Control of early cell death by BDNF in the chick retina

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SUMMARY

The developing chick retina undergoes at least two discrete periods of programmed cell death. The earlier period coincides with the main onset of neuron birth and migration (embryonic day 5-7), whereas the latter one corresponds to the well-documented process of retinal ganglion cell death following tectal innervation (embryonic day 10-14; Rager, G. H. (1980) Adv. Anat. Embryol. Cell Biol. 63, 1-92). In the early period, apoptosis is induced by nerve growth factor (NGF) acting via its p75 receptor (Frade, J. M., Rodríguez-Tébar, A. and Barde, Y.-A. (1996) Nature 383, 166-168). Here, we show that the application of brain-derived neurotrophic factor (BDNF) to chick embryos in ovo prevented retinal cell death in the early period, whereas exogenously applied NGF and neurotrophin-3 had no such effect. The addition of BDNF to embryos resulted in about 70% increase in the number of retinal ganglion cells in both E6 and E9 retinas relative to controls. BDNF is first expressed in both the pigment epithelium and neural retina of embryonic day 4 embryos, and at the same stage of development, its TrkB receptor is expressed in the neural retina. Our data indicate that early cell death is an important process in the neurogenesis of retinal ganglion cells and is regulated by locally produced BDNF.

Key words: chick/retina development, retinal ganglion cells, apoptosis, BDNF, cell death

INTRODUCTION

Cell death has long been recognised as a key feature of normal animal development (Glucksman, 1951; Saunders, 1966). Although the molecular mechanisms underlying this phenomenon have been elusive for a long time, in recent years it has become apparent that cells die by apoptosis, activating a suicide programme that may be regulated by interactions with other cells (Raff, 1992). In the nervous system, a well-known period of programmed cell death (PCD) takes place at relatively late stages of neuron maturation, following the projection of the axons onto their innervation areas (Oppenheim, 1991, review). According to the neurotrophic theory (Purves, 1988), the death/survival destiny of a neuron depends on the capability of its outgrowing axon to obtain an adequate neurotrophic input from the target area. Hence, target-derived neurotrophins are essential for the survival and maintenance of developing neurones in vertebrates (Lewin and Barde, 1996). However, it is now known that the role of neurotrophins is not restricted to this developmental period, since a number of studies indicate that these growth factors play critical roles in other earlier fundamental processes of development of the nervous system, including cell proliferation and neuron differentiation (Lewin and Barde, 1996). For instance, recent studies in our laboratory have shown that NT-3 promotes neuron differentiation in the chick retina in vivo (Bovolenta et al., 1996). Since an early phase of PCD has been found to be associated with cell differentiation in the CNS (Källén, 1965; Homma et al., 1994; Blaschke et al., 1996), and neural progenitors in the PNS suffer an excessive degree of apoptosis in nt-3 (−/−) mice (ElShamy and Ernfors, 1996a,b), we have addressed the question as to whether neurotrophins could also act as cell survival factors in the early development of the CNS.

In the chicken retina, neuron precursor cells have been shown to undergo PCD early in development (Silver and Hughes, 1973; García-Porrero and Ojeda, 1979; Cuadros and Ríos, 1988). Recently, it was shown that endogenous NGF acting through its p75 receptor induces cell death in early (E6) chick retinas (Frade et al., 1996). We have further addressed this issue and, in this communication, we report that cell death affects neuron precursor cells at E5-7, a period coincident with retinal neuron differentiation (Prada et al., 1991), and constitutes an important process limiting the number of differentiated retinal ganglion cells (RGCs). We have also analysed the effects of different neurotrophins on this process and show that BDNF acts as a survival factor for cells undergoing early cell death in the chick retina.

MATERIALS AND METHODS

Embryos

White Leghorn chicken embryos were used throughout this study and were staged according to Hamburger and Hamilton (1951).

Antibodies

Chick anti-Islet-1/2 was obtained from the Hybridoma Bank, Univer-
Retinas from several developmental stages were dissected free from the brain. Measurement of BDNF high-affinity receptors in retinal cells in the absence of reverse transcriptase were RT-PCR amplified and gave rise to DNA contamination in the cDNA preparations. Reactions performed with the primers provided by Frade et al. (1996). To check genomic contamination in the cDNA preparations, reactions performed in absence of reverse transcriptase were RT-PCR amplified and gave no reaction products (data not shown).

