

Immunocytochemical study of substance P-like cell bodies and fibres in the brain of the rainbow trout, *Salmo gairdneri**

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INTRODUCTION

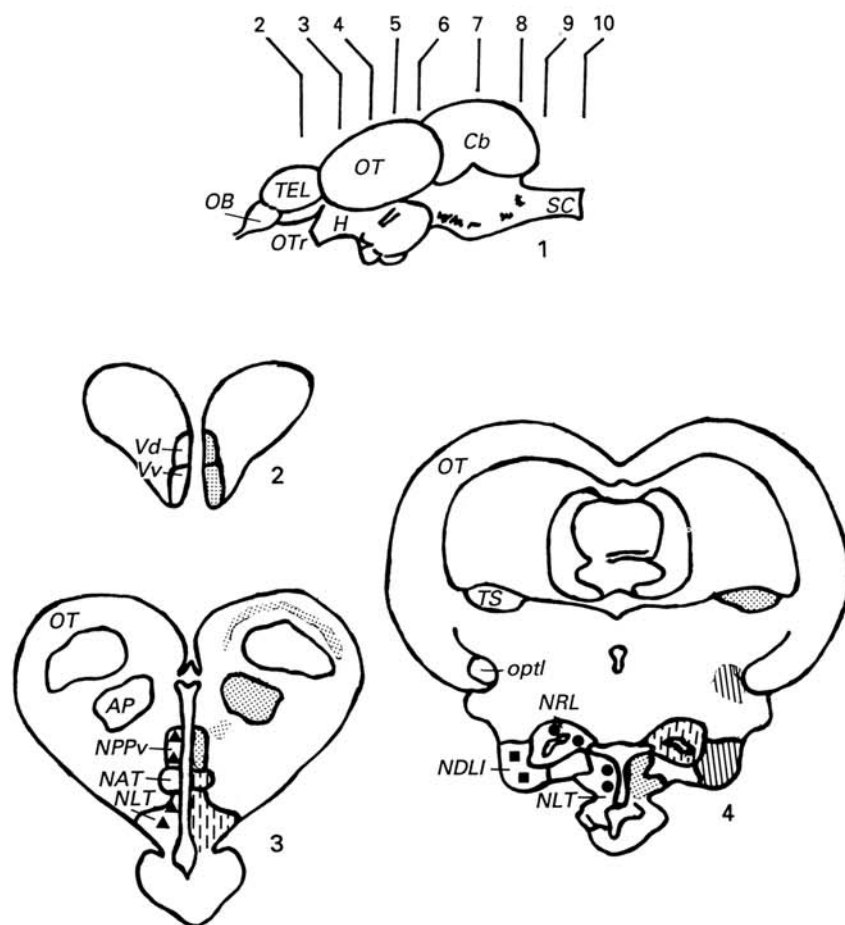
Since substance P (SP) was discovered (von Euler & Gaddum, 1931), many functional implications have been attributed to it, such as pain transmission (Henry, 1980; Haigler, 1987), regulation of the cardiovascular system (Unger *et al.* 1981; Bayorh & Feuerstein, 1985), as well as modulation of other neurotransmitters (Bouvet, Delaleu & Holley, 1984; Holmgren, Grove & Nilsson, 1985; Olpe, Heid, Bittiger & Steinmann, 1987). Several immunohistochemical and radioimmunological studies have been carried out in fish on the distribution of SP in the gastrointestinal tract (Holmgren, Vaillant & Dimaline, 1982; Holmgren, 1985; Bjenning & Holmgren, 1988), pineal organ (Ekström & Horst, 1986), retina (Li, Marshak, Douling & Lam, 1986; Marshak, Carraway & Ferris, 1987) and also in the olfactory and electrosensory cells (Szabo, Blahser, Denizot & Veron-Ravaille, 1987). However, little information is available on the substance P-immunoreactivity in the central nervous system of fish (Ritchie & Leonard, 1983; Reiner & Northcutt, 1987). We were therefore prompted to study the distribution of fibres and cell bodies staining for substance P-like activity in the brain of a teleost (*Salmo gairdneri*) by means of intraventricular injections of colchicine and using the indirect immunohistochemical peroxidase-labelled antibody method (Coons & Kaplan, 1950).

