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The spatial and temporal pattern of βNGF receptor expression in the developing chick embryo

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To gain insight into the developmental program of nerve growth factor (NGF) receptor expression, the binding of [^{125}I]βNGF to frozen chick sections was investigated autoradiographically between embryonic day 3 (E3) and post-hatching day 3. Strong NGF receptor expression was observed as early as E4, throughout embryonic development and in the post-hatching period at the classical NGF target sites: the paravertebral sensory and sympathetic ganglia, the paraaortal sympathetic ganglia as well as the cranial sensory ganglia with neurons of neural crest origin and their respective nerves. Only weak [^{125}I]βNGF binding was observed during a restricted time span in the parasympathetic ciliary ganglion. Clear differences were observed in the intensity and in the developmental time course of [^{125}I]βNGF binding to the dorsomedial and ventrolateral aspects of the dorsal root ganglia. NGF receptors were also found to be expressed on central axons of the dorsal root entry zone and the dorsal tract in the spinal cord. A transient expression of specific NGF binding sites of the same high affinity as measured at the classical NGF targets, was detected in the lateral motor column and in muscle at the time of motoneuron synapse formation and elimination.

Key words: βNGF/receptor expression/chick embryo/peripheral ganglia/muscle/lateral motor column

Introduction

The study of neurotrophic factors and their specific receptors on responsive neural target cells offers a key to the understanding of nervous system development. β Nerve growth factor (βNGF) is unique in its role as a model for neurotrophic factors. Sensory and sympathetic neurons are the classical targets of this well-characterized protein (Levi-Montalcini and Angeletti, 1968). The *in vivo* effects of NGF depletion were seen early on, when it was found that injection or induction of anti-NGF antibodies in mice, rats and guinea pigs resulted in the degeneration of sympathetic (Levi-Montalcini and Booker, 1960) and sensory (Johnson *et al.*, 1980) neurons. When βNGF was administered during the time of naturally occurring cell death within the sympathetic and sensory ganglia, neurons which normally would have died were rescued (Hendry and Campbell, 1976; Hamburger *et al.*, 1981). These *in vivo* experiments amply demonstrated a requirement for NGF for the survival of these neurons. A series of later *in vitro* experiments showed that the survival effect is a direct one exerted *via* binding of βNGF to a high affinity receptor in the cell surface membrane of the responsive neurons (Sutter *et al.*, 1979a). *In vitro* experiments also allowed a detailed analysis of the pleiotrophic effects of βNGF (Greene, 1984). More recently, *in vitro* systems have supplied the information that not only peripheral neurons but also peripheral glial cells express specific binding sites for βNGF at the time when neuron-glial cell engagement is initiated (Zimmermann and Sutter, 1983). NGF receptors were found in the developing CNS, too (Frazier *et al.*, 1974; Zimmermann *et al.*, 1978), and *in vitro* and *in vivo* studies have indicated that NGF can influence the levels of acetylcholine transferase there (Honegger and Lenoir, 1982; Gnahn *et al.*, 1983). NGF seems to affect parasympathetic neurons and even mast cells (Collins and Dawson, 1983; Bruni *et al.*, 1982). These newly detected potential targets appear not to be dependent on NGF in terms of survival. Instead, NGF or an NGF-like factor seems to control or modulate certain cellular differentiation characteristics. In order to study the potentially more widespread involvement of NGF in the program of neural development it is essential to obtain data on the timing and location of NGF production as well as on NGF receptor expression *in vivo*.

We have addressed the question of the when and where of NGF action during development from the site of the target cell by analysing the temporal and spatial distribution of NGF receptors in the developing chick embryo. The data described confirm and extend available information on sensory, sympathetic and parasympathetic ganglion development and provide a framework for the discussion of the NGF dependency of the development of these ganglia. They also reveal specific high affinity binding sites for NGF on embryonal tissues which have so far not been considered as targets for NGF action, namely skeletal muscles and the lateral motor column.

Results

βNGF receptors in sensory, sympathetic and parasympathetic ganglia and in peripheral nerves

Frozen sections from chicken taken at various stages of development between embryonic day 3 (E3) and post-hatching day 3 (P3) were autoradiographed following incubation with [^{125}I]βNGF as described in Materials and methods. Adjacent sections were used for immunofluorescence staining with anti-SGII-1 and anti-SGIII-1, two nervous system-specific monoclonal antibodies produced in our laboratory (Sutter and Zimmermann, 1983; and in preparation). These antibodies served as probes for sensory, sympathetic and parasympathetic neuronal/glial somata and for tracing nerve fibers in the periphery and in the spinal cord.

Already at E4, [^{125}I]βNGF labelling of the sensory and sympathetic ganglia was observed. As can be seen on the photomicrograph in Figure 1a, dorsal root ganglia (drg), dorsal roots as well as ventral roots and vertebral and paraaortal sympathetic ganglia were specifically labelled with [^{125}I]βNGF. The degree of non-specific labelling is shown in Figure 1b. Dorsal and ventral roots and sensory and sympathetic ganglia were delineated with anti-SGIII-1 antibody on an adjacent section (Figure 1b, insert). The binding of [^{125}I]βNGF to receptors in the ganglia described in this section and to receptors in muscle and spinal cord described in the following sections was determined to be of high affinity (approximate K_d 2 x 10^{-10} M, see Materials and methods). Bound [^{125}I]βNGF was displayed only by excess of unlabelled