Measurement of BDNF high-affinity receptors in retinal cells

Retinas from several developmental stages were dissected free from the brain. RNA from E4 and E8 pigment epithelium and from E4, E6, E8 and E14 neural retinas were extracted by the method of Chomczynski and Sacchi (1987). 0.5 µg of RNA was reverse transcribed with SuperScript II RNase H- reverse transcriptase ( Gibco/BRL) following the manufacturer’s instructions. PCR was carried out using 25 ng of each cDNA as template and the primers corresponded to either nucleotides 1413-1432 and complementary to nucleotides 1798-1817 of the chicken trkB cDNA, which amplify the kinase domain (Dechard et al., 1993) (30 cycles; 59°C), or nucleotides 57-76 and complementary to nucleotides 562-581 of the chicken BDNF cDNA (Maisonpierre et al., 1992) (35 cycles; 59°C). As internal controls, glyceraldehyde-3-phosphate dehydrogenase mRNA was RT-PCR co-amplified as described by Frade et al. (1996). To check genomic DNA contamination in the cDNA preparations, reactions performed in absence of reverse transcriptase were RT-PCR amplified and gave no reaction products (data not shown).

Immunohistochemistry

Immunohistochemistry was carried out on frozen tissue sections. Briefly, chicken embryo heads were fixed in immersion in 4% paraformaldehyde/PBS [w/v] for 5 hours at 4°C and then washed in 100 mM sodium phosphate, pH 7.3, containing 30% sucrose [w/v] for 36 hours. 12 µm cryostat sections were mounted on 2% 3-amino-propyltriethoxy-silane-coated slides. Sections were incubated in phosphate buffer saline (PBS) containing 0.1% Tween-20 [w/v] and 10% goat serum [v/v] (PTG) for 1 hour. Immunostaining was performed using either anti-Islet-1/2 mAb (ascitis fluid, 1:1000) diluted, or RA4 mAb (culture supernatant 1:1000) in PTG for 16 hours at 4°C. After rinsing, sections were incubated with goat anti-mouse IgG conjugated to Cy3 (Amersham) diluted 1:1000 in PTG. Sections were mounted with PBS/glycerol and analysed with a TCS laser scanning confocal imaging system equipped with a krypton-argon ion laser (Leica). Comparison was performed analysing equivalent fields of dorsocentral retinas taken from frontal sections through the optic nerve.

Cell death determination and quantification

To reveal pyknotic nuclei, retinal sections from E6 embryos were stained with either propidium iodide as described previously (Voyvodic et al., 1995) or bisbenzimide (1 µg/ml in PBS, 10 minutes incubation at room temperature). Slides were mounted in PBS glycerol and labelled nuclei were observed under epifluorescence microscopy at 1000× magnification. Only clearly condensed and/or fragmented nuclei were counted and their position within the retina determined. In addition, cells containing fragmented DNA were determined by TUNEL assay as previously described (Frade et al., 1996). Briefly, cryopreserved retina sections were washed with PBS and incubated with 0.1% Tween 20 in 0.1% sodium citrate. After washing with PBS, terminal nucleotidy transferase was used to incorporate a mixture of fluorescein-conjugated nucleotides into the nicked DNA (Boehringer Mannheim; cat. no. 1684817). After incubation with a horseradish peroxidase (HRP)-conjugated anti-fluorescein antibody, HRP products were visualized as described above. Cell death was quantified by ELISA (Boehringer Mannheim, cat. no. 1544 675) as described previously (Frade et al., 1996). Briefly, a combination of antibodies recognising histones and DNA, which allows the quantification of soluble nucleosomes in cell lysates, was used. Retinae were homogenised in 200 µl PBS containing 1 mM phenylmethylsulfonyl-fluoride and centrifuged at 20,000 g for 10 minutes. An aliquot of the supernatant was used to quantitate proteins by standard methods and the rest was diluted 1:10 in the supplied buffer and processed as described by manufacturers. Absorbance values ranged between 0.1 and 0.7, and were normalised with respect to the values obtained with control retinas.

