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**Somatostatin, Neuropeptide Y and Galanin: Immunohistochemical
Detection and Mapping of Neuropeptide Distributions in Goldfish
Brain, with Reference to Rat**

by

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BSc. Hons.

Thesis submitted in partial fulfillment of the requirements for the degree Master of
Science in Anatomy with Specialization in Neuroscience

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Lucy Cecilia Pickavance, Ottawa, Canada, 1990



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SUMMARY

The biochemical neuroanatomy of the goldfish brain, with some reference to that in the rat brain, was investigated. Initial interests in the connections and neurotransmitter content of the basal ganglia in mammals led to the use of retrograde tracing in combination with immunofluorescence techniques to reveal that the efferents of the somatostatin (SRIF)-positive cells of the entopeduncular nucleus of the rat do not project to the ventroanterior/ventrolateral nuclei of the thalamus. A related set of experiments, not documented in this thesis, involved an attempt to identify primordia of the basal ganglia in a lower vertebrate, the goldfish, using histochemical and immunohistochemical criteria. These experiments did not lead to conclusive evidence that homologues of the basal ganglia exist in the goldfish, but they did reveal a possible basal ganglia homologue identified by the presence of both SRIF and neuropeptide Y (NPY) immunoreactivity in a particular set of forebrain neurons. These data also encouraged the pursuit of the overall neuroanatomical distribution of these and other peptide neurotransmitters in the goldfish brain.

Through the further use of immunohistochemical staining techniques, the distributions of the neurotransmitters SRIF, NPY and galanin (Gal) were mapped in detail throughout the entire brain of the goldfish. In the case of SRIF and NPY, special emphasis was placed on determining their codistribution through a double-labelling immunofluorescence procedure. SRIF and NPY were found to be colocalized in various regions of the goldfish brain, including cell bodies of the ventrolateral telencephalon and medial column of the vagal motor complex and in fibres and terminals of the dorsolateral telencephalon. To an even greater extent, the distributions of these two neuropeptides exhibited striking coincidence. This was particularly

true of the fibres and terminal fields of the telencephalon and vagal lobe.

Single-label immunohistochemistry was used to determine the distribution of Gal in the goldfish brain and revealed that Gal is present primarily in fibres and terminals but in only a few cell bodies restricted to the preoptic and tuberal hypothalamus and the reticular formation. Gal-immunoreactive fibres apparently arose from the Gal-immunoreactive cell bodies of the hypothalamus, coursed along the base of the forebrain and branched off into dense terminal fields in the mid-ventral telencephalon. This suggested homology to the tuberomammillary nucleus of the hypothalamus in mammals and its projections to the septal area. As was seen for the distribution of SRIF and NPY, Gal immunoreactivity was found throughout the goldfish brain and was seen in a number of structures comprising sensory systems, particularly taste and audition. In addition, the density of Gal-immunoreactive fibres in the optic tectum of the goldfish was noted to be sexually dimorphic, and statistical analysis of this dimorphism revealed it to be highly significant.

When the distributions of SRIF, NPY and Gal in the goldfish brain were compared to the literature on the distributions of all of these neuropeptides in the brains of other teleost fish and in the mammalian brain, it was clear that they have altered little across species. Coexistence and coincidence of neuronal messengers is a conserved feature of the nervous system, and immunohistochemical techniques are valid methods in marking the similarities and differences between the central nervous systems of diverse species.

GENERAL INTRODUCTION

The study of neuroanatomy, like other morphological work, involves examination of the results of an experiment and not only attempting to answer the questions which prompted the acquisition of these data, but also remaining open to the possibility that new and even better questions may be triggered by what is observed. Morphology, the study of the form and structure of organisms, is a primarily natural science, and it is a passively exploratory and somewhat non-experimental approach that has been taken in the present neuroanatomical investigations. Rather than testing an hypothesis within the restrictive boundaries of a single experiment, the components of a thesis can follow the natural progression of the experimenter's interests and findings. In this way, one can avoid the constraints of technical or theoretical inadequacy in keeping within the limits of a particular experimental design or the boundaries of a single experimental question. One is then able to present positive and novel results that have arisen from the careful observation of the native state rather than its response to experimental perturbation. This can be worth the sacrifice of the cohesion with which the rigid pattern of a predetermined set of experiments may be presented in thesis form.

My main interests were in the comparative aspects of neuroanatomy, specifically in the areas of neuropathology and evolution. I thought that from these two areas of study could be deduced some general themes of how the brain functions and whether or not the similarities and differences between the central nervous systems of diverse species are random or appear to be adaptive. Species commonly examined in neuroanatomical studies are the rat, cat, monkey and human. About 175 million years passed between the emergence of the most primitive teleosts, bony fish of which goldfish are one example, and the most primitive mammals (for

review, see Sarnat and Netsky, 1981), and it was thought that it would be of interest to examine neuroanatomical phenomena in a sub-mammalian species that is relatively far removed from mammals on the phylogenetic scale. The approach used in the experiments comprising this thesis was encouraged by the easy access to both the rat and goldfish species and to the local expertise on these species within the Department of Anatomy. These experiments were conducted within the field of biochemical neuroanatomy (the localization of neurochemicals) through the use of immunohistochemical techniques. These techniques are a way to define and mark the components of the nervous system, in a way that is supplementary to traditional morphological methods such as cytoarchitectonics, enzyme histochemistry and degeneration. Lower vertebrates such as the goldfish are not to be excluded from neuroanatomical study using modern techniques because they may even demonstrate some neuroanatomical phenomenon that makes theirs a better system than a mammal's, for example, in their hypothalamus and pituitary organization. However, this thesis does not strictly address questions of evolution, the interest in neuropeptide distributions in the goldfish and rat brains being mostly for their own sakes, regardless of comparative states.

The experiments comprising this thesis were prompted by a number of interests together with the most convenient ways in which to satisfy those interests simultaneously. The two main areas of interest, seemingly disparate, were in evolution and neuropathology. Although I have not studied evolutionary and neuropathological phenomena directly in this thesis, the comparative neurobiological approach and the training in neuroanatomical techniques that have helped to produce this thesis prepare me for future investigations in both fields. The latter interest was in keeping with the ongoing research in the laboratory of Dr. Wm. Staines into neurodegenerative diseases (motor disorders) of the basal ganglia, a group of subcortical nuclei

comprising a motor circuit intrinsic to the brain. The investigation into one of these nuclei, specifically, the somatostatin (SRIF)-containing neurons of the entopeduncular nucleus (Experiment 1), led me to the subsequent projects on the distributions of SRIF and other neuropeptides, neuropeptide Y (NPY) and galanin (Gal), in the goldfish brain.

Examining the neuroanatomy of different species can suggest homologous regions of the brain. In particular, the study of the colocalization of neuropeptides within the same neurons and the coincidence of neuropeptides within similar regions of the brains of both higher and lower vertebrates can help to establish possible synergistic relationships between neuronal messengers and general principles of neuronal organization.

The importance of neurotransmitters in the context of species divergence becomes evident when we consider how the study of these signalling molecules is imperative to an understanding of how the brain functions and, therefore, how this function remains constant or varies across species. Neurotransmitters are important because it is through their recognition by particular receptors on other cells that they can carry selective messages between groups of cells (for review, see Wolstencroft, 1987). The study of transmitters occurring in the nervous system is one of the fastest growing areas in neurobiology today, in part because the identification of these molecules, present in very small amounts in the brain, has recently been made possible through simple and widely available immunohistochemical techniques (for review, see Hökfelt et al., 1984).

The experiments described in this thesis are united by a theme having its methodological basis in immunohistochemistry and its intellectual goal in comparison and contrast between species and sexes. Defining the biochemical anatomy of the brain across species can be approached (1) through the study of neuronal connectivity, using anatomical tracers, as in

Experiment 1, in which the entopeduncular projection to the motor thalamus of the rat was found to be devoid of SRIF, (2) through the study of distributions of neuronal messengers, as in Experiments 2 and 3, in which distributions of the neuropeptides SRIF, NPY and Gal were mapped throughout the goldfish brain, and (3) statistically, as in Experiment 4, in which a significant sexual dimorphism in the density of Gal-immunoreactive fibres was found in the goldfish optic tectum.