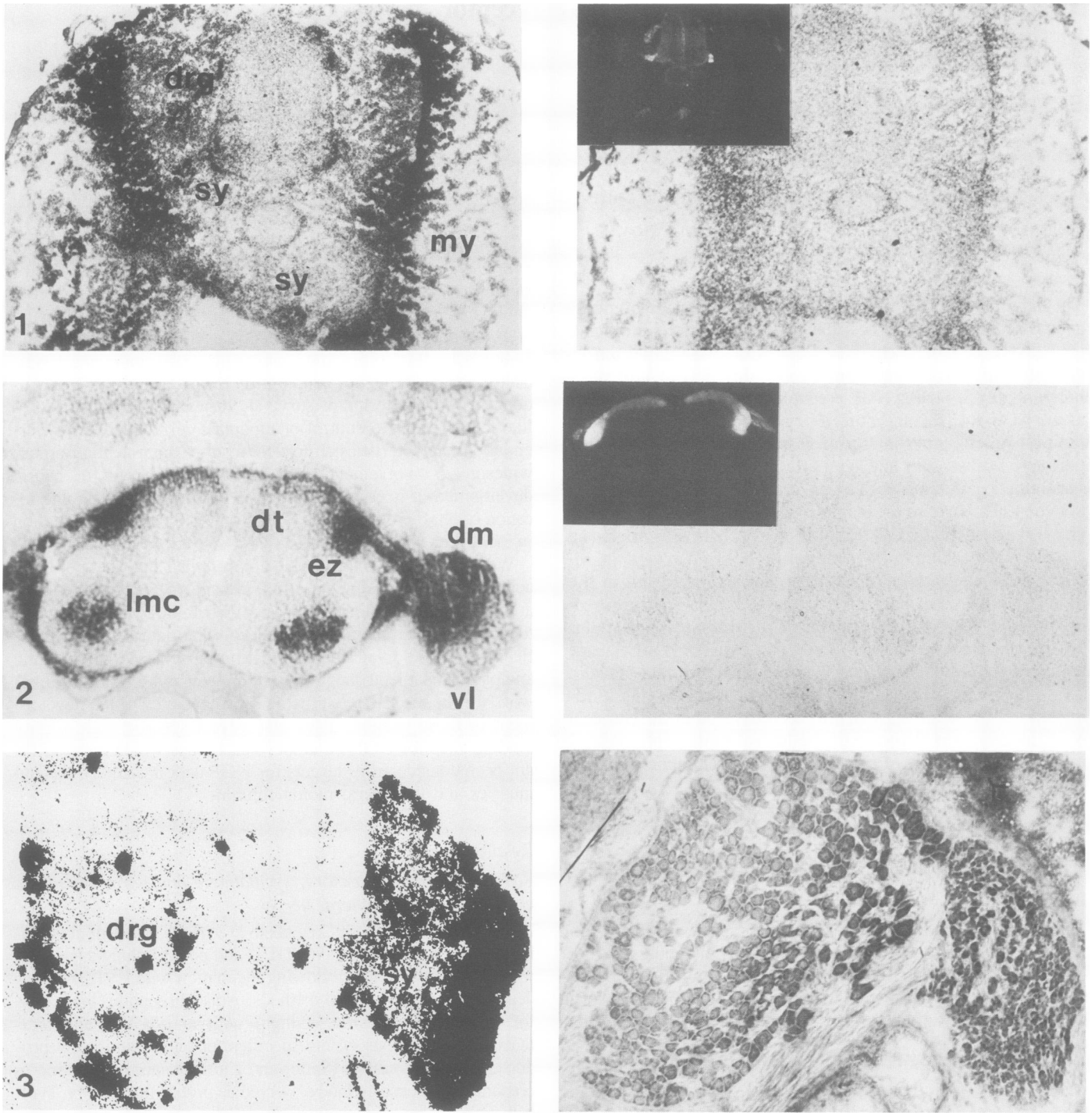


Fig. 1. E4. Transversal thorax sections. **(a)** [^{125}I]βNGF autoradiography as described in Materials and methods. [^{125}I]βNGF binding is especially strong to myotome (my), weaker to drg and sympathetic ganglia (sy) (paravertebral and paraaortal chain) (40 x). **(b)** [^{125}I]βNGF autoradiography of an adjacent section. [^{125}I]βNGF labelling was carried out in the presence of a 100-fold excess of unlabelled βNGF (control) (40 x). Insert: SG-III-1 immunofluorescence staining of an adjacent section. The sensory and sympapthetic ganglia are clearly delineated (25 x). Anti-SGIII-1 is a monoclonal antibody binding to a cell surface antigen represented on neurons and glial cells of the ganglia. Aside from the peripheral nervous system, anti-SGIII-1 also labels cells in the CNS.

Fig. 2. E8. Transveral lower cervical section. **(a)** [^{125}I]βNGF autoradiography. Strong labelling of the lmc, the ventral and dorsal roots, the dorsomedial part of the spinal ganglion (dm) and the dorsal root entry zone (ez). Weak to moderate labelling of the dorsal tract (dt), muscle, and the ventrolateral part of the spinal ganglion (vl) (40 x). **(b)** Control (40 x). Insert: SGII-1 immunofluorescence delineates the dorsal roots, the dorsal root entry zones and the dorsal tracts. Other parts of the spinal cord are unstained (25 x). Anti-SGII-1 is a monoclonal antibody binding to neurons of the ganglia only. At E8 essentially all sensory neurons are SGII-1 positive.

Fig. 3. P3. Transversal section of the spinal and sympathetic ganglia (paravertebral chain) at the upper thoracal level. **(a)** [^{125}I]βNGF autoradiography (100 x). Labelling of the drg is restricted to a fraction of the neurons of predominantly dorsomedial positions. More homogeneous labelling of the sympathetic ganglion (sy). **(b)** Giemsa staining of an adjacent section (100 x).

Table I. Relative intensities of specific [^{125}I]βNGF binding to various tissues of the developing chick embryo

	E3	E4	E6	E8	E10	E12	E14	E16	E20	P0	P3
Peripheral ganglia and nerves											
dm				+++	+++	+++	++	++	nd	+	+
drg	nd	+[a]	++[a]								
vl				+	+	+	+	(+)	nd	0[b]	0[b]
Sympathetics	nd	++	+++	+++	+++	+++	+++	+++	++	++	++
Ciliaris (parasympathetic)	nd	nd	nd	(+)	nd	0	nd	0	nd	0	0
Somatic nerves	nd	++	+++	+++	++	++	+	(+)	0	0	0
Visceral nerves	nd	++	+++	+++	+++	++	++	++	+	+	+
Adrenal anlage	nd	nd	+++	+++	+++	nd	nd	nd	nd	nd	nd
Spinal cord											
Meninges	0	0	+	+	+	+	+	+	+	+	+
Dorsal root entry zone	0	+	+++	+++	+++	+++	++	++	+	+	+
Dorsal tract	0	0	+	+	(+)	(+)	0	0	0	0	0
Lateral motor column	0	+	+++	++	+	(+)	0	0	0	0	0
Anterior roots	0	+	+++	+	(+)	0	0	0	0	0	0
Muscle											
Skeletal muscle	(+)	+++	+++	+	(+)	0	0	0	0	0	0
Skeletal muscle circumference	0	0	0	0	(+)	+	+++	++	0	0	0