MATERIAL AND METHODS

Seven adult rainbow trout (*Salmo gairdneri*), obtained from commercial sources (Fisheries La Flecha, Salamanca), were used in this study. Under deep anaesthesia with MS-222 (Sandoz) at 0.03%, the animals were placed in a stereotaxic apparatus and colchicine was injected intraventricularly (25–75 µg in 0.15–1.5 µl of distilled water).

One to two days after the injection, the animals were re-anaesthetised and perfused transcardially with physiological saline solution for several minutes, followed by a fixative containing 4% paraformaldehyde in phosphate buffer 0.1 M pH 7.2 (PB). The brains were postfixated in the same fixative for 12 hours and then placed in a 30% sucrose bath until they sank. Serial transverse sections (50–70 µm) were cut on a Vibratome and processed for substance P immunocytochemistry. Tissue sections were incubated in PB containing 1% normal sheep serum and 0.3% Triton X-100 for 30 minutes. The sections were then incubated overnight in the same buffer containing substance P antibody (Cambridge Research Biochemicals, Cambridge, U.K.) diluted

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1:500 or 1:1000 in PB. The sections were washed for 30 minutes with PB and incubated for 2–3 hours with sheep anti-rabbit IgG coupled to HRP (Pasteur, Paris) as a second antibody, diluted 1:250 in PB. Finally, the sections were washed in PB and the HRP was revealed by the DAB method. The specificity of the immunostaining was checked with the following controls: (a) the anti-SP serum was omitted, (b) the anti-SP serum was pre-incubated with synthetic SP (200 μ g per ml diluted antiserum) or with other antigens (neurokinins A and B, Kassinin, eledosin).

Neuronal size was determined by measuring the major axis with a Zeiss ocular micrometer. In order to carry out the mapping of the substance P-like immunoreactivity the atlases of Billard & Peter (1982), Nieuwenhuys & Powels (1983), and Northcutt & Davis (1983) were used.

RESULTS

SP-positive cell bodies and fibres are scarce in the rainbow trout brain (Figs. 1–10). Thus, perikarya staining for SP were only observed in a few diencephalic nuclei such as: nucleus posterior periventricularis, nucleus lateralis tuberis, nucleus saccus vasculosus, nucleus recessus lateralis and nucleus diffusus lobi inferioris. The highest