Electron microscopy

E9 embryo heads were fixed in cacodylate buffer, pH 7.3, containing 3% glutaraldehyde and 1% formaldehyde. The heads were washed in PBS and the optic nerves dissected out together with the optic fissure, processed for plastic embedding according to De Felice and Fairén (1993) and Bovolenta et al. (1996). Nerve cross-sections were obtained from the region midway from the optic stalk and the entrance to the cranial cavity. The total number of optic nerve fibres of E9 embryos was calculated by multiplying the cross-sectional area of the sections by the mean fibre density (Rager, 1980). The cross-sectional area was measured on semi-thin sections with the aid of an Argus-10 Image Processor System coupled to a Diaphot inverted microscope (Nikon). Approximately 50 micrographs were taken for each sample at the same magnification of 15,000×. The number of axonal profiles present in each micrograph was determined and the mean density calculated.

RESULTS

Early and late periods of cell death in the developing retina

To determine precisely the occurrence of cell death during the development of the neural retina, we first used a detection method based on a two-site immunoassay to measure the levels of nucleosomes released to the cytoplasmatic fraction (Frade et al., 1996). As shown in Fig. 1A, cell death occurs in two distinct periods during development. The early period, peaking at E6-7, coincides with the main onset of neuron differentiation (Prada et al., 1991), whereas the second phase, peaking at E12, is coincident with the well-described period of PCD undergone by RGCs at the time when their axons invade the optic tectum (reviewed in Rager, 1980). Further, we applied the TUNEL assay to detect apoptotic cell death on retinal sections of three representative ages (Fig. 1B-D). At E6, interspersed TUNEL-positive nuclei were detected across the thickness of the pseudostratified epithelium, being more abundant in the inner half of the retina, but affecting few layered RGCs (Figs 1B, 2). These results, when compared with the morphological data available (Prada et al., 1991),
BDNF prevents early neural death

indicate that cells undergoing death are either proliferating neuroepithelial cells or postmitotic neuroblasts migrating to their final destination, but not fully differentiated neurones. By contrast, E8.5 retinal sections (Fig. 1C) showed very few labelled nuclei in accordance with the ELISA assay (Fig. 1A). As expected, the number of TUNEL-positive nuclei increased later in development and thus, at E12, labelled nuclei appeared in the RGC layer and in the inner nuclear layer (Fig. 1F), where amacrine and bipolar neurones are located. Interestingly, neither horizontal nor photoreceptor cells seemed to be affected by cell death at this stage since no apoptotic nuclei were observed in these layers. Although PCD has been detected in the E6-7 retinas (Hughes and McLoon, 1979), these studies were designed to detect only the death of layered RGCs and not that of other cells within the thickness of the retina. However, labelling of E6 sections with either propidium iodide or bisbenzimide revealed pyknotic nuclei within the thickness of the retina (Fig. 2A). Such nuclei appeared preferentially located in the central part and in the inner half of the retina, similar to the location of the TUNEL-positive cells (Fig. 2B). However, the number of TUNEL-positive nuclei was greater than that of pyknotic nuclei (216±52, n=3 versus 137±35, n=3) in each frontal section of central retina. Thus the TUNEL assay appears more sensitive since it is able to detect DNA fragmentation before morphological alterations are clearly visible in the nuclei.
Exogenously added BDNF prevents early PCD in living embryos

Much of the early retinal cell death at E6 is mediated by NGF acting through its p75 receptor (Frade et al., 1996). To investigate whether this early wave of cell death is also regulated by other neurotrophins, we treated young embryos with BDNF and NT-3. After treatment, the embryos were killed at E6 and the retinal extracts immunoassayed to quantify cell death. As shown in Fig. 3A, NT-3 did not affect the amount of cell death detected in control embryos. In contrast, treatment of the embryos with BDNF significantly reduced the degree of cell death in the neural retinas. This degree is similar to the one observed after neutralisation of endogenous NGF with specific antibodies (Frade et al., 1996). The survival activity of BDNF on retinal cells was confirmed by a dramatic decrease of TUNEL-positive cells in the E6 chick retina (Fig. 3B,C).

Finally, similarly to what was previously reported (Frade et al., 1996), the application of exogenous NGF did not affect cell death (Fig. 3A; see Discussion).