An arbitrary grading from +++ = very strong, ++ = strong, + = moderate to (+) = faint to weak was used to describe the rise and fall of specific NGF binding sites in the various tissues. This grading reflects both the intensity of the labelling and the size of the labelled area (e.g., as detailed in the text, + in the case of the vl region stands for a moderate diffuse labelling of the whole vl area, while + in the dm region stands for a strong labelling of scattered individual cell profiles).

[a]At E4 and E6 the labelling the vl and dm region of the drg was not evaluated separately.

[b]The vl region appears unlabelled at P0 and P3 with the exception of very few strongly labelled cell profiles.

βNGF but not by proteins which are structurally related to NGF like insulin and relaxin or similar in isoelectric point and mol. wt. like cytochrome C. In parallel with the development of the peripheral nervous system, the specific binding of [^{125}I]βNGF to sensory and sympathetic ganglia and peripheral nerves increased. The labelling was very prominent with respect to the size of the labelled area and the labelling intensity between E6 and E12 with a peak around E8. Table I lists the relative levels of specific [^{125}I]βNGF binding to the ganglia and other receptor-positive tissues throughout development.

From developmental studies it is known that there are two populations of neurons in the sensory ganglia which develop on a different time scale. One positioned dorsomedially and the other ventrolaterally (Levi-Montalcini and Angeletti, 1968; Hamburger *et al.*, 1981). It was interesting to note that the distribution of NGF binding sites within the drg was not uniform. Figure 2a shows the labelling pattern of the ganglion at E8. In the dorsomedial (dm) region of the ganglion heavily labelled, densely packed profiles could be seen while the ventrolateral (vl) region was only moderately and diffusely labelled with the exception of a small number of strongly labelled individual cell profiles. The moderate and diffuse labelling component of the vl region was all but gone at E16. The strong labelling component persisted into the post-natal period when heavily labelled individual cell profiles could be observed. From E14 onward strong labelling became gradually restricted to a smaller population of cells predominantly distributed in the dm region with only few NGF receptor-positive cells to be found in the vl region. Figure 1c shows the distribution of NGF receptor-positive ganglion cells at P3.

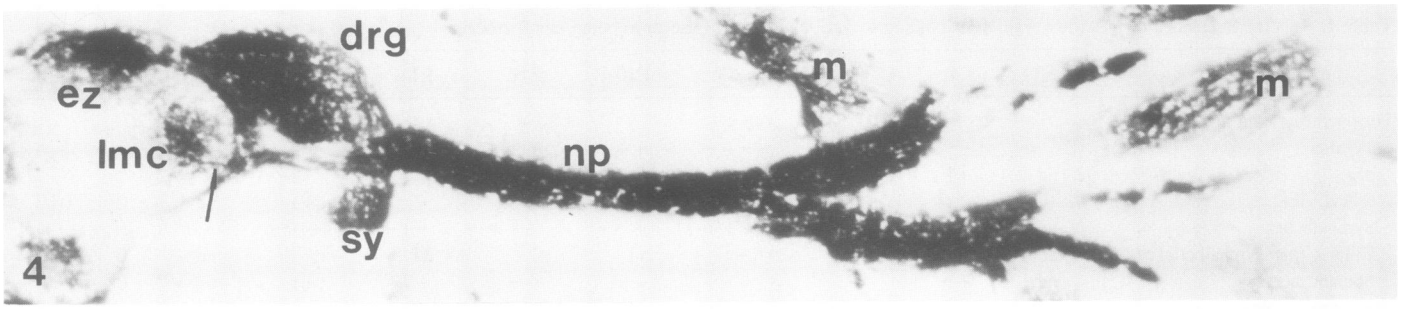
The same temporal and spatial pattern of NGF receptor expression seen in the drg, was also observed in the cranial sensory ganglia, with neurons of neural crest origin, but not in the ganglia containing neurons of placodal origin. A detailed report of the results obtained for the various cranial sensory ganglia will be published separately.

Sympathetic ganglia were also found to be NGF receptor positive from E4 to P3, the latest stage tested. In contrast to the uneven distribution of NGF receptors in the drg, the sympathetic ganglia showed a relatively homogeneous labelling throughout embryonic development. Sections of post-natal stages (see Figure 3) showed strong to moderate labelling of the ganglion in the area of the cell somata, and weak or no labelling of the nerve fibers entering the ganglion. The adrenal anlage was found to be strongly labelled at E6, E8 and E10 (Table I).

Purely parasympathetic ganglia (e.g., ciliaris), which are not considered to be classical NGF targets, appeared NGF receptor negative at E12 and E20. A slight [^{125}I]βNGF labelling above background was observed, however, at E8 in the ciliary ganglion (Table I).

Receptors on peripheral nerves could be traced both ways, from the ganglia into the spinal cord and out into the peripheral target areas. Figure 4 depicts NGF receptors of the upper limb nerve plexus on a longitudinal wing section at E8. In Figure 5a, a wing cross-section of the same developmental stage, several sectioned nerves are visible as [^{125}I]βNGF-labelled dark spots. The series of dark spots below the skin represent NGF receptor-positive skin nerves. They could all be stained with anti-SGIII-1 antibody in indirect immunofluorescence (data not shown). Cross-sections of peripheral nerves often showed strongly labelled fiber bundles surrounded by weakly labelled fibers (data not shown). In contrast to the persistent labelling of the ganglia, somatic nerves and skin nerves were no longer labelled at late developmental stages. At E14, their labelling was clearly reduced, it was barely discernible at E16 and it was gone by E20 (Figure 8b). Binding of [^{125}I]βNGF to visceral nerves and to nerves innervating the meninges (Figure 8a) could still be seen at the time of hatching.