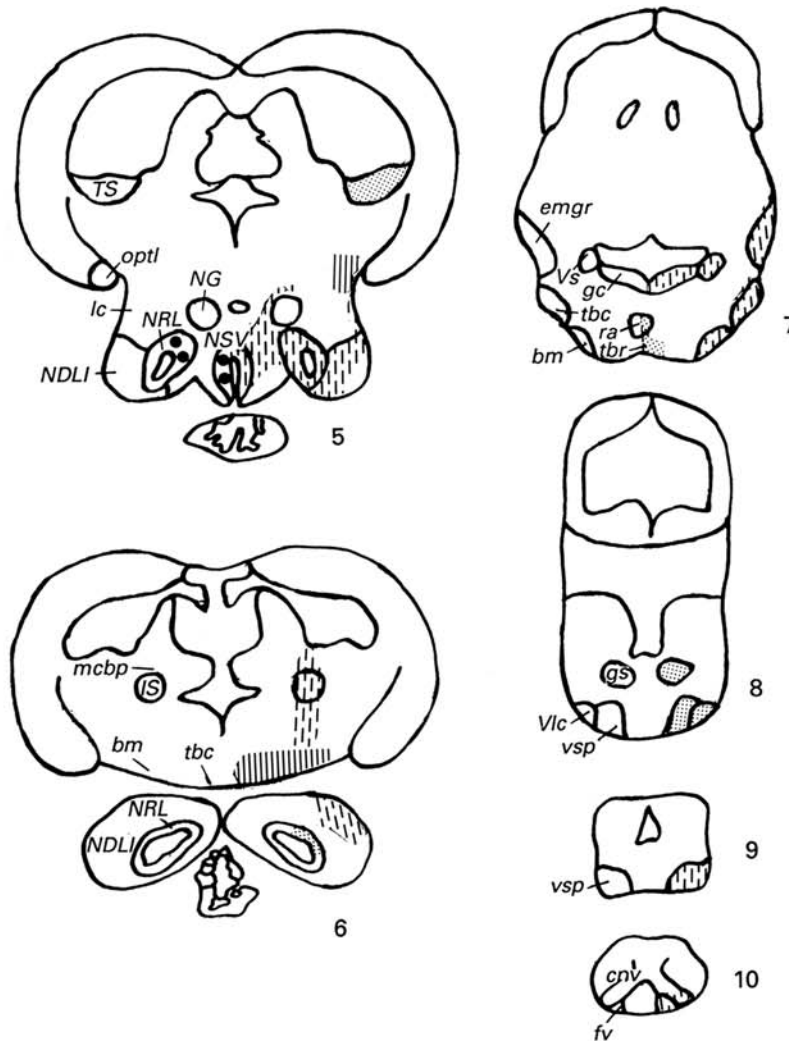


Fig. 1. Outline drawing of the trout brain showing the levels of transverse sections illustrated in Figures 2-10 inclusive. *Cb*, cerebellum; *H*, hypothalamus; *OB*, olfactory bulb; *OT*, optic tectum; *OTr*: optic tract; *SC*: spinal cord; *TEL*: telencephalon.

Figs. 2-10. Show the distribution of SP-immunoreactive fibres and perikarya in the rainbow trout brain, from the most rostral part (2) to the most caudal (10). SP-immunoreactive fibres are represented on the right side of each diagram by different symbols relating to their density (▨ high; ▩ moderate; ▪ low). SP-immunoreactive perikarya are represented on the left side, indicating by symbols the density of cell bodies per section in the area of a particular nucleus. (■), high density: more than 20 cell bodies; (▲), middle density: 10-20 cell bodies; (●), lower density: 1-10 cell bodies). The presence of symbols of one kind in one area means that this area contains the level of density of cells referred to.

AP, area prepectalis; *bm*, tractus bulbomesencephalicus; *cnv*, cornu ventrale; *emgr*, eminentia granularis; *fv*, funiculus ventralis; *gc*, griseum centrale; *gs*, tractus gustatorius secundarius; *IS*, nucleus isthmi; *lc*, tractus lobocerebellaris posterior; *mcbp*, tractus mesencephalo-cerebellaris posterior; *NAT*, nucleus anterior tuberis; *NDLI*, nucleus diffusus lobi inferioris; *NG*, nucleus glomerulosus; *NLT*, nucleus lateralis tuberis; *NPPv*, nucleus posterior periventricularis; *NRL*, nucleus recessus lateralis; *NSV*, nucleus saccus vasculosus; *optl*, tractus opticus marginalis lateralis; *OT*, optic tectum; *ra*, nucleus raphe; *tbc*, tractus tectobulbaris cruciatus; *tbr*, tractus tectobulbaris rectus; *TS*, torus semicircularis; *Vd*, dorsal nucleus of the area ventralis telencephali; *Vs*, nucleus sensibilis nervi trigemini; *vsp*, tractus vestibulospinalis; *Vv*, ventral nucleus of the area ventralis telencephali; *Vlc*, nucleus motorius nervi abducentis caudalis.

density of immunoreactive perikarya were observed in the latter, whereas in the other nuclei the number of cell bodies was moderate in the nuclei posterior periventricularis and lateralis tuberis (rostral part) and scarce in the nuclei recessus lateralis, saccus vasculosus and lateralis tuberis (caudal part).

Immunoreactive fibres were found in the telencephalon, diencephalon, mesencephalon and medulla oblongata, the diencephalon showing the largest number of immunoreactive nuclei. Thus, such fibres were found in the area pretectalis, nucleus anterior tuberis, nucleus lateralis tuberis, nucleus recessus lateralis and nucleus diffusus lobi inferioris. In this latter nucleus, we observed the highest density of immunoreactive fibres in the rainbow trout brain. Similar fibres were observed in the tractus mesencephalo-cerebellaris posterior, bulbomesencephalicus, gustatorius secundarius, tectobulbaris rectus, vestibulospinalis, lobocerebellaris posterior and tectobulbaris cruciatus. In the telencephalon, mesencephalon and medulla oblongata a lower density of fibres containing SP was observed.