Experiment 1 was the result of interest in the limbic and motor components of the entopeduncular nucleus; that is, the projections of the entopeduncular nucleus to the lateral habenula and ventroanterior/ventrolateral thalamus, respectively, and the striatal inputs to these entopeduncular subdivisions. The limbic components of the brain are generally accepted as being phylogenetically older, and attempts to identify limbic components of the basal ganglia in the more primitive goldfish brain, while receiving training in the immunohistochemical mapping of a simpler central nervous system, led to Experiment 2. The mammalian neostriatum is compartmentalized into a "patch-matrix" organization that is revealed by various histochemical and immunohistochemical stains. These stains include those for SRIF, NPY, enkephalin, substance P, dynorphin, glutamic acid decarboxylase and acetyl cholinesterase (for review, see Heimer, 1985). All of these staining procedures were applied to goldfish forebrain, including an iron stain designed to distinguish pallidal and nigral regions (Hill, 1985), the globus pallidus and substantia nigra being components of the basal ganglia, from other regions of the brain. Of all of these potential basal ganglia markers, only one yielded results that could be interpreted with even limited confidence as representing a basal ganglia homologue in goldfish brain. This marker was the high degree of colocalization of SRIF and NPY immunoreactivity in cell bodies of the ventrolateral telencephalon, reminiscent of the

complete colocalization of these two neuropeptides in cell bodies of the rat striatum (Vincent et al., 1983). In addition to this finding was the interesting degree of overlap of SRIF and NPY immunoreactivity in terminal distributions throughout the entire goldfish brain. Findings such as these made the goldfish brain worthy of study on its own, with extensive comparison to the literature on the distributions of these same neuropeptides in the mammalian brain.

There are a number of evolutionary considerations to make when discussing a characteristic held in common by different species, such as the localization of a neuropeptide to a particular brain nucleus. We must always keep in mind that it may be an homologous trait, in which case it has been inherited from a common ancestor, or a homoplastic trait, in which case it has evolved independently in each of the species that carry it. Goldfish are teleosts, a subclass of the bony fish group, the actinopterygians (ray-finned fishes). Mammals and other tetrapods are not linearly evolved from the teleosts, but are evolved almost in parallel with them from the common ancestral group of the gnathostomes (jawed vertebrates). We cannot know for certain if structures in goldfish and rats are homologous because there are no existing gnathostomes to investigate for the presence of a similar structure. The only other modern representatives of gnathostomes are the chondrichthians, including sharks and rays, which arose at about the same time in vertebrate phylogeny as the bony fish, such that neither are ancestral to the other. This means that the examination of a cartilaginous fish for a structure common to the goldfish and rat would not help to answer questions of homology versus homoplasy (for review, see Northcutt, 1981). However, if peptidergic distributions were found common to the brains of all three groups, this would argue strongly for the presence of this same distribution in their common ancestor, and therefore homology between the structures containing the peptides in all three groups.

Nevertheless, comparison of neuropeptide, or, more generally, neurotransmitter distributions across species can always allow for speculation on common neuronal functional, if not strictly anatomical, characteristics. Even if there are discrepancies in the neurotransmitter contents of suspected homologous nuclei, these nuclei may still be of homologous origin. Primordial nuclei may have existed in a common ancestor of goldfish and rats, but the mediation of the same function in the two vertebrate classes may have been taken over by different sets of neurotransmitters. During the evolution of a species, the transcription of the genes encoding particular neuroactive substances may come under the constraint of a newly evolved set of regulatory peptides, themselves resulting from mutations in the base sequence of DNA, changing the final state of neurochemical differentiation of these nuclei, but retaining their function. If the changes are major, they result in completely different transmitters, specialized for functions unique to that organism. The other possibility is that the nuclei are homoplastic and evolved independently to possess the same transmitters, as a similar response in both vertebrates to a similar set of environmental pressures.

The study of neuropeptides in two vertebrates such as the goldfish and rat, despite their relatively wide evolutionary separation, is a legitimate pursuit. Similarities in the localizations of peptides in these two animals will begin to establish the extent of conservation of these peptides throughout evolution. In fact, SRIF is already on the way to being designated as an evolutionarily old peptide: A SRIF-related material has been found in a number of bacteria and higher plants (for review, see Roth et al., 1986). The NPY-like material of goldfish co-elutes with synthetic porcine NPY (Pontet et al., 1989), suggesting that the peptide has altered little from that of the common ancestor to fish and mammals. Gal is a more recently discovered peptide and the degree to which its amino acid sequence is conserved across species may also

be revealed in the future. Its distribution in the goldfish brain has been helpful in suggesting homologies between lower and higher vertebrate brains, as discussed in Experiment 3.

One of the most important things to be aware of in comparative studies is the tendency to overinterpret data. Homologies can certainly be suggested, but, as with any scientific pursuit, suggestions on the basis of similar anatomical locations, biochemical make-up or internuclear connectivity cannot stand alone. For this reason, an alternate approach to neuroanatomy, the statistical approach, was employed in determining the degree of significance in the difference between the densities of Gal-immunoreactive fibres in the optic tecta of male and female goldfish in Experiment 4. This difference has been very recently confirmed by ourselves in the rat brain in that a dimorphism has been visually observed in the densities of Gal-immunoreactive fibres in the superior colliculus and visual cortex (homologous to the optic tectum of the goldfish), and this qualitative difference merely awaits statistical evaluation.

Inquiries into interspecies biochemical neuroanatomy through the use of immunohistochemistry may provide data that for evolutionary researchers may yield new perspectives through the comparative observations they can make. Study of more than one species allows evaluation of the possibilities that (1) the findings in any one species may not be typical of or generalizable to other species and so (2) we cannot afford to be so narrowly focussed as to suggest that questions of one nervous system heritage, age or phylogenetic rank can be answered using a few taxonomic exemplars (Bullock, 1983).

Experiment 1

The Somatostatinergic Neurons of the Entopeduncular Nucleus do not Project to the Ventroanterior/Ventrolateral Thalamus of the Rat

ABSTRACT

Combined retrograde tracing and immunofluorescence techniques revealed that the entopeduncular neurons projecting to the ventroanterior/ventrolateral thalamus of the rat do not contain somatostatin whereas other neurons in this nucleus do. These findings suggest that the neurons projecting to the ventroanterior/ventrolateral thalamus form a unique subpopulation of entopeduncular neurons neurochemically as well as anatomically and that somatostatin can be used as a marker for the differential projections of the entopeduncular nucleus.

INTRODUCTION

The entopeduncular nucleus (EP) is a component of the basal ganglia, the subcortical nuclei controlling motor activity. The EP is the subprimate equivalent of the internal (medial) segment of the globus pallidus (GPi). The EP gets input from the caudate-putamen (CPu) and projects via the ansa lenticularis and Forel's field H to the ventroanterior and ventrolateral nuclei (VA/VL) of the thalamus. This projection has been known for some time to be GABAergic. The VA/VL in turn projects to the premotor cortex, and, in this way, information funnelled from widespread areas of the neocortex into the neostriatum can influence movement. The EP

is worthy of study because of its unique status, along with the substantia nigra, pars reticulata, as the major output pathway, or 'escape route' for information from this intrinsic circuit (Schmued et al., 1989; for review, see Faull and Mehler, 1985; Heimer et al., 1985).

The EP is divided into regions along its rostrocaudal axis that are defined by the particular targets to which the EP neurons project. The EP has both limbic and motor connections, its rostral two thirds projecting to the lateral habenula (LHb) and CPu, and its caudal third projecting to the VA/VL, centromedian-parafascicular complex (CM-Pf) of the thalamus and pedunculopontine tegmental nucleus, pars compacta (TPC; Takada and Hattori, 1987).