Neural crest derived cranial skeletal and mesenchymal elements did not show specific binding of [^{125}I]βNGF (E8-P3) (data not shown).



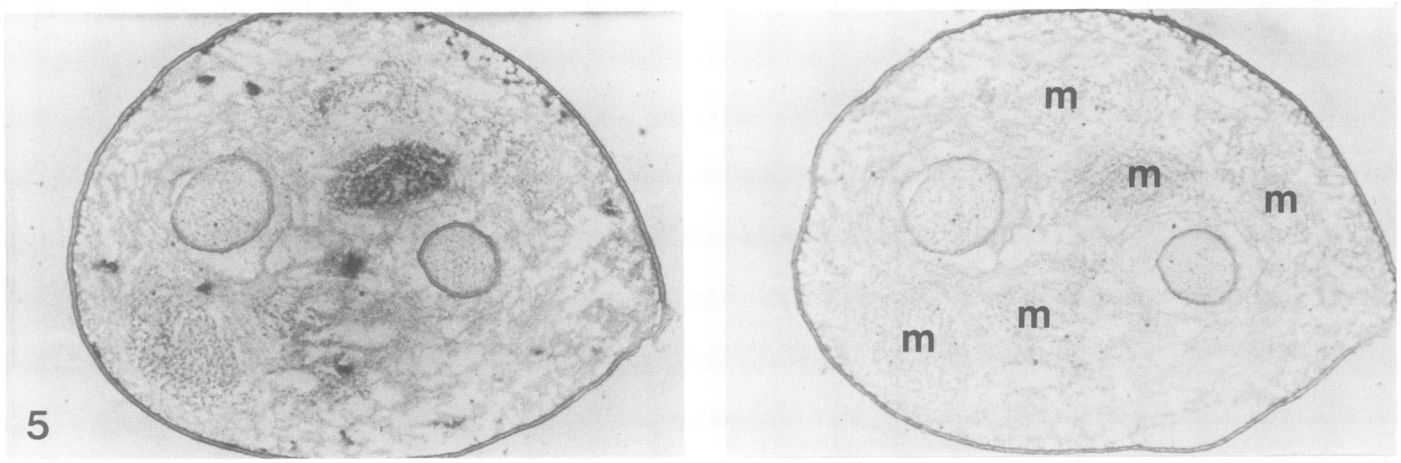


Fig. 4. E8. Transversal neck section including part of the wing. [^{125}I]βNGF autoradiography (30 x). The upper limb nerve plexus (np) is strongly labelled along its entire length. The wing muscles are more or less homogeneously labelled. Labelling of the lmc is stronger in its medial aspect. The arrow points to a labelled motoneuron fascicle.

Fig. 5. E8. Transversal section of distal wing. **(a)** [^{125}I]βNGF autoradiogrpahy (40 x). Note the labelling of small, purely cutaneous and larger, mixed nerves. Of several muscles (m) only one appears clearly labelled. **(b)** Control (40 x).

Fig. 6. E15. Part of a transversal section of distal wing. **(a)** [^{125}I]βNGF autoradiography (45 x). Note the strong labelling at the muscle-connective tissue border. **(b)** Control (45 x). **(c)** SGIII-1 immunofluorescence staining (see Figure 1) of cutaneous and muscular nerves (45 x).

Expression of βNGF receptors in muscle

The most intensively labelled tissue on autoradiographs of E4 embryo sections was in the position of the myotomes (Figure 1a). Already at E3 some labelling could be observed there. NGF binding to muscle was found to be developmentally regulated. NGF receptor expression was transient and a remarkable change in the pattern of receptor distribution was observed (Table I). [^{125}I]βNGF was found to bind to skeletal muscle in all parts of the chick embryo. Following a period of heavy labelling between E4 and E6, [^{125}I]βNGF binding to muscle declined. At E8, the labelling of different muscles was graded from strong over weak to none. Examples of muscle labelling at E8 are given in Figures 2a, 4 and 5). As can be seen on the autoradiograph of an E8 wing section, one muscle is strongly labelled, while others appeared only weakly labelled or unlabelled (Figure 5). At E12 labelling of skeletal muscle was all but gone. With the disappearance of the binding sites on the muscle mass, specific binding of [^{125}I]βNGF to a defined zone at the muscle-connective tissue interface became discernible. This is demonstrated in Figure 6a which shows an autoradiograph of an E15 wing section. The immunofluorescence staining with anti-SGIII-1 antibody in Figure 6c delineates the peripheral nerves on an adjacent section. [^{125}I]βNGF and anti-SGIII-1 antibody do not bind to the same sites. This, together with the observation that the circumference of the muscle seems to be labelled at all levels sectioned, indicates that it is not nerves or nerve endings but rather a more ubiquitous structure at the border of muscle and connective tissue which is labelled. As this structure does not appear to be cellular, it may represent basement membrane. [^{125}I]βNGF binding at the muscle-connective tissue border was especially prominent between E14

