgrowth. *Neuron* **24**, 585-593.
- Yoon, S. O., Casaccia-Bonnel, P., Carter, B. and Chao, M. V.** (1998). Competitive signaling between TrkA and p75 nerve growth factor receptors determines cell survival. *J. Neurosci.* **18**, 3273-3281.