Rostral EP neurons contain the neuropeptide somatostatin (SRIF; Vincent et al., 1985), and lesions of the EP abolish dense SRIF-positive terminal fields in the LHb, demonstrating that the SRIF-containing EP neurons project to the LHb (Vincent and Brown, 1986). Whether or not the SRIF-ergic neurons of the EP also project to its other targets remains to be determined. SRIF-positive terminals have also been reported in all the other EP targets (Vincent et al., 1985), but the TPC and VA/VL. We ourselves saw very little evidence of SRIF-positive fibres in the VA/VL (L. Pickavance, unpublished observations). Nonetheless, it is possible that these targets do indeed receive afferents arising from SRIF-positive neurons in the EP, but that SRIF is simply not being expressed in the terminals of these projection neurons. There is some precedent for this notion: All of the interneurons of the CPu that contain neuropeptide Y (NPY) also express SRIF (Vincent et al., 1983), and yet NPY-positive fibres are found within some compartments of the CPu where SRIF-positive fibres are not. From this we know that it is indeed possible for some forms of the SRIF molecule to be expressed in cell bodies but not in the processes of these same neurons (Wm. Staines, personal communication). It was thought that if the EP neurons shown experimentally to project to the VA/VL could be found

devoid of SRIF in their cell bodies, the latter possibility could be refuted.

MATERIALS AND METHODS

Adult male albino rats (Sprague Dawley, 300-350g) were used in this study. All animals were deeply anesthetized with sodium pentobarbital (6.5 mg/kg, i.p.) prior to surgery.

Retrograde tracing

Fluorogold (Fluorochrome, Inc.; 2% in 0.9% sodium chloride) was injected stereotaxically into the VA/VL according to the co-ordinates of Paxinos and Watson (1986). Injections were unilateral and performed by iontophoresis using a micropipette of 40 μ m outer tip diameter. An alternating current of 5.0 μ A was used at 7s on/7s off for 10 min. Pressure injections were also performed whereby 0.2 μ l of the tracer was delivered over 5 min through a 10 μ l Hamilton syringe. Animals were allowed to survive for 18 to 24 hours following surgery.

SRIF immunofluorescence

Rats were reanesthetized and perfused transcardially with 10 mM phosphate buffered saline (PBS) followed by fixative consisting of 4% paraformaldehyde and 0.2% picric acid in 0.16 M phosphate buffer, pH 6.9, at room temperature. Brains were removed and post-fixed in the same fixative for 3 h at 4^o C then transferred to 10% sucrose, 0.01% sodium azide in 100 mM

phosphate buffer for 1h then stored in a change of sucrose at 4° C for at least 12 h. After fast-freezing brains with CO₂, sections of 15 µm were taken through injection sites and through the EP using a cryostat (Hacker Instruments, Inc.) and thaw-mounted onto chrome alum-coated slides.

Sections were washed for 15 min in 10 mM PBS, pH 7.2, then incubated overnight at 4° C in mouse anti-SRIF antibody (Dr. J.C. Brown) at a concentration of 1 in 200 in 10 mM PBS, 0.3% Triton X-100. After a second 15 min wash in PBS, sections were incubated for 30 min at 37° C in secondary antibody, FITC-conjugated rabbit anti-mouse (Dakopatts) diluted to 1 in 20. Sections were washed a final time and coverslipped under glycerol containing 0.1% *p*-phenylenediamine. Cell bodies in the EP could be simultaneously evaluated for the presence of retrograde fluorescent labelling and SRIF immunoreactivity using separate filters on the same reflector slide of a Zeiss Axioplan microscope. Microphotographs were taken using Kodak Tri-X 400 ASA film.

RESULTS

Neurons projecting to the VA/VL from the caudal EP contained no SRIF-immunoreactivity (Fig. 1.1 A,B). In contrast, more rostral EP neurons which did not project to the VA/VL were indeed SRIF-immunoreactive (Fig. 1.2 A,B), and the immunoreactivity was localized to perinuclear regions of the cell bodies. These regions appeared to be subcellular compartments and the perikaryal cytosol itself was unstained. The distribution of SRIF-positive neurons within the EP gradually changed from rostral to caudal levels: At the most anterior levels of the EP, these neurons filled the entire mediolateral and

Fig. 1.1. The posterior entopeduncular nucleus after injection of Fluorogold into the ventrolateral nucleus of the thalamus. **A.** Neurons in the caudal entopeduncular nucleus retrogradely labelled by thalamic injection of Fluorogold. **B.** The same neurons do not show SRIF-immunofluorescence. Note the SRIF-positive neuron on the right does not exhibit Fluorogold labelling in **A**, further emphasizing that neurons projecting from the entopeduncular nucleus to the ventrolateral nucleus of the thalamus are not those containing SRIF. Scale bar = 50 μm .

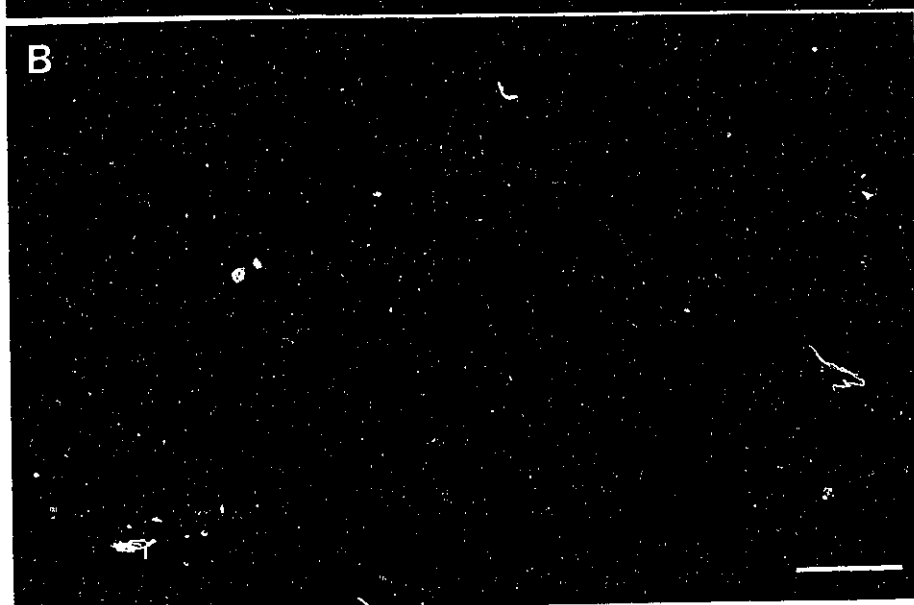
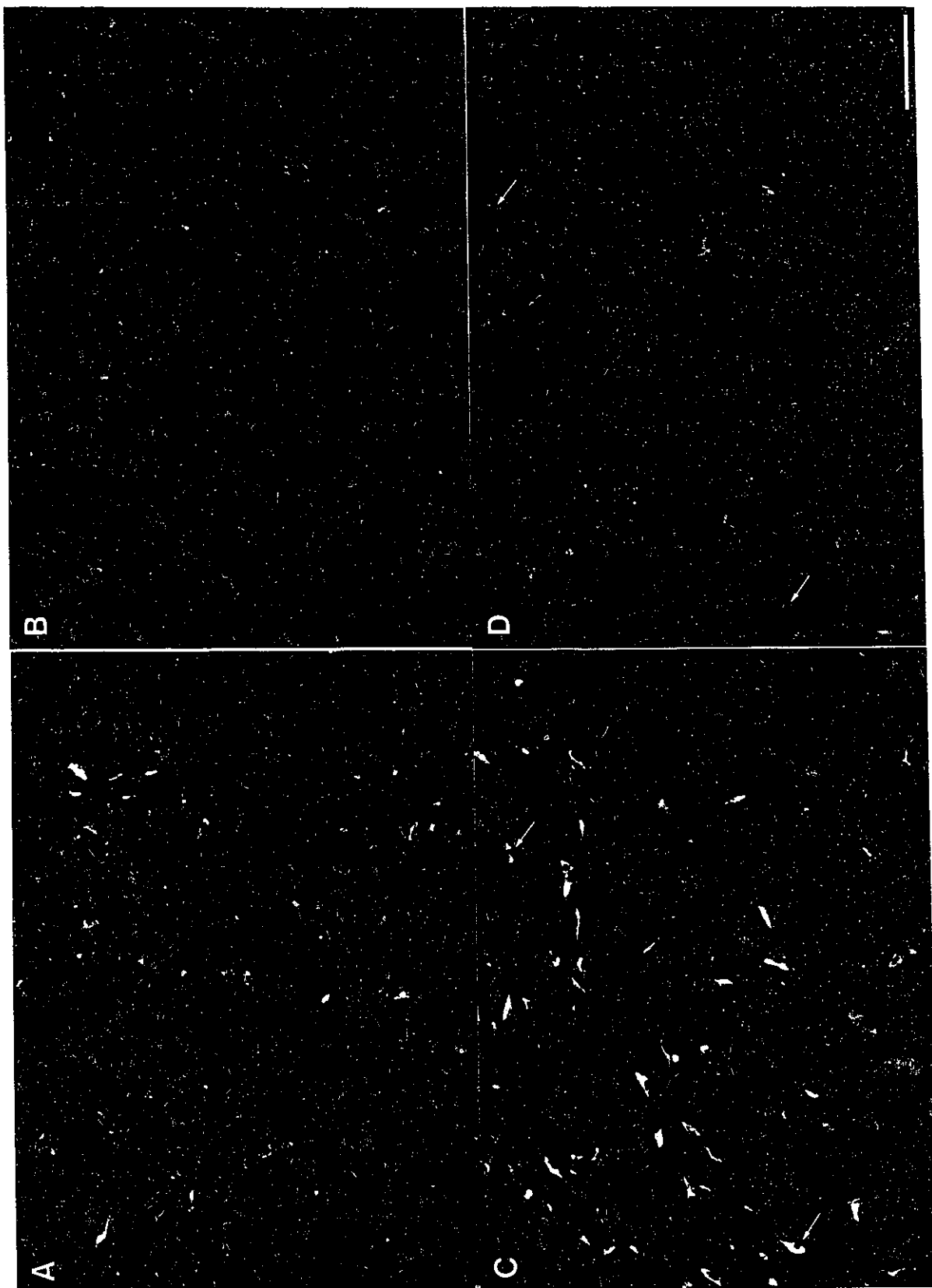