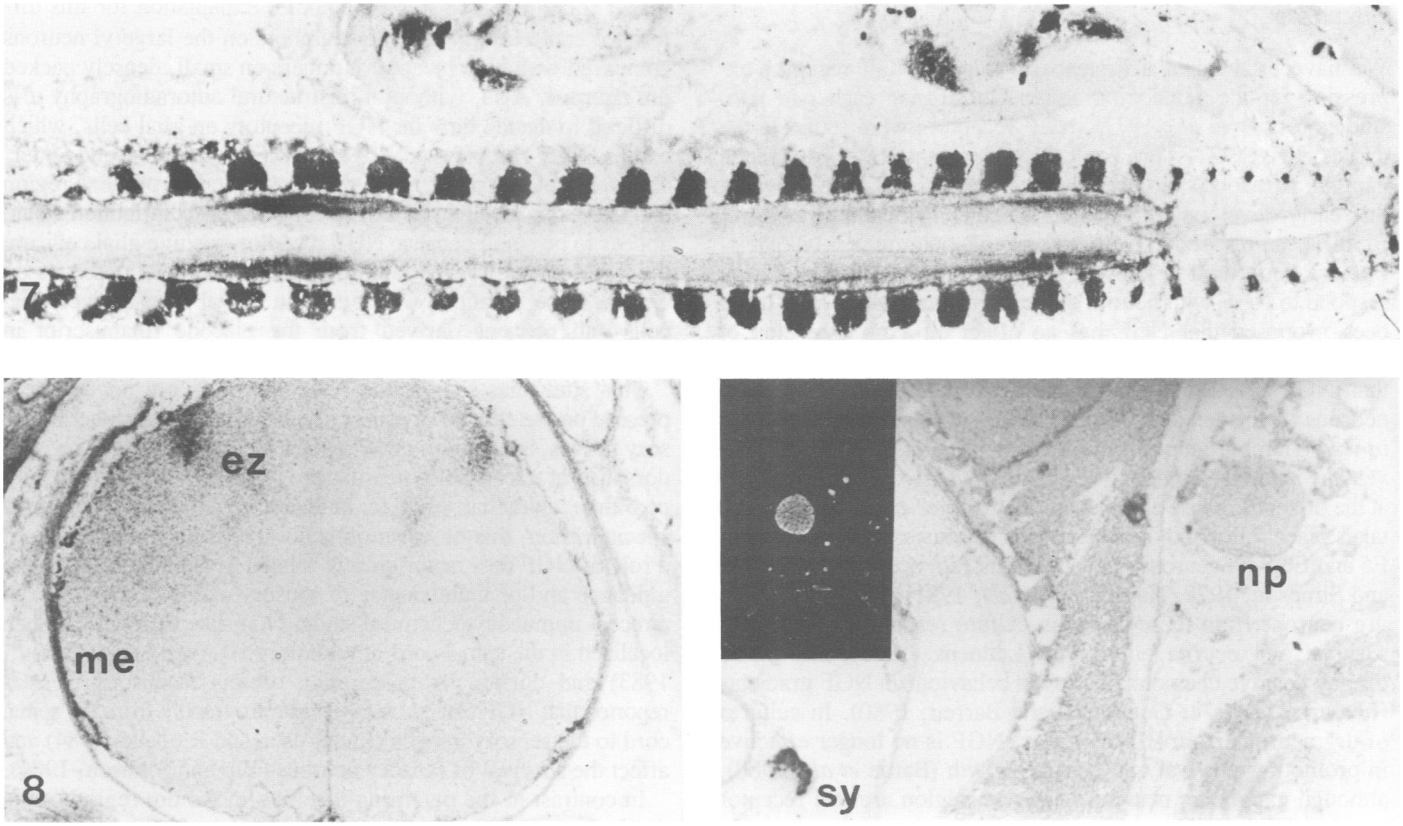


Fig. 7. E8. [^{125}I]βNGF autoradiography of a frontal whole body section through spinal cord and drg (12 x). Note the expansion of the labelled lmc in the region of the upper and the lower limb segments.

Fig. 8. E20. Transversal lower neck section. **(a)** [^{125}I]βNGF autoradiography (40 x). Note the loss of labelling of the lmc and the dorsal tract, and the persisting [^{125}I]βNGF binding to the lateral aspect of the dorsal root entry zone (ez) and to the meninges (me). **(b)** [^{125}I]βNGF autoradiography (40 x). The upper limb nerve plexus (np) is unlabelled; the sympathetic ganglion (sy) (paravertebral chain) is strongly labelled. Insert: SGIII-1 immunofluorescence staining (see Figure 1) of the nerve plexus and several smaller nerves in the direct vicinity (16 x).

and E16. It was no longer discernible at E20 (Figure 8b).

Expression of βNGF receptor in the spinal cord

Already at E4, a first indication of specific [^{125}I]βNGF binding was observed on autoradiographs of transverse sections in both the anterior and the posterior parts of the spinal cord (Figure 1a). Figures 2a and 4 show three areas in the spinal cord distinctly labelled at E8, the dorsal root entry zones, the dorsal tracts and the areas of the lateral motor columns (lmc). In the posterior spinal cord the dorsal root entry zones and dorsal tracts, delinated by anti-SGII-1 antibody (Sutter and Zimmermann, 1983; and in preparation) in immunofluorescence (Figure 2b), were differentially labelled with [^{125}I]βNGF. The moderate to weak labelling of the dorsal tracts disappeared later in development (E14). Labelling of central drg axons in the dorsal root entry zone of the spinal cord, which was strong at E8, persisted throughout development. Figure 8a depicts a spinal cord section at E20. Similar to the reduced [^{125}I]βNGF binding to the drg late in development, labelling of the dorsal root entry zones decreased at later stages. Throughout development neither [^{125}I]βNGF autoradiography nor anti-SGII-1 immunofluorescence depicted the sensory afferents passing through the grey matter to the lmc.

The size of the lmc area in the anterior spinal cord labelled with [^{125}I]βNGF was variable along the rostral caudal axis of the embryo (see Figure 7). Transverse sections showed that the labelled area was particularly large at the level of the cervical and lumbar intumescence while at other levels little or no labelling was to be found. The representation of [^{125}I]βNGF binding sites thereby appeared to correlate with the pattern of motoneuron representation in the anterior spinal cord. No labelling of the visceral motoneuron area was seen at any time in development. The pattern and the intensity of [^{125}I]βNGF binding in the anterior spinal cord was found to be developmentally regulated. It is interesting to note that the time course of [^{125}I]βNGF binding to the lmc paralleled the time course of receptor expression in muscle, and that, with the sequential loss of NGF receptors from various muscles, the pattern of labelling in the lmc appeared inhomogeneous. Labelling of the lmc was prominent between E6 and E8, reduced at E10, and weak by E12. It disappeared at the stage at which the labelling pattern on muscle switched from a rather ubiquitous distribution within the muscle to a restricted distribution at the muscle-connective tissue border (Table I).