Expression of BDNF and the trkB receptor in early retinas

If BDNF has a physiologically relevant role in the control of the earlier phase of cell death, then it should be expressed together with its specific receptor TrkB at the appropriate location and time in the developing retina. To address this issue, we analysed the expression of bdnf and trkB genes at early stages of eye development. To date, both TrkB and BDNF mRNA have only been studied after E6 in the embryonic avian retina (Herzog et al., 1994; Hallböök et al., 1996; Garner et al., 1996; Strohmaier et al., 1996). By using RT-PCR, BDNF...
mRNA was detected predominantly in the pigment epithelium of E4 embryos, although expression at lower levels was also detected in the neural retina (NR) (Fig. 4A). RT-PCR amplification also showed that TrkB mRNA was expressed in the neural retina from E4 onwards (Fig. 4B). The primers used amplified the tyrosine kinase domain of TrkB and, therefore, it is expected that, from early in development, functional TrkB receptors exist in the neural retina.

To quantify the levels of BDNF high-affinity receptors, neural retinas from different embryonic ages were dissociated to single cell and used for binding experiments with 125I-BDNF. As shown in Fig. 4C, the number of high-affinity binding sites significantly changed during the course of retinal development. Between E5 and E8 binding remained at moderate levels and then decreased after E8. Receptors were increased again from E10 onwards. Thus, the expression of high-affinity BDNF receptors closely parallels the developmental pattern of cell death (Fig. 1A). These data together strongly suggest a physiological link between BDNF high-affinity receptor expression and cell death.

**BDNF controls the number of RGCs**

Based on morphological criteria, it was clear that many cells undergoing early cell death were not differentiated stratified neurons. Our data rather show that the pattern of cell death is consistent with that of late migrating RGCs in the central part of the retina (Prada et al., 1991). To verify that the cell rescued from death by BDNF are, at least in part, RGCs, we quantified these cells in retinal sections from control and BDNF-treated embryos using two specific RGC markers. Thus sections of E6 retinas were immunolabelled with either RA4 mAb specific for nascent RGCs (McLoom and Barnes, 1989; Waid and McLoom, 1995) or the anti-Islet-1/2 mAb specific for layered RGCs (Austin et al., 1995). The RA4 mAb detects RGCs at the time that they exit their last mitotic cycle (Waid and McLoom, 1995) although part of the labelling is lost as RGCs migrate to the vitreal surface. BDNF treatment did not affect the number of differentiated RGCs at the ventricular surface of the retina (Fig. 5A,B; Table 1). In contrast, BDNF treatment produced a 80% increase of the number of Islet-1/2 positive cells (Fig. 5A,B; Table 1). Hence, BDNF did not affect the generation of RGCs but, instead, it did increase the number of cells arriving at the vitreal surface. Taken together, these data indicate that BDNF rescued some of the migrating RGCs from death.

To further assess whether young RGCs are the target for the survival activity of BDNF, this factor was applied to embryos daily from E2.5 through E8 which were then killed at E9, by which time the RGC layer is nearly completely formed in the central retina. As shown in Fig. 6, the most evident alteration in retinas from BDNF-treated embryos was an increase in the thickness of the ganglion cell layer due to 90% increase in the number of cell bodies in this layer (Table 1). To find out how many of these cells were RGCs, the number of axons in the optic nerves of both control and BDNF-treated embryos were determined. The numbers of RGC axons increased by about 60% in the BDNF-treated embryos (Table 1), suggesting that the number of displaced amacrine cells was also increased in the RGC layer (see Discussion).

**DISCUSSION**

The data presented show that early cell death in the embryonic chick retina is regulated by BDNF via its TrkB receptor, to act as a survival factor for newly born RGCs before they project axons to their target area. These data, together with the findings that NT-3 regulates neuron differentiation (Bovolenta et al., 1996) and that NGF exerts a killing activity on retinal cells (Frade et al., 1996), reveal that these three neurotrophins exert a decisive control on retinal neurogenesis.