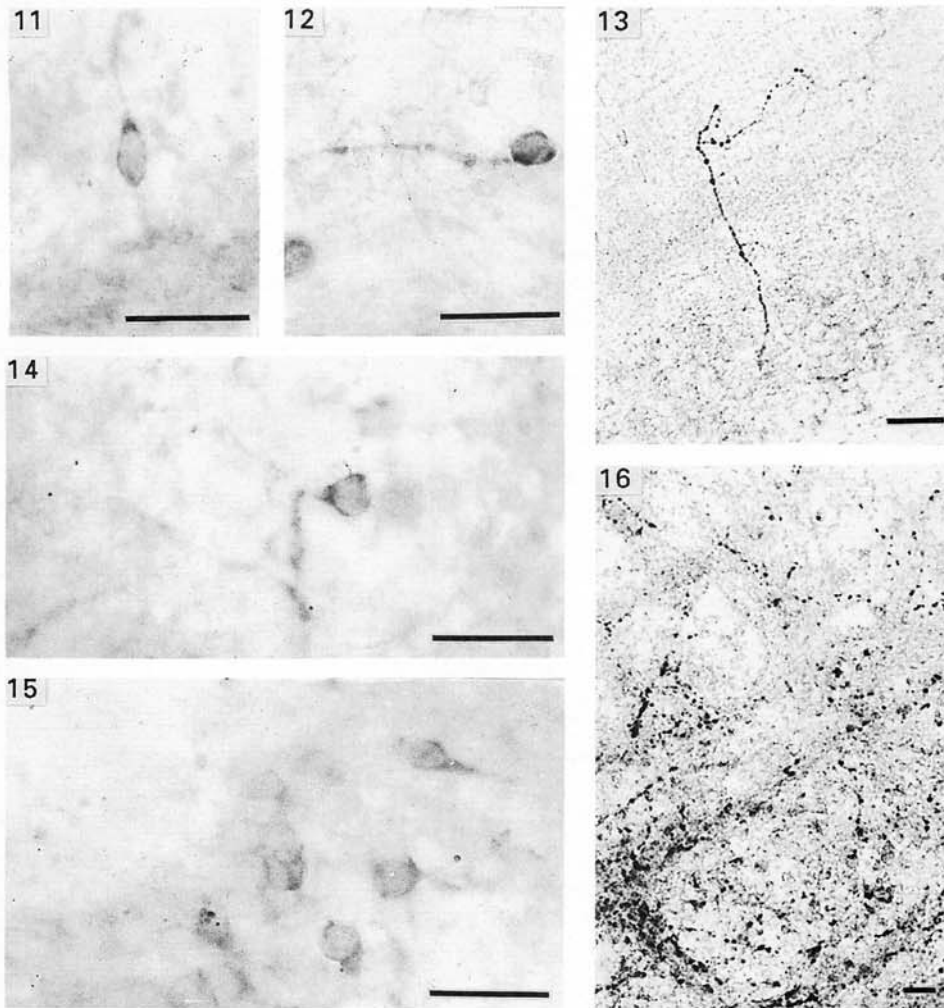
In the telencephalon a low density of SP fibres was found in the dorsal and ventral nuclei of the area ventralis telencephali (Fig. 2).

More caudally, SP perikarya were observed in the nuclei posterior periventricularis (Figs. 3, 11) and lateralis tuberis (Figs. 3, 12). In both nuclei were observed immunoreactive middle-sized neurons (10–15 μm), round or fusiform; at the same level scanty SP-immunoreactive fibres were observed in the area pretectalis, between this area and the nucleus posterior periventricularis and in the latter nucleus.