The representation of specific binding sites for NGF in the brain is not the topic of this report. Specific though weak labelling was observed in various parts of the brain during early development (data not shown). The intensity of the labelling was comparable with the labelling seen in parasympathetic ganglia (e.g., ciliaris). The relatively high non-specific binding of [^{125}I]βNGF to brain sites makes the analysis there more difficult. In contrast to the weak specific binding of [^{125}I]βNGF in various brain regions, the entry zones of the axons of cranial neural crest derived sensory neurons into the brain stem were labelled as strongly as the dorsal root entry zones in the spinal cord.

Discussion

We have established a time/space map of NGF receptor expression for the developing chick. Confirming earlier *in vitro* studies (Sutter *et al.*, 1977), NGF receptors were found in the drg as early as E4. We could also show that the sympathetic ganglia, paraaortal and paravertebral, express NGF receptors at this early developmental stage. Whether sympathetic neurons from E4 can respond to NGF with neurite outgrowth is unknown. For drg neurons it is known, however, that at E4 they do not respond to NGF with neurite outgrowth (Luduena, 1973). It has been proposed that NGF has no effect on axon extension of neurons prior to target cell innervation and that a factor other than NGF is required for the initial axonal outgrowth of these neurons (Lumsden and Davies, 1983), which takes place at E4 *in vivo* (Hamburger and Levi-Montalcini, 1949). NGF can prevent the naturally occurring cell death of vl as well as dm neurons at the time of synapse elimination (Hamburger *et al.*, 1981) which takes place following the innervation of sensory targets between E5 and E6.5 (vl neurons) and around E9 (dm neurons) (McMillan and Simpson, 1978; Hamburger *et al.*, 1981). In concordance, drg neurons from these stages in culture respond to NGF with survival and neurite outgrowth (Luduena, 1973), and at E8 display positive chemotactic growth behaviour in NGF gradients (Letourneau, 1978; Gundersen and Barrett, 1980). In cultures of drg neurons from E16 and later, NGF is no longer effective in promoting survival and neurite growth (Barde *et al.*, 1980), although ganglionic neurons in the dm region are still receptor positive. With NGF receptor expression on drg neurons as early as E4 and as late as P3, this study has shown the presence of receptors *in vivo* before, during and after target cell innervation. Which of the pleiotrophic effects of NGF are exerted *via* NGF receptors expressed on sensory neurons early – prior to target cell innervation – remains to be seen. In the period following the formation of the proper synaptic connections, NGF still seems to be necessary for maintaining neuropeptide production in certain sensory neurons (Otten *et al.*, 1980).

It is of interest to note that *in vitro* on dissociated drg neurons two forms of the NGF receptor have been characterized: the high affinity form ($K_{d(I)} = 2 \times 10^{-11}$ M) and a form of lower affinity ($K_{d(II)} = 2 \times 10^{-9}$ M) (Sutter *et al.*, 1979b). At E4 sensory neurons bind NGF with the characteristics of site II (Sutter *et al.*, 1977). At the time, when drg neurons *in vitro* become responsive to NGF in terms of neurite growth (E6) the receptor binding characteristics change from simple (site II binding) to complex (site II and site I binding) (Sutter *et al.*, 1977). In its high affinity form (site I) the NGF receptor is coupled to the cytoskeleton and following solubilization with detergent exhibits a higher mol. wt. than site II (Sutter *et al.*, 1984). NGF bound to site I might exert a direct influence on cytoskeletal organization in neurite growth and chemotaxis. For reasons detailed in Materials and methods, it was not possible to differentiate on tissue sections between the two binding affinities of NGF found on viable neurons. The approximate K_d of the strong [^{125}I]βNGF binding on ganglia, lmc and muscle was 2×10^{-10} M, a value in between the K_d values determined for site I and site II. The labelling intensity of the dm region of the drg is much higher than that of the vl region. One could speculate that NGF binds with simple (site II) characteristics to vl neurons and with complex (site II and site I) characteristics to dm neurons – thus affecting neurite outgrowth and neurite guidance only in the latter. A difference in the K_d of [^{125}I]βNGF binding could be one reason for the difference observed in the labelling intensities of the vl *versus* the dm region in drg. Another explanation for this difference could be low receptor numbers on the large vl neurons compared with high receptor numbers on small, densely packed dm neurons. Also, without ultrastructural autoradiography it is difficult to decide how far NGF receptors on glial cells, which express NGF receptors (site II) *in vitro* (Zimmermann and Sutter, 1983), might contribute to the moderate labelling of the vl region and the strong labelling of the dm region. This contribution would appear to be small because NGF receptor-positive glial cells are the likely source for the faint to weak labelling we observed on cranial sensory ganglia which contain neural crest-derived glial cells and neurons derived from the placode (manuscript in preparation).

This study has shown that NGF receptors are not only expressed on the somata of sensory neurons and on peripheral sensory nerves, but also on growing central axons of the dorsal and dorsolateral tracts. Since neurons are probably even better equipped than epithelial cells to maintain a polarized membrane specialization, this observation is not trivial but is indicative of a role of NGF or a factor closely related to NGF in the growth, guidance and/or maintenance of sensory axons in the CNS. In a recent immunocytochemical study, NGF-like material has been localized in the spinal cord of rat embryos (Ayer-Lelievre *et al.*, 1983) and during the preparation of this manuscript it was reported that NGF can be transported retrogradely from the spinal cord to the sensory ganglia (Richardson and Riopelle, 1984) and affect the survival of sensory neurons (Yip and Johnson, 1984).

In contrast to the persisting labelling in the dm region of the drg and on central sensory axons in the dorsal root entry zone, peripheral somatic nerves as well as skin nerves were virtually unlabelled late in development. This could be due to an asymmetric distribution of NGF receptors or, maybe more likely, to an insufficient sensitivity of the autoradiographic method used for recording the label of the reduced number of NGF receptor-positive thin unmyelinated fibers of nociceptive and thermosensitive neurons, scattered among the many NGF receptor negative myelinated fibers in the developed peripheral nerve. The developmental stability of NGF receptor expression on some dm neurons in the drg, also observed in adult rats (Raivich, unpublished data), may be a prerequisite for the observed retrograde axonal transport of NGF from the periphery to a small fraction of sensory neurons in adult animals (Stoeckel *et al.*, 1975) and the effect the injection of anti-NGF antibodies has on the production of neuropeptides in sensory neurons of newborn rats (Otten *et al.*, 1980).