Fig. 1.2. SRIF-immunoreactive neurons in the rostral entopeduncular nucleus. **A.** Rostral entopeduncular neurons are SRIF-positive. **B.** These SRIF-labelled neurons are not retrogradely labelled by Fluorogold injection into the ventrolateral nucleus of the thalamus. **C.** SRIF-immunoreactive neurons in the anterior entopeduncular nucleus in an animal in which Fluorogold was injected into the lateral habenula. **D.** Rostral entopeduncular nucleus neurons retrogradely labelled from the lateral habenula are the same as those containing SRIF in **A**. A few examples are shown with arrows. Scale bar = 100 μm .



dorsoventral dimension of the nucleus. Posteriorly, SRIF-immunoreactive neurons became increasingly scant in the centre of the nucleus and were observed only at its outer boundaries as seen in coronal section. EP neurons retrogradely labelled from the VA/VL were clustered in this central region of the posterior nucleus (Fig. 1.1 A). In some cases, in which the Fluorogold had been delivered by pressure injection, the injection site had spread to include more of the LHb than of the VA/VL. In these instances, retrogradely labelled neurons were distributed extensively throughout anterior as well as posterior levels of the EP. With the exception of only a few cells at mid-levels of the EP, all of the Fluorogold-labelled neurons distributed at anterior and mid-levels in the EP of these animals also showed SRIF immunofluorescence (Fig. 1.2 C,D).

DISCUSSION

These results indicate that SRIF is not present in that population of EP neurons that project to the VA/VL. In addition, these data provide some independent confirmation that SRIF is present in the projection from the EP to the LHb (Vincent and Brown, 1986). That pallidothalamic and pallidohabenular projections do not arise from the same class of EP neurons gains some support from the fact that the terminals found in the LHb and VA/VL of the cat are ultrastructurally different in terms of the elongation and area of synaptic vesicles (Tokuno et al., 1988).

The localization of SRIF to the peripheral cytoplasm in EP perikarya is not unique. Many, although not all, SRIF-immunoreactive neurons in the brain show this staining pattern (data not shown), reflecting the restriction of SRIF to this subcellular compartment in a way not seen

with most other peptides. Contrasting intracellular peptide distributions have been shown in the feline reticular nucleus of the thalamus. SRIF was found restricted to perinuclear structures, skeins of immunoreactivity, identified by electron microscopy as Golgi apparatus and multivesicular bodies. Immunoreactivity for glutamic acid decarboxylase, on the other hand, was broadly dispersed throughout the cytoplasm (Oertel et al., 1983), suggesting differential processing for different components of neurotransmitter systems. Perinuclear and broadly dispersed SRIF staining in the cell body are cytoarchitectonic characteristics which may be used to sort out different types of SRIF-containing cells. Observations made in Experiment 2 (data not shown) revealed that this staining pattern is also characteristic of SRIF-immunoreactive neurons in some regions of the goldfish brain. This feature may in fact be of some value in identifying homologous nuclei in divergent species.

The centroposterior EP was devoid of SRIF-containing neurons, and this is exactly where the neurons projecting to VA/VL were concentrated. It is not that this centre is devoid of perikarya, but that the cell bodies within it are a select group devoid of the neuropeptide that many others contain. The central portion of the homologous GPi in the squirrel monkey also projects to the VA/VL, as shown by injection of the retrograde tracer, Evans Blue, into the VA/VL (Parent and de Bellefeuille, 1982). Therefore, the EP may not only be homologous to the GPi in terms of its function and connectivity in the basal ganglia but also in terms of its internal anatomical organization. These workers also found, through concomitant injection of another fluorescent retrograde tracer, DAPI-primuline, into the TPC that 70-75% of these central cells were double-labelled, meaning that pallidothalamic and pallidotegmental fibres arise largely from the same neurons in the core of the GPi. These collateral projections to VA/VL and TPC from the EP also exist in the rat (Takada and Hattori, 1987). This would

explain the lack of SRIF-immunoreactive terminals in the TPC.

The subpopulation of EP neurons projecting to the VA/VL must contain some transmitter other than SRIF. GABA is thought to be present in this projection because there are glutamic acid decarboxylase (GAD)-immunoreactive neurons in the caudal 1/3 of the rodent EP (Murakami et al., 1989), GAD being the synthetic enzyme for GABA, and there are GAD-positive boutons in the motor thalamus of the cat (Kultas-Ilinsky et al., 1985). The inhibitory action of GABA neurons projecting from the EP to the VA/VL is suggested by physiological analyses in the cat whereby orthodromic stimulation of the EP afferents inhibits VA/VL neurons (Gazzara et al., 1986; Jinnai et al., 1987). SRIF in the pallidohabenular projection, on the other hand, may somehow modulate the inhibitory action of this input because part of this projection is also GABAergic (for review, see Heimer et al., 1985). Substance P is another candidate for this projection because some of the GAD-positive neurons also contain substance P (Murakami et al., 1989).

From the results of this experiment, it can be inferred that GABA and SRIF must not coexist in VA/VL afferents. Examples of differential colocalization abound in the central nervous system. For example, a marked regional variation in coexistence exists in the ventral mesencephalon of the rat, with dopamine and cholecystokinin exhibiting almost total coexistence in the pars lateralis of the substantia nigra but virtually no coexistence within the set of dopaminergic neurons which are displaced within the pars reticulata. The finding in the EP indirectly supports the common principle that rarely does coexistence of a certain combination of messengers occur in all of the neurons of a particular nucleus (for review, see Hökfelt et al., 1986), albeit when the nucleus is defined as a homogeneous structure, which, in terms of its connectivity, the EP is not. It is actually a group of subnuclei when we consider that its

subdivisions project to different targets. How many other nuclei will be found to possess differential coexistence of transmitters corresponding to their differential projections?