Immunoreactive fibres were found in the rostral optic tectum. These fibres were scanty but showed a strong degree of immunoreactivity. In the caudal optic tectum, SP-immunoreactive fibres were rarely observed. The fibres were confined to the stratum album centrale, stratum griseum centrale and stratum fibrosum et griseum superficiale (nomenclature according to Vanegas, Laufer & Amat, 1974). Most of the fibres were branched in the stratum griseum centrale (Fig. 13). In the area pretectalis scanty fibres were found but these showed a high degree of immunoreactivity. In addition, very weakly immunoreactive fibres were observed between the area pretectalis and the nucleus posterior periventricularis. A moderate density of SP-immunoreactive fibres was found in the nuclei anterior tuberis and lateralis tuberis.

More caudally (Fig. 4) SP-immunoreactive perikarya (10–15 μm) were observed in the nucleus recessus lateralis (Fig. 14), nucleus diffusus lobi inferioris (Fig. 15) and nucleus lateralis tuberis. In the latter, the neurons were round or fusiform, whereas in the others most of them were round.

In the nucleus recessus lateralis, SP-immunoreactive cell bodies were observed in the dorsal part of the nucleus, whereas in the nucleus lateralis tuberis the immunoreactive neurons were medially placed. In both nuclei, the immunoreactive cell bodies were located next to the recessus lateralis of the third ventricle. In the nucleus diffusus lobi inferioris, a large number of immunoreactive neurons was seen in its rostral part (Fig. 4), whereas no immunoreactive perikarya were found in the caudal part of this nucleus (Fig. 5). In addition, a lower density was observed in the nucleus recessus lateralis, in which SP-positive perikarya were mainly found in its rostral part (Figs. 4, 5), since in the caudal part immunoreactive cell bodies were not observed (Fig. 6). At the level represented in Figure 4, immunoreactive fibres were found dorsolaterally in the dorsal part of the torus semicircularis, laterally in the nucleus diffusus lobi inferioris, next to the tractus opticus marginalis lateralis, and surrounding the recessus lateralis in the nuclei lateralis tuberis and recessus lateralis. In the nuclei torus semicircularis and lateralis tuberis, scanty fibres were observed, whereas moderate



Figs. 11–16. SP-like immunoreactive cell bodies and fibres in the rainbow trout brain.

Fig. 11. Fusiform SP-immunoreactive neuron location in the nucleus posterior periventricularis.

Fig. 12. SP-immunoreactive perikarya located in the nucleus lateralis tuberis showing a round appearance. Note an immunoreactive dendrite.

Fig. 13. Immunoreactive fibre located in the stratum album centrale and griseum centrale of the optic tectum. The fibre is branching in the latter stratum.

Fig. 14. SP-immunoreactive perikarya in the nucleus recessus lateralis.

Fig. 15. Cluster of SP-immunoreactive neurons in the nucleus diffusus lobi inferioris.

Fig. 16. SP-immunoreactive fibres in the tractus vestibulospinalis.

Scale bar = 30 μ m.

numbers of fibres were observed in the nucleus recessus lateralis. A high density of SP-immunoreactive processes was found in the nucleus diffusus lobi inferioris and in the area next to the tractus opticus marginalis lateralis.

In more caudal sections (Fig. 5), there were few SP-immunoreactive fibres in the nucleus torus semicircularis, whereas a moderate degree of immunoreactivity was found in the following nuclei: saccus vasculosus, recessus lateralis and diffusus lobi inferioris. Immunoreactive fibres were also located in the tractus lobocerebellaris posterior, between the nuclei saccus vasculosus and recessus lateralis, between both

nuclei and the nucleus glomerulosus and partially surrounding this latter nucleus. A high degree of immunoreactivity was found next to the tractus opticus marginalis lateralis. Scanty SP-immunoreactive cell bodies were observed in the nucleus saccus vasculosus and in the nucleus recessus lateralis. In both nuclei, the neurons were round and of medium size (10–15 μm).

More caudally (Fig. 6), SP-immunoreactive fibres were found dorsally in the tractus mesencephalo-cerebellaris posterior (moderate density), ventrally in the tractus tectobulbaris cruciatus and in the tractus bulbomesencephalicus (both with a high density). Moreover, SP-immunoreactive fibres extending from ventral to dorsal regions were observed crossing the nucleus isthmi. Immunoreactive fibres were also found in the nuclei diffusus lobii inferioris and recessus lateralis. In the former, moderate numbers of processes were observed in its dorsolateral part, whereas in the latter scarce fibres were located in the dorsolateral part of the nucleus.