Unlike sensory ganglia, sympathetic ganglia and nerves were found to sustain quite high levels of [^{125}I]βNGF binding until late in embryonic development and post-hatching. This is consistent with the observed prolonged sensitivity of rat sympathetic neurons to the injection of βNGF antibodies. Exposure of newborn rats to anti-NGF antibodies dramatically reduces the number of neurons in the sympathetic ganglia, but does not affect neuron numbers in the sensory ganglia (Levi-Montalcini and Angeletti, 1968). With the demonstration of NGF receptors on central sensory afferents these results can be interpreted. Late in development, when the blood brain barrier has formed, sensory neurons, in contrast to sympathetic neurons (the processes of which do not reach into the spinal cord), could tap a central source of NGF which cannot be neutralized by circulating anti-NGF antibodies.

NGF transforms embryonic adrenal medulla cells into sympathetic neurons (Luigi and Levi-Montalcini, 1979). It was not unexpected, therefore, to observe NGF receptor expression in

the suprarenal gland anlage. Neither was it unexpected to find faint to weak diffuse labelling in the ciliary ganglia and in the cranial sensory ganglia whose neurons are derived from the placode, and which, though not containing NGF-dependent neurons, have been shown *in vitro* to contain NGF receptor-positive non-neuronal, flat cells with neuronal and glial properties (Rohrer and Sommer, 1983). The absence of receptors on neural crest-derived cranial skeletal and mesenchymal elements underlines that NGF receptors are not a general marker of neural crest-derived cells.

The strong and developmentally regulated NGF receptor expression in the lateral motor column and in muscle was unexpected. Particularly, the [^{125}I]βNGF labelling of muscle, which is a mesodermally derived tissue, breaks with the so far exclusively neuroectodermal pattern of NGF receptor expression. The [^{125}I]βNGF binding sites on muscle and lmc could be due to a receptor system closely related, but not identical, to the NGF receptors on sympathetic and sensory neurons. It should be stressed, however, that in competition experiments the binding affinity for [^{125}I]βNGF on muscle and lmc did not seem to be different from the one on drg. Final proof for the identity or relatedness of the receptors on muscle, motoneurons and sensory and sympathetic neurons can only come from a molecular analysis of the receptors.

NGF receptors are co-expressed on interacting cell types, namely on sensory neurons and glial cells at the time when neuron-glial cell engagement is initiated (Zimmermann and Sutter, 1983). From the data described here it appears that during the time of synapse formation and elimination, NGF receptors are represented on three interacting systems: sensory neurons, motoneurons and skeletal muscle. Sensory endings might contribute to the [^{125}I]βNGF labelling observed in muscle and lmc. They do not appear, however, to be the only or even the main cause for the [^{125}I]βNGF binding observed there. At the time when NGF receptors were lost from muscle and lmc during development, no corresponding change in [^{125}I]βNGF labelling of sensory ganglia and central and peripheral axons was observed. [^{125}I]βNGF binding on the mytome was already seen at E3. The first sensory fibers reach the myotomes between E3.5 and E4 (Hamburger and Levi-Montalcini, 1949) and the first retrograde axonal transport of HRP injected into muscle is observed around E4.5 (Openheim and Heaton, 1975). It is unlikely that the sensitivity of the autoradiographic method used is sufficient to visualize the first sensory endings reaching the myotome. Only if the myotome and early muscle Anlage contained a rather homogeneous, profusely arborized web of sensory endings – for which there is no evidence so far – with an exceptionally high NGF receptor density compared with that of the neuronal somata in the ganglia, could the labelling of muscle be envisaged to be due to sensory nerve endings. Later in development the immunofluorescence studies with nervous system specific monoclonal antibodies showed that the [^{125}I]βNGF labelling of the muscle-connective tissue border could not be due to sensory nerves or their terminals. There also appears to be no significant contribution of sensory nerve endings to the strong [^{125}I]βNGF binding to the lmc, though extrapolating from HRP backfilling experiments in rat embryos (Smith, 1983) sensory afferents should have reached the lmc in the chick at the time, when strong [^{125}I]βNGF binding was observed there. In the spinal cord sensory axons were equally well visualized by [^{125}I]βNGF autoradiography and by SGII-1 immunofluorescence. Since at E8, SGII-1 stains essentially all neurons of the sensory ganglion, one would have expected similarly strong labelling of the lmc with anti-SGII-1 as with [^{125}I]βNGF, if the labelling with [^{125}I]βNGF was due to sensory nerve endings. This, however, is not the case. Anti-SGII-1 strongly binds to the dorsal root entry zone and dorsal tract but not to the lmc. A further argument for NGF receptor expression on motoneurons and their axons is the heavy [^{125}I]βNGF labelling of ventral roots which parallels that of the lmc. If the labelling of the ventral roots was due to sensory afferents, which have been shown to be represented there in adult cats (Chung *et al.*, 1983) and not to motoneurons, such sensory afferents in the ventral roots must constitute a sizeable proportion of drg-derived axons. This would be in contradiction to the evidence from immunofluorescence stainings with anti-SGII-1 which showed no labelling of the ventral roots.

It is intriguing that NGF receptors are expressed in the lmc and in muscle at the time of motoneuron-muscle synapse formation and motoneuron cell death following synapse elimination. Published data suggest that NGF does not function as a survival factor, since application of βNGF during the time of motoneuron cell death did not cause increased motoneuron survival (Oppenheim *et al.*, 1982). Brunso-Bechtold and Hamburger (1979) have reported that NGF is transported retrogradely from the muscle into the drg at E10. NGF did not reach the motoneurons in the spinal cord in these experiments. However, at this stage, NGF binding sites are already lost from most muscles and motoneurons. It would be interesting to obtain results from such a retrograde transport experiment at an earlier time in development such as E6. It should also be investigated whether NGF has a myotrophic effect or influences muscle differentiation during early development. Such a NGF effect on muscle has been suggested by Radeva (1978) in the newt following the observation that NGF enhances muscle differentiation prior to innervation. The NGF receptor-positive lining which appears transiently at the muscle-connective tissue border but not at other locations rich in extracellular matrix is intriguing. Its appearance does not seem to be an immediate consequence of the loss of labelling of the muscle because at E8 some muscles can already be seen devoid of NGF receptors and no [^{125}I]βNGF positive border at the interphase to the connective tissue has formed. In the context of neuromuscular differentiation NGF receptors become represented at the muscle-connective tissue interface at a time when major changes in the physiology of the developing muscle can be followed by electromyograms of chick embryos (Böethius, 1967). It will be of interest to determine which cells produce the NGF receptors localized in the basement membrane-like structure positioned between muscle and connective tissue and to analyze what their physiological significance might be.