In future, SRIF can be used as a marker for that class of EP neuron which projects to the LHb. If similar studies confirm that some portion of the SRIF-terminals in the CPu and CM-Pf arise from the SRIF-containing population of EP neurons, this subset can be easily distinguished from that projecting to the VA/VL without going to the trouble of using a retrograde tracer. For example, a fluorescent counterstain such as ethidium bromide, used in conjunction with immunofluorescent labelling for SRIF, would designate with a fair degree of certainty those neurons projecting to the VA/VL as those that are counterstained but do not also have the immunofluorescent label. Thus, SRIF may become a marker for the differential projections of the EP. Ever since the first findings on the connective compartmentalization of the EP (Van der Kooy and Carter, 1981), it has not been sufficient to talk of the EP as a single structure, and now it is even more appropriate to consider the EP as a compartmentalized structure because its separate divisions have different peptide contents as well.

Experiment 2

Distributions and Colocalization of Neuropeptide Y and Somatostatin in the Goldfish Brain

ABSTRACT

The distributions of single- and double-labelled neuropeptide Y (NPY) and somatostatin (SRIF)-containing perikarya and processes were determined in the goldfish brain using immunoperoxidase and immunofluorescence techniques, respectively.

Observation of double-labelling distributions in the goldfish brain led to the conclusion that coincident distribution of NPY and SRIF was far more extensive than the colocalization of these two neuropeptides. In the forebrain, a particularly striking degree of colocalization was found in the neurons of the ventrolateral telencephalon (VL). In the brainstem, SRIF and NPY were colocalized only in the large cell bodies of the medial column of the vagal motor complex. In the midbrain, single-labelled NPY-positive cell bodies were found in the locus coeruleus. Single-labelled SRIF-positive cells were found within the hypothalamus, and most SRIF-positive cell bodies found throughout the reticular formation appeared to be abutted by SRIF-positive boutons. Neurons immunoreactive for both peptides were found in the thalamus and intermingled in the periventricular layer of the optic tectum, but none showed colocalization.

Colocalization was also noted within fibres in a number of regions. Fibres found within the telencephalon showed high degrees of colocalization of NPY and SRIF. Some fibres in

the swim bladder, one of the peripheral organs to which the medial column of the vagal motor complex projects, were also colocalized. Processes in the torus semicircularis and in the sensory and motor layers of the vagal lobe showed single-labelled immunoreactivity for both SRIF and NPY in distinct laminar patterns. The only cerebellar region which showed NPY and SRIF immunoreactivity was the eminentia granularis. Here, very large single-labelled SRIF-positive terminals appeared to form pericellular baskets. Single-labelled NPY- and SRIF-positive fibres were also found in the secondary gustatory nucleus and tract, the facial lobe and the descending trigeminal tract.

Because of the colocalization and high degree of coincidence of SRIF and NPY in the goldfish brain, these two neuropeptides appear to act together in a variety of sensory and motor systems. The high degree of colocalization of SRIF and NPY in the cell bodies of the VI as well as in the terminals of dorsolateral regions of the telencephalon, suggest intrinsic telencephalic projections from the VI to most of the telencephalon. From our understanding of the function of various neuroanatomical regions of the goldfish brain, we can conclude that SRIF and NPY may function within the gustatory system and may mediate both auditory and mechanoreceptive sensation in the octavolateral system.

INTRODUCTION

Neuropeptide Y (NPY) is a 36-amino acid peptide containing five tyrosine residues and was first purified from porcine brain. The primary structure of NPY is well-conserved across species, with only one amino-acid substitution at position 17: Leucine in pig and cow is replaced by methionine in human, rat, guinea-pig and rabbit (for review, see Tatamoto, 1989).

The nucleotide sequences encoding the precursors to both human and rat NPY have been deduced from cDNAs, and the translated regions of the rat and human genes show about 75% homology. There are two potential sites for proteolytic processing of the rat NPY precursor, generating three peptides: a 29-amino acid signal peptide, the 36-amino acid NPY and the 30-amino acid C-terminal peptide (CPON). CPON is also highly conserved, with only two amino acid substitutions between rat and human (for review, see Allen, 1989).

The concentration of NPY in the brain is higher than that of any other known peptide (for review, see Tatamoto, 1989), and its distribution in the central nervous systems of mammals (Chronwall et al., 1985), amphibians (Danger et al., 1985; Cailliez et al., 1987) and goldfish (Pontet et al., 1989) is widespread. Functionally, NPY is best known for its vascular control, as NPY-containing fibres abundantly supply blood vessels in the peripheral nervous systems (for review, see Tatamoto, 1989). Centrally, NPY has neuroendocrine functions among others, in both higher and lower vertebrates. Kah et al. (1989) have discovered that in goldfish, NPY causes a dose-dependent release of gonadotrophin from the anterior lobe of the pituitary, as it does in rabbits (Pau et al., 1989). NPY is colocalized with noradrenaline in the central and peripheral nervous systems (for review, see Hökfelt et al., 1986) and is functionally best characterized to date in the hippocampus of the rat (Haas et al., 1987). Generally speaking, colocalization of neuropeptides can increase functional diversity at the synapse.

Somatostatin, or somatotropin release inhibiting factor (SRIF), was first isolated from ovine hypothalamus, and its primary structure of 14 amino acids, including the sulfide linkage between its two cysteine residues, was determined (Brazeau et al., 1973). Later, ovine and porcine hypothalamic extracts of an NH₂-terminally extended somatostatin, containing 28

residues, were isolated and sequenced (Esch et al, 1980; Schally et al., 1980). Two distinct somatostatin molecules are also present in the pancreas of the anglerfish and catfish, but they are encoded by separate genes, apparently because of a gene duplication event in the common ancestor of these two fish (Hobart et al., 1980; Su et al., 1988). In contrast to this, a single gene encodes both SRIF-14 and -28 in mammals, and the form of SRIF expressed may be due instead to differential expression or regulation of the endopeptidases that cleave the precursor molecule (McDonald et al., 1987).

In all animals, cleavage of SRIF-28 to yield SRIF-14, which comprises the amino acids 15-28, takes place at the same site, between the arginine and lysine residues (Argos et al., 1983). More importantly, the isolation from rat medullary thyroid carcinoma of a cDNA encoding SRIF-28 has led to observations that there is strict conservation of the SRIF-28 and SRIF-14 amino acid sequences across mammals, and even between the nucleotide sequences encoding SRIF-14 of fish and mammals. This high degree of conservation suggests that strong selective pressures were exerted during evolution to maintain the sequence over the 400 million years since fish and mammals diverged, that SRIF-14 has stringent structural requirements for its function and that important biological functions for the NH₂-terminal extension of SRIF exist in addition to the regulatory actions of SRIF-14 (Goodman et al., 1982; Su et al., 1988).

SRIF also has an extensive cerebral distribution outside the hypothalamus in mammals such as the rat (Johansson et al., 1984; Vincent et al., 1985) and so is considered to have neurotransmission functions in addition to hormonal ones. SRIF and NPY appear to act together in neurotransmission and are both colocalized with GABA in selective subsets of neurons in the caudate nucleus, hippocampus and cerebral cortex as has been seen in rat and

most other mammals (Staines et al., 1988).

Although whole brain immunohistochemical mappings of teleosts exist for several neuroactive substances or their synthetic enzymes (choline acetyltransferase: Brantley and Bass, 1988; Ekström, 1987; substance P: Sharma et al., 1989; tyrosine hydroxylase: Hornby et al., 1987), the overall central distribution of SRIF has not yet been determined. This is because although immunocytochemical localization of SRIF has been given generous attention in a wide variety of amphibians (Laquerriere et al., 1989; Olivereau et al., 1987) and teleosts including the goldfish (Batten et al., 1985; Grau et al., 1985; Kah et al., 1982; Olivereau et al., 1984a; 1984b), the focus of this work has been on forebrain structures controlling pituitary function. This bias is also present in immunohistochemical mappings of SRIF in the brains of several avian and reptilian species (for review, see Peter, 1986). The whole brain mapping of SRIF immunoreactivity was completed in this experiment partly for the above reason and also for the opportunity to include the brainstem which has been virtually ignored in distribution studies of the goldfish brain. It is only very recently that SRIF immunoreactivity in an entire teleost brain has been studied, in a gymnotid (Sas and Maler, 1988), and a map of the goldfish brain can allow for future comparison and contrast between gymnotids and goldfish. The whole brain distribution of NPY has already been determined, including the brainstem (Pontet et al., 1989), but its inclusion in the present experiment began with an interest in its colocalization with SRIF in a possible striatal primordium and continued with curiosity in other brain regions in which there is an interaction between the two peptides.