**Retinal cells undergo two developmental phases of death**

PCD has been previously observed at various ages of NR development and the presence of pyknotic nuclei in the NR has been shown in chick embryos from E2.5 until E4.5 (Cuadros and Rios, 1988). Later in development, up to 40-45% of RGCs die at E10-14 after their axons have arrived at the optic tectum (Rager, 1980). We have studied retinas between E4 and E14 during which period we could clearly resolve two distinct phases of retinal cell death. The first phase, peaking at E6 has not been studied before. Indeed, early studies on retinal cell death focused exclusively on layered RGCs where it is low at E6-7 compared with E12-14 (Hughes and McLoom, 1979). The availability of the TUNEL assay has enabled us to detect interspersed apoptotic nuclei in the central E6 retina during this early phase of cell death as previously reported (Frade et al., 1996). The validity of the TUNEL assay for the detection of cell death has been confirmed by a number of previous studies (Gavielli et al., 1992; Wood et al., 1993; Wijssmann et al., 1993; Surh and Sprent, 1994; Ben-Sasson et al., 1995). Furthermore, we have also measured cell death at this age by counting pyknotic nuclei stained by classical methods. Our result show that the distribution pattern of pyknotic nuclei is similar to that of TUNEL-positive nuclei, although the higher number of the

**Table 1. Retinal ganglion cells and optic nerve axons in control and BDNF-treated embryos**

<table>
<thead>
<tr>
<th></th>
<th>Control embryos</th>
<th>BDNF-treated embryos</th>
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<tbody>
<tr>
<td>E6 retinal sections; RA4-positive cells*</td>
<td>14.4±4.1 cells/field (n=2)</td>
<td>14.3±4.3 cells/field (n=2)</td>
</tr>
<tr>
<td>E6 retinal sections; Islet-1/2-positive cells</td>
<td>34.2±7.0 cells/field (n=2)</td>
<td>57.7±4.1 cells/field (n=2)</td>
</tr>
<tr>
<td>E9 retinal sections; cells in the ganglion cell layer</td>
<td>137.2±21.1 cells/field (n=4)</td>
<td>273.5±62.4 cells/field (n=4)</td>
</tr>
<tr>
<td>E9 optic nerve; retinal ganglion cell axons</td>
<td>2,877.024±51.576† (n=3)</td>
<td>4,596.546±193.270 (n=3)</td>
</tr>
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Cells were counted in frontal sections of the retinas. Sections selected contained the optic nerve. All cells were counted in similar zones of all retinas: ten different microscope fields (250 μm diameter) in the dorsocentral region were counted and their numbers, with less than 5% s. d., were averaged. The numbers obtained from different number of embryos (n) were averaged and are given in the table (±s.d.). The experimental details for counting optic nerve axons are given in Materials and Methods. Student’s t-test results are included in the table; N. S. (non significant).

*RA4-positive cells counted corresponded to those newly generated ganglion cells at the ventricular surface of the retina. At or after the last mitotic cycle the RA4 mAb labels most of the surface of the cells and this allows the counting of discrete cells, whereas during migration the label is partially lost and identification of individual cells would make the counting inaccurate (see Fig. 5A,B). Observe that BDNF treatment did not alter the number of newly generated ganglion cells.

†These numbers are in agreement with those of Rager (1980).
latter may reflect the onset of DNA fragmentation before pyknosis is evident (discussed in Vojvodic, 1996).

According to the data available on chick retinal morphogenesis (Prada et al., 1981, 1991), the position occupied by the apoptotic cells corresponds to either proliferating neuroepithelial cells or migratory postmitotic neuroblasts and not to differentiated stratified neurones. However, because the developmental profile of the early phase of cell death is coincident with the main onset of neuron birth (Prada et al., 1991), we conclude that PCD is likely to be associated with the differentiation of neurones shortly after they have left the cell cycle. After their last mitosis at the ventricular surface of the neuroepithelium, the postmitotic neuroblasts migrate across the NR to their final destination, which, in the case of RGCs, is the inner zone of the neuroepithelium. During differentiation, neurones go through a transition period characterised by the repression of genes associated with the cell cycle. Concomitantly, genes must be expressed that are necessary for the development or maintenance of the differentiated phenotype including the expression of specific receptors for survival factors (reviewed by Kageyama et al., 1995). Interestingly, Kranenburg et al. (1996) established a correlation between cyclin D1 activity and neuronal apoptosis and concluded that ‘neuronal apoptosis results from an aborted attempt to activate the cell cycle in terminally differentiated neurones’. Therefore, it is most likely that the early cell death in E6 retinas affects a fraction of postmitotic neuroblasts during migration to their respective layer. In this context, this death process would limit and provisionally control the initial number of postmitotic neurones. A substantial fraction of these dying cells are likely to be young RGCs, since the prevention of cell death by application of BDNF is paralleled by a substantial increase of RGCs in both E6 and E9 embryos without affecting their generation (see below).