In conclusion, autoradiographic procedures applied to tissue sections provide insight into the spatial and temporal expression of NGF receptors in the developing chick embryo. We have described the rise and fall of NGF receptor levels in the classical target tissues of NGF – the sympathetic ganglia and the sensory ganglia and their central and peripheral nerves – and have shown developmentally regulated specific high affinity binding of [^{125}I]βNGF to muscle and the lmc, tissues which are thought not to be affected by NGF or an NGF-like factor in their development. The data on the timing of NGF receptor expression in the sensory ganglia add substance to the discussion that the role of NGF in the development of its neuronal targets is not restricted to its function as a neurotrophic factor, when synaptic contacts to peripheral and central targets have already been made. The finding of specific high affinity binding sites for NGF in muscle and in the lmc certainly warrants a detailed analysis and may well add new perspectives to research on neuromuscular differen-

tiation. A better understanding of how the production of NGF or other growth and differentiation factors and their respective receptors is regulated will play a key role in solving some of the puzzles of developmental neurobiology.

Materials and methods

Embryo sections

Fertilized chicken eggs (White Leghorns, Hühnerfarm von Bernuth, Berlin) were incubated in an egg incubator (Vomo 5) at 38°C and the development of the chick embryos staged according to Lillie and Hamilton (1952). Embryos were positioned on a metal block and frozen with expanding CO_2 gas. Sections of 10 μm were cut at −23°C in a Kryostat (SLEE, Mainz, FRG), collected on warm glass slides, stored at −30°C and used within 1 week.

Materials

βNGF was purified from the saliva of NMRI mice according to the procedure of Burton *et al.* (1978). The radioiodination of βNGF was performed as described (Sutter *et al.*, 1979b) with lactoperoxidase (Sigma) and ^{125}I (Amersham). The [^{125}I]βNGF preparations were used within 2 weeks. Relaxin was a gift from I. Greenwood (University of Hawaii). Insulin was purchased from Farbwerke Hoechst AG, bovine serum albumin (BSA) and cytochrome C from Sigma. Monoclonal antibodies against drg cells (SGII-1 and SGIII-1) were employed in the form of culture supernatants from the corresponding hybridoma clones raised in our laboratory (Sutter and Zimmermann, 1983).

Autoradiography

The sections, dried for 3 min at room temperature, were incubated in phosphate buffered saline (PBS), containing 0.1% BSA (PBS/BSA) for 30 min at 37°C. The sections were then incubated for 1 h at 37°C with 20 ng/ml [^{125}I]βNGF (sp. act. 50−60 c.p.m./pg) in PBS/BSA. The level of non-specific binding was tested in incubations with 20 ng/ml [^{125}I]βNGF and 5 μg/ml added unlabelled βNGF. Relaxin and insulin (20 μg/ml) and cytochrome C (1 mg/ml) did not compete for [^{125}I]βNGF binding. Following the incubations the slides were washed three times each with PBS/BSA and PBS at room temperature, fixed in 1% paraformaldehyde/ 2.5% glutaraldehyde for 30 min at 37°C, washed once each in PBS, PBS/BSA and PBS, dried and dipped in photoemulsion NTB2 (Kodak) diluted 1:1 with H_2O. The slides were exposed for 2 weeks at4°C, developed in Kodak D-19, and fixed in Kodak Rapid Fix. For storage at 4°C the slides were covered with 60% glycerin in PBS. Photographs were taken with Panatomic-X film (Kodak) on a Zeiss Photomicroscope III. For an approximate estimate of the affinity of specific [^{125}I]βNGF binding longitudinal sections of chick embryos (E8) with muscle, drg and spinal cord lmc were incubated with 0.6 ng/ml [^{125}I]βNGF and increasing amounts of unlabelled βNGF (0.3−72.9 ng/ml). A sharp drop in grain density over the [^{125}I]βNGF binding areas was observed between concentrations of 2.7 ng/ml and 8.1 ng/ml added unlabelled βNGF when compared with the grain density on autoradiographs of incubations without added unlabelled βNGF. The grain density of the strongly labelled areas of the sections (sympathetics, drg, lmc, muscle) decreased in parallel with half maximal competition observed at ~5 ng/ml βNGF. From these competition data an apparent K_d of 2×10^{-10} M was calculated. Without the use of stripping film and quantitative densitometry this value is a rough estimate, showing an affinity intermediate between the affinities for the βNGF receptors site I and site II measured on viable chick sensory ganglion cells (Sutter *et al.*, 1979a, 1979b) in suspension. The approximation of this value is not as good for tissues displaying low levels of [^{125}I]βNGF binding (vl region of the drg and parasympathetic ganglia). Partial denaturation of the receptors, when the sections were dried before the [^{125}I]βNGF incubation, loss of the cytoskeletal organization in frozen sections and diffusion barriers within the sections are causes which may contribute to the different binding properties of sections when compared with those on viable cell suspensions.

Immunofluorescence

For immunofluorescence, sections were dried for 3 min at room temperature, placed in covered Petri dishes on wet filter paper with undiluted hybridoma supernatants anti-SGII-1 and anti-SGIII-1 for 30 min. Following three washes in PBS, they were incubated for 30 min at room temperature with FITC labelled goat anti-mouse IgG antibodies (Paesel/Antibodies Incorp.) diluted 1:200 in PBS. After three more washes with PBS they were fixed in 1% paraformaldehyde at 4°C for 15 min. Photographs were taken with Kodak Tri-X Pan film on a Zeiss PMQ2 photomicroscope.

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