Colocalization of neurotransmitters refers to the existence of multiple neuronal messengers within a single neuron (for review, see Hökfelt et al., 1986). Although the study of coexistence of neurotransmitters across phylogeny has been studied to only a limited extent,

and mostly in mammals, it is known that colocalization of neural substances is also characteristic of bony fish, such as goldfish. For example, Fryer and Lederis (1988) have used immunocytochemistry to locate corticotropin-releasing factor and arginine vasotocin in the same hypothalamic neurons of the goldfish, and Moons et al. (1988) have found vasotocin and growth hormone-releasing factor to coexist in a population of nerve fibres in the sea bass pituitary. These experiments all employed the staining of the same cells in adjacent sections by the immunoperoxidase method. The double-immunofluorescence method has not yet been used to examine coexistence in fish brain. The coexistence of SRIF and NPY in particular has been documented by Vincent and his colleagues (1982; 1983) in the forebrain of mammals. It was thought that this same phenomenon in the goldfish could help to place the importance of colocalization in an evolutionary context, particularly with reference to the striatum (see General Introduction).

The use of the double-label immunofluorescence technique in the current mapping study was especially helpful in differentiating between coincidence and coexistence. Coincidence refers to the existence of multiple neuronal messengers in separate populations of neurons or nerve fibres that are similarly distributed. Double-labelling was the best, and in the case of nerve fibres, the only way of differentiating between a high degree of coincidence of NPY and SRIF and actual coexistence of the two neuropeptides.

The single-labelling immunoperoxidase method, on the other hand, was included to facilitate the mapping of NPY and SRIF and as some confirmation of the double-immunofluorescence results and also because it is a more sensitive technique than that of immunofluorescence and could reveal labelling in regions that the other approach could not.

MATERIALS AND METHODS

Goldfish (*Carassius auratus*) of approximately 35 g were perfused transcardially with 0.6% saline followed by fixative consisting of 4% paraformaldehyde and 0.2% picric acid in 0.16 M phosphate buffer, pH 6.9, at room temperature. Brains and swim bladders were removed and post-fixed in the same fixative for 3 h at 4° C then transferred to 10% sucrose, 0.01% sodium azide in 100mM phosphate buffer for 1 h and, finally, stored in a change of sucrose at 4° C until cut. After freezing with crushed dry ice, brain and swim bladder sections of 15 µm thickness were cut on a cryostat (Hacker Instruments Inc.) and thaw-mounted onto chrome alum-coated slides. Adjacent brain sections were processed for SRIF and NPY immunoperoxidase (single-labelling), immunofluorescence (double-labelling) and cresyl violet (Nissl) staining.

Single-labelling (Immunoperoxidase)

The slide-mounted sections were washed for 15 min in 10 mM phosphate buffered saline (PBS), pH 7.2, then incubated overnight at 4° C in mouse anti-SRIF antibody (Dr. J.C. Brown) or rabbit anti-NPY antibody (Amersham) at concentrations of 1 in 500 and 1 in 1500, respectively, in 10 mM PBS, 0.3% Triton X-100. After another 15 min wash in 10 mM PBS, sections were incubated in biotinylated sheep anti-mouse antibody (Amersham) or biotinylated donkey anti-rabbit antibody (Amersham), depending on the primary antibody, both at concentrations of 1 in 100 in 10 mM PBS, 0.3% Triton X-100 at room temperature for 1 h. Sections were washed a third time for 15 min in 10 mM PBS and then incubated in

streptavidin-horseradish peroxidase (HRP; Amersham) at 1 in 100 in 10 mM PBS for 1 hr at room temperature. After a 10 min wash in 10 mM PBS followed by a 10 min wash in 50 mM Tris buffer, pH 7.4, immunostaining was visualized in the following way: Sections were first pre-incubated for 5 min in a 0.02% solution of 3, 3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) in 50 mM Tris and then reacted for 5 min in the same solution with 30% H_2O_2 at a final concentration of 0.009%. Another aliquot of H_2O_2 was added and the reaction continued until specific staining was judged to be sufficiently above background. Sections were then washed in progressive 1:1 dilutions of 50 mM Tris, for 10 min each wash, left to air dry overnight and coverslipped with Permount.

Controls

Specificity of primary antisera was tested by incubating control sections, cut adjacent to experimental sections, in pre-adsorbed antibody overnight at 40°C. Pre-adsorption was carried out by mixing about 8 µg of peptide (Peninsula) per ml of antibody diluted to 1 in 500 in the case of SRIF and 1 in 1500 in the case of NPY and gently agitating at room temperature for several hours. Experimental (unsaturated) primary antisera were agitated in the same way. Specificity of secondary antisera was tested by omitting primary antisera from the first incubation medium. All subsequent steps in both control situations were exactly as described under "Single-labelling". The rationale behind pre-adsorption controls in immunohistochemistry is discussed in Appendix B.

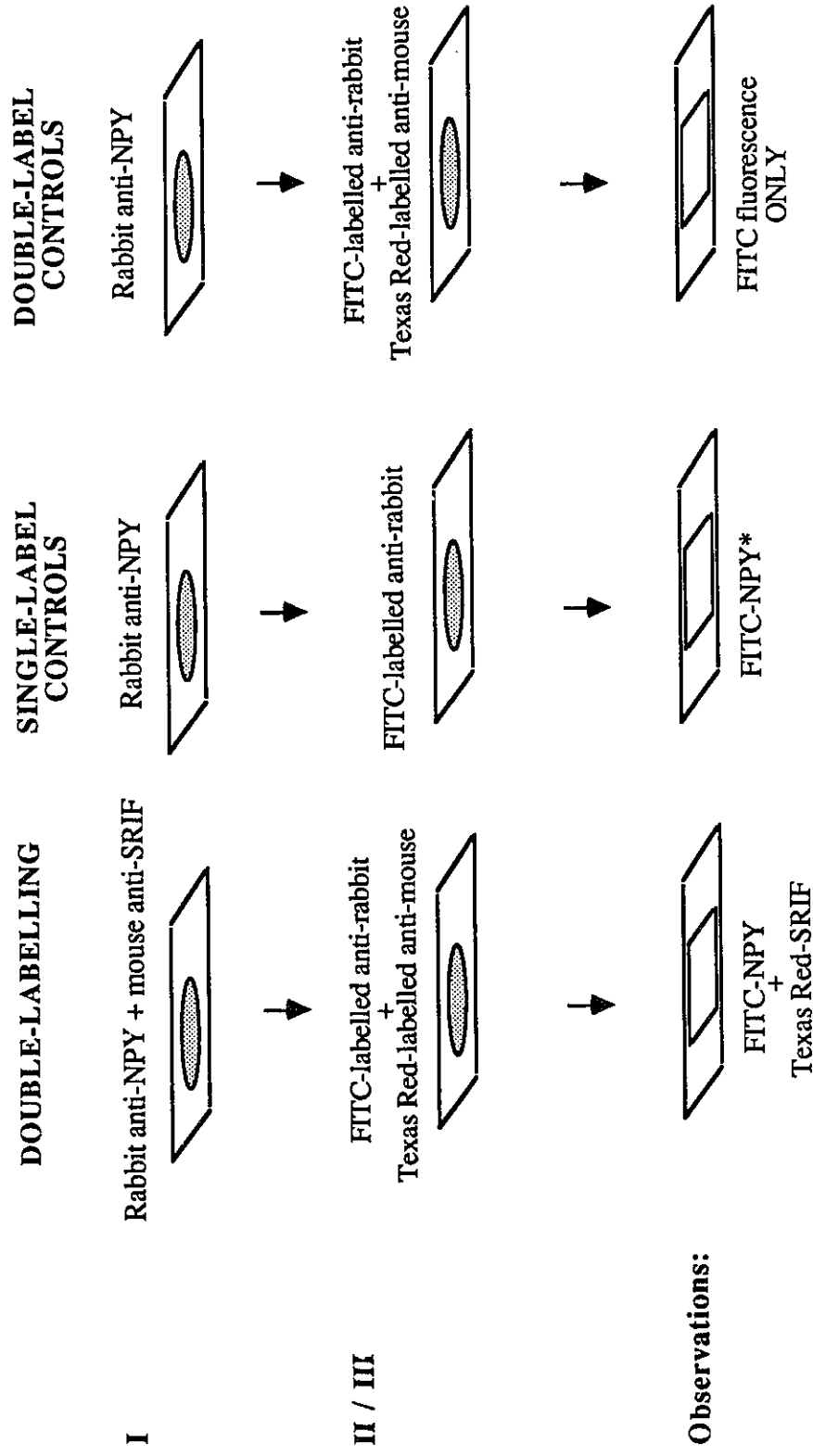
Double-labelling (Immunofluorescence)