In the retina, the early phase of cell death may counterbalance the overproduction of RGCs and ensure a correct number of stratified postmigratory neurones. Cell death associated with neuron differentiation has been also described across the dorsoventral axis of the developing neural tube by Homma et al. (1994), who interpreted these data in terms of phenotypic selection of specific progenitor cells. A biphasic occurrence of cell death has also been recently reported in the chick cervical spinal cord, affecting motor neurones (Yaginuma et al., 1996). However, the first wave of cell death appears to affect motor neurones at a stage of development different from that of RGC. Thus in the spinal cord, the first phase of PCD occurs at E4-5 and apoptotic nuclei were detected in the ventral horns, the final location of postmitotic motor neurones, whereas retinal cells seem to die before they reach their definitive position in the retina. Furthermore, all the factors known to rescue motor neurones from death in the late phase (purified brain and muscle extracts, neurotrophins, bFGF, CNTF, etc) failed to prevent the early phase of motor neuron apoptotic death. In contrast, our present data show that BDNF is clearly involved in the regulation of the early phase of PCD in the retina.

**BDNF, a survival factor for newly born RGCs**

We have concluded that BDNF activity rescues migratory RGCs from death based on the following observations: first, BDNF decreased early retinal cell death as determined by both the TUNEL and the nucleosome release assays; second, the increased number of RGCs is not the result of greater generation, since the number of RA4-positive newly generated RGCs at the vitreal surface was not affected by BDNF treatment; and, third, application of BDNF resulted in a 60-90% increase of the number of RCCs and ganglion cell axons in the optic nerve. Our data do not rule out the possibility that other retinal neurones may be also rescued by BDNF from early death.
Indeed, we have observed that, in E9 retinas from BDNF-treated embryos, the cells of inner nuclear layer were more compact and more densely stained by cresyl violet. Additionally, the number of migratory displaced amacrine cells also appeared higher in the treated embryos. The fact that we determined a 90% increase in the number of cells in the RGC layer and only a 60% increase of axon number in the optic nerve, supports the notion that the number of displaced amacrine neurones also increased upon BDNF treatment. However, here we have restricted our study to the differentiation of RGCs and only a 50% increase of axon number in the optic nerve.

By comparison with other neurotrophins, the pharmacological effect of BDNF in the prevention of the early phase of cell death appears to be specific. The other neurotrophins tested, NGF (Frade et al., 1996; this study) and NT-3 (this study), did not display trophic activity on cells from early developing retinas. The fact that exogenously applied NGF did not increase the number of dead cells has been discussed (Frade et al., 1996). It seems that this factor is able to kill cells that otherwise predispose to die, likely because of BDNF deprivation or the downregulation of TrkB expression that is apparent at the end of the early death period.

The survival action of exogenously added BDNF might be indirect. Indeed, exogenous BDNF might inhibit the binding of endogenous NGF to p75, since the neurotrophins bind to this receptor with similar affinity (Rodríguez-Tébar et al., 1990, 1992). However, this possibility can be ruled out since neither the application of NGF, nor that of NT-3, affects the levels of cell death (Frade et al., 1996; this study). Therefore, one of the causes triggering cell death in the retina, may be the low local concentrations of BDNF so that cells either unable to interact with sufficient amounts of BDNF, or having very low levels of its specific receptor TrkB become susceptible to the killing action of NGF. In this context, it is interesting to note that TrkB is downregulated at the end of the first phase of cell death.

In conclusion, early cell death in the retina appears to be regulated by the antagonistic actions of BDNF and NGF: NGF provokes PCD by interacting with p75, whereas BDNF prevents it, presumably by interacting with TrkB. While NT-3 does not affect PCD (this study), this factor also plays a crucial role on retinal neurogenesis (Bovolenta et al., 1996). Thus the orchestrated interactions of multiple neurotrophins govern the embryonic development of the retina.

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