At the level of the isthmus (Fig. 7), a moderate density of immunoreactive fibres was seen in the following areas: griseum centrale, nucleus sensibilis nervi trigemini, eminentia granularis, tractus tectobulbaris rectus and tractus bulbomesencephalicus. In the midline area, scanty immunoreactive fibres staining for SP were located in the nucleus raphes and in the tractus tectobulbaris cruciatus.

At medulla oblongata level (Fig. 8), immunoreactive fibres were found in the tractus gustatorius secundarius, tractus vestibulospinalis and in the nucleus motorius nervi abducentis caudalis. In the three regions, a low density of immunoreactive fibres was found. More caudally, SP fibres were also found in the tractus vestibulospinalis (Figs. 9, 16).

Finally, in the spinal cord (Fig. 10), a moderate network of fibres was found in the funiculus ventralis and in the ventral region of the cornu ventrale.

DISCUSSION

The present study demonstrates that SP-positive structures are scantily distributed in the rainbow trout brain. However, their distribution in other vertebrates is denser, e.g. amphibia (Inagaki *et al.* 1981; Kuljis & Karten, 1982), reptiles (Brauth, Reiner, Kitt & Karten, 1983), birds (Reubi & Jessel, 1978) and mammals (Cuello & Kanazawa, 1978; Ljungdahl, Hökfelt & Nilsson, 1978; Bouras *et al.* 1986; Mai, Stephens, Hopf & Cuello, 1986).

In comparison with previous studies on the localisation of SP in the telencephalon of the African lungfish *Protopterus annectens* (Reiner & Northcutt, 1987) and in the spinal cord of the Atlantic stingray *Dasyatis sabina* (Ritchie & Leonard, 1983), it seems that the distribution of the undecapeptide is wider in both these fish than that found in our study. Thus, in *Salmo gairdneri* SP fibres were only found in two telencephalic regions, the dorsal and ventral nuclei of the area ventralis telencephali. These data are in agreement with the observations of Reiner & Northcutt (1987), carried out in *Protopterus annectens*, since these authors also observed immunoreactive fibres in the same nuclei. Moreover, in the ventral region they found SP perikarya, whereas we have not observed them in the rainbow trout. In the Atlantic stingray, Ritchie & Leonard (1983) observed immunoreactive fibres in the substantia gelatinosa, nucleus proprius, intermediate zone and ventral horn, whereas in the rainbow trout immunoreactive fibres were only found in the ventral part of the cornu ventrale and in the funiculus ventralis. In both animals (*Protopterus* and *Dasyatis*) no SP perikarya were observed in the spinal cord. The presence of immunoreactive fibres in the ventral horn of the rainbow trout is in agreement with previous studies on the Atlantic

stingray (Ritchie & Leonard, 1983), and on the chick embryo (Du, Charnay & Dubois, 1987). Furthermore, the coexistence of serotonin and SP in immunoreactive fibres has been found in the ventral horn of the rat (Wessendorf & Elde, 1987).

The present work is the first report revealing the distribution of both fibres and cell bodies containing SP in a teleost fish. This study does not deal with SP-containing pathways. However, according to the localisation of SP fibres and perikarya, it could be suggested that immunoreactive cell bodies located in the nucleus diffusus lobi inferioris may be interneurons rather than projecting neurons, since in this nucleus we found a high density of both fibres and cell bodies staining for SP. Alternatively, the nucleus might send distant SP projections and/or receive SP afferents. In the other four regions in which immunoreactive cell bodies were found (nucleus posterior periventricularis, nucleus lateralis tuberis, nucleus recessus lateralis and nucleus saccus vasculosus) data to confirm whether such perikarya are interneurons or projecting neurons are lacking. Further experimental studies are thus necessary to elucidate such questions.