Sections were washed for 15 min in 10 mM PBS then incubated overnight at 4° C with a mixture of the primary antisera, at final concentrations of 1 in 400 (NPY) and 1 in 200 (SRIF) in 10 mM PBS, 0.3% Triton X-100. After a second 15 min wash in PBS, sections were incubated for 30 min at 37° C with secondary antibodies also diluted in 10mM PBS, 0.3% Triton X-100. The secondary antisera employed were FITC-labelled donkey anti-rabbit (Amersham) and biotinylated sheep anti-mouse (Amersham) at final concentrations of 1 in 20 and 1 in 100, respectively. The mixture of secondary antisera was prepared the night before and left at 4° C to allow any potential cross-reaction to occur and precipitate. After a further wash, sections were incubated for 30 min at 37° C with streptavidin-Texas Red (Vector) diluted to 1 in 100 in PBS alone. After a final wash, sections were coverslipped under glycerol containing 0.1% *p*-phenylenediamine.

Controls

Fig. 2.1 shows the single- and double-label control procedures for the double-labelling immunofluorescence technique. Single-label controls for the double-labelled material (according to the method of Staines et al., 1988) were incubated with either FITC-labelled donkey anti-rabbit (1 in 20; Amersham) or rabbit anti-mouse (1 in 20; Dakopatts) alone, depending on the primary antibody. This was to ensure that labelling patterns for each substance in double-stained sections agreed with those observed in sections incubated with only one primary antiserum.

Fig. 2.1. Schematic diagram of the steps involved in double-labelling and single- and double-label control procedures. Steps involving incubation with antisera solutions are followed by observation of coverslipped sections under the microscope.



*FITC fluorescence distributions in single-label controls are the same as the FITC distributions in double-label controls

The species specificity of secondary antisera was tested using double-label controls. These were incubated first in only the primary antiserum raised against NPY or SRIF, then in the double secondary antibody solution and finally streptavidin-Texas Red, the expectation being that only the appropriate fluorescence of the two possible would result if neither primary antiserum cross-reacted with the inappropriate secondary in the mixture.

Microscopy

Tissue processed by the immunoperoxidase method was studied under both bright field and dark field conditions. Cell bodies and processes were simultaneously evaluated for NPY and SRIF immunoreactivity by visualizing FITC and Texas Red with separate filters on the same reflector slide of a Zeiss Axioplan microscope. Microphotographs were taken using Kodak Pan-X 32 ASA and Tri-X 400 ASA film for bright field and fluorescent conditions, respectively. Distributions were mapped onto drawings taken from the forebrain atlas of Peter & Gill (1975), and drawings of hindbrain sections adapted from the sections themselves. These hindbrain drawings were not necessarily obtained from sets continuous with the forebrain sections. Consequently, forebrain and hindbrain drawings may appear to be in slightly different planes. The terminology used in naming brain regions is that of Peter and Gill (1975), Morita and Finger (1987) and Prasada Rao et al. (1987).

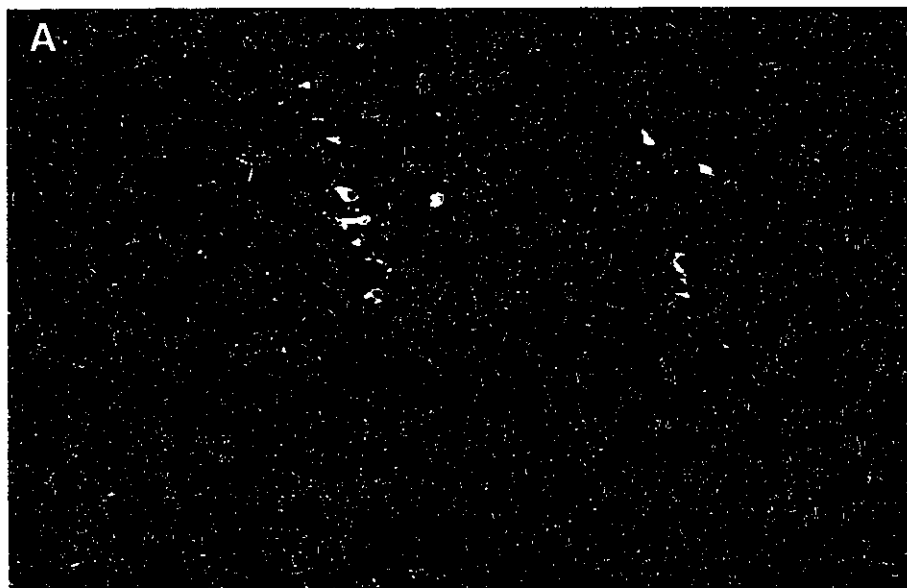
RESULTS

Controls

No specific staining was found in controls in which the primary antiserum was omitted from the immunoperoxidase staining procedure. Pre-adsorption controls abolished SRIF staining but some pale brown non-specific staining did remain in NPY primary controls. In spite of this, it was easy to discriminate between specific and nonspecific labelling because the specific staining was so much sharper and darker than the latter. The antiserum must have had receptors available to bind not only to NPY but to some other neural compound as well. For further discussion of antibody specificity, see Appendix B.

The controls run for the double-labelling experiments were similarly problematic. In addition to these results (not shown), Fig. 2.2 depicts an example of an internal control, that is, a demonstration within the experimental tissue itself that distributions of NPY and SRIF immunoreactivity are different from one another and, therefore, the two primary antisera do not cross-react. The complete absence of NPY-positive neurons where SRIF-immunoreactive neurons were found shows that NPY/SRIF coexistence was not a general phenomenon. Distribution patterns of NPY and SRIF in double-immunofluorescent sections corresponded to those in single-labelled controls, and double-labelled controls met the criteria for species specificity in the secondary antisera, with each control showing only the one expected fluorescence.

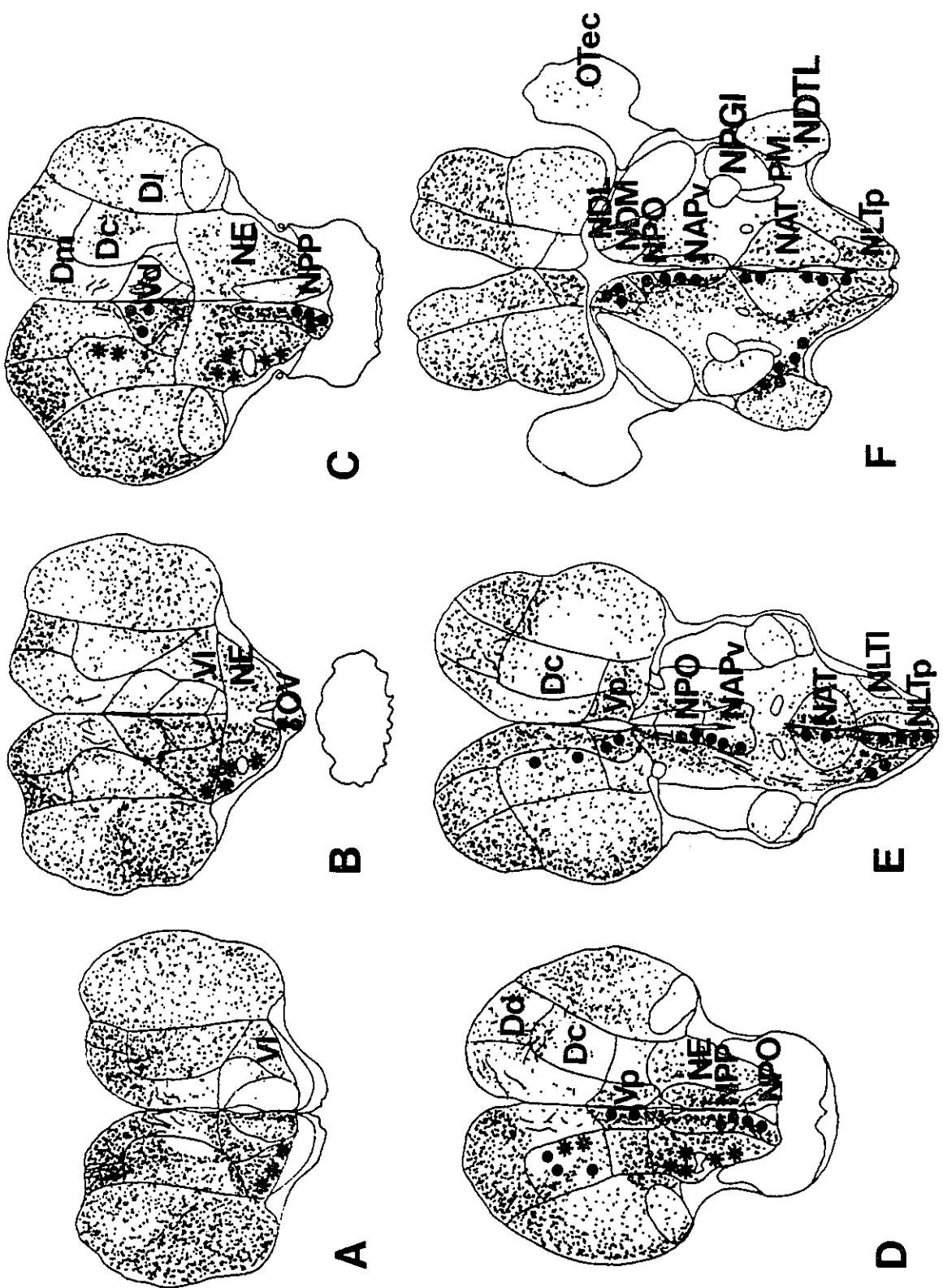
Fig. 2.2. Two-colour immunofluorescence photomicrographs depicting SRIF and NPY immunoreactivity in the same section. **A** and **B** differ only in the fluorescent filter conditions used to examine the section. **A.** SRIF-immunofluorescent dorso-ventral arcades of cell bodies in the dorsal thalamus. **B.** These same cells show no NPY-immunofluorescence, providing an example of an internal control for antibody specificity. Scale bar = 100 μm .

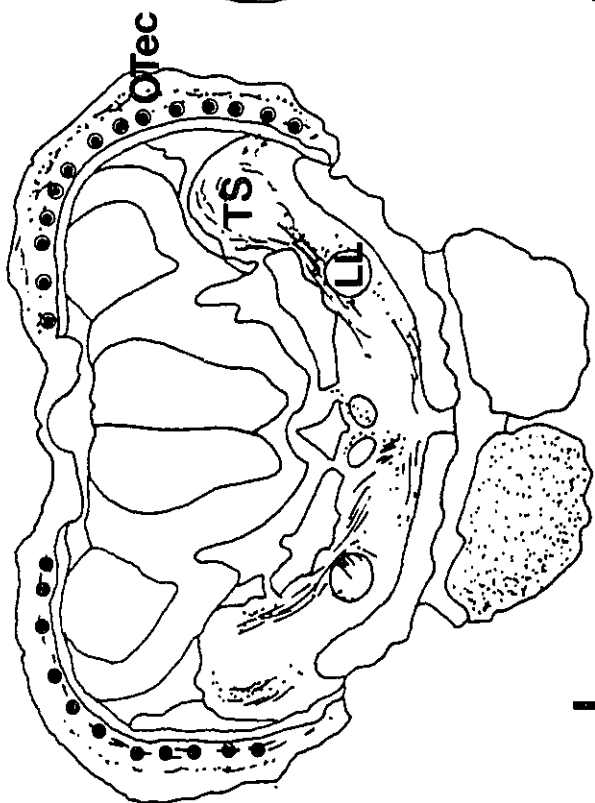
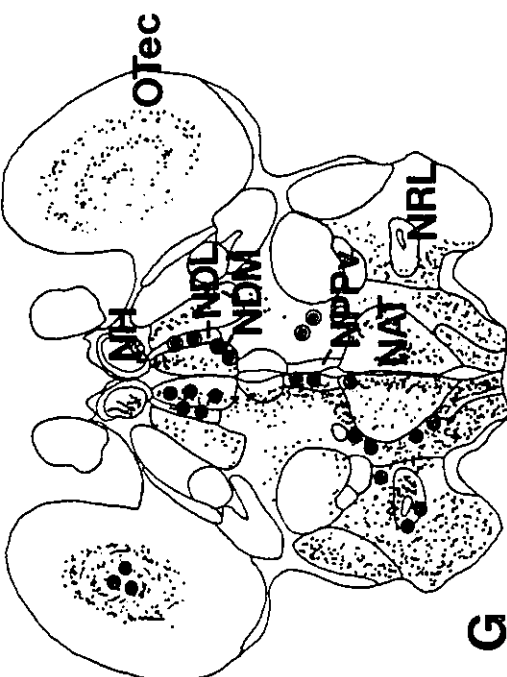
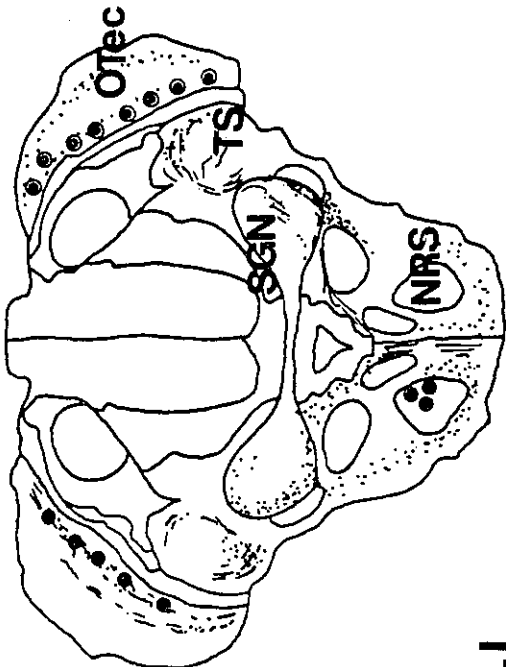
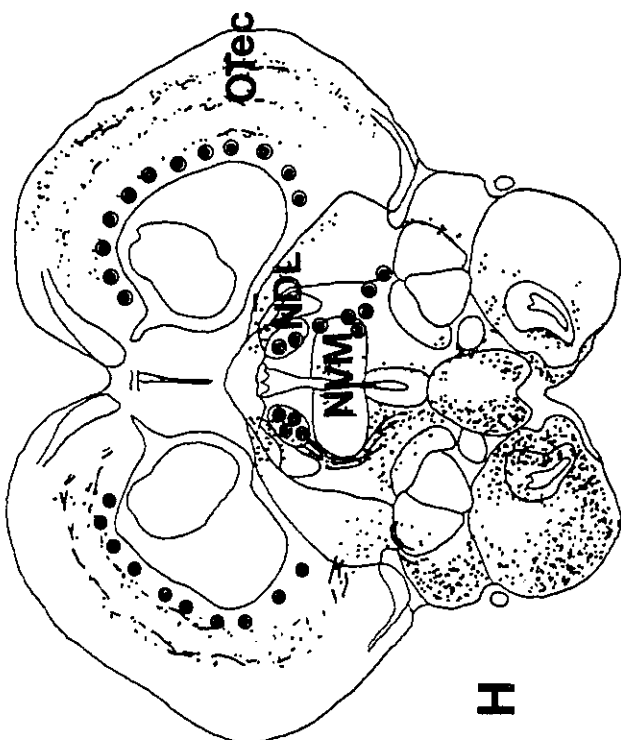