The hypothalamus is the region of the rainbow trout brain in which the richest network of SP-immunoreactive fibres was found. Furthermore, SP-immunoreactive perikarya were only observed in this diencephalic region, in nuclei located next to the ventricles. In the brain of *Salmo gairdneri*, a wider distribution of SP-immunoreactive fibres than perikarya was found. According to this observation it thus appears that more cell bodies containing the undecapeptide might be located in other areas of the brain of the rainbow trout that were not found in the present study. More experimental work such as the intratissue injection of colchicine would be necessary to confirm this idea. However, in view of the small width of the rainbow trout brain, it is possible that intraventricular colchicine diffuses through the brain, since other peptidergic perikarya, distant from the ventricles (unpublished results), were found when intraventricular injections of colchicine at a similar concentration of the drug were performed.

The localisation of SP-immunoreactive perikarya and fibres in the nuclei lateralis tuberis and recessus lateralis suggests that the peptide might play a role in the control of the hypophysis and in particular may be involved in reproductive processes, since it has been pointed out that both nuclei innervate the hypophysis (Holmes & Ball, 1974; Simon & Reinboth, 1974), regulating its function (Ekengren & Terlou, 1978; Terlou & Ekengren, 1979). Thus, lesions of the nucleus lateralis tuberis cause a decrease in gonadotropin levels in both male and female rainbow trout, suggesting that this nucleus releases gonadotropin-releasing factor (Peter & Crim, 1978). Furthermore, in the same nucleus of the rainbow trout, cell bodies containing somatotropin-release inhibiting factor have been observed. Thus, the presence in the nucleus lateralis tuberis of SP suggests that the peptide might regulate the neural activity of such neurons. On the other hand, we have also observed immunoreactive fibres in the area pretectalis and optic tectum of the rainbow trout. These data suggest the possibility of some participation of the SP in visual circuits. The location of SP fibres in the stratum griseum centrale and in the nucleus sensibilis nervi trigemini suggests that the peptide could play a role in somatosensory transmission.

Finally, the presence of SP in the nucleus raphes would indicate some influence of the undecapeptide on serotonergic structures, since in teleosts immunoreactive cell bodies in this nucleus have since been found (Ekström & Van Veen, 1984; Nagatsu, Karasawa, Kawakami & Yoshida, 1984). It seems that the distribution of SP in the rainbow trout brain is less extensive than the distribution of both serotonergic and catecholaminergic structures in the brain of other teleosts (Parent, Dube, Bradford &

Northcutt, 1978; Terlou, Ekengren & Hiemstra, 1978; Ekström & Van Veen, 1984; Kah & Chambolle, 1983; Nagatsu *et al.* 1984). A similar suggestion has been made by other authors concerning the frog brain and spinal cord (Inagaki *et al.* 1981). Thus, the distribution of SP-immunoreactive structures in the brain of *Salmo gairdneri* suggests that the neuropeptide may be involved in neuroendocrine, visual and somatosensorial mechanisms.

SUMMARY

The distribution of substance P-like immunoreactive cell bodies and fibres in the rainbow trout brain was studied using an indirect immunoperoxidase technique. Previously, intraventricular injection of colchicine had been made. Substance P-immunoreactive perikarya were located in the hypothalamic nuclei posterior periventricularis, lateralis tuberis, saccus vasculosus, recessus lateralis and diffusus lobi inferioris. In the latter, a high density of immunoreactive cell bodies was observed, whereas in the other nuclei the density observed was moderate and/or low. The most dense network of immunoreactive fibres was observed in the nucleus diffusus lobi inferioris, whereas the dorsal and ventral nuclei of the area ventralis telencephali, optic tectum, area pretecalis, nucleus posterior periventricularis, torus semicircularis, nucleus raphes and nucleus motorius nervi abducentis caudalis had a low density of immunoreactive fibres. In other nuclei such as anterior tuberis, recessus lateralis, saccus vasculosus, isthmi, griseum centrale, sensibilis nervi trigemini, eminentia granularis, funiculus ventralis and cornu ventrale, the density of substance P-like fibres was moderate. Finally, the tractus mesencephalo-cerebellaris posterior, lobocerebellaris posterior and tectobulbaris rectus showed a moderate density of immunoreactive fibres; the tractus gustatorius secundarius had a low density and the tractus bulbomesencephalicus, vestibulospinalis and tectobulbaris cruciatus showed a variable density of immunoreactivity from low to high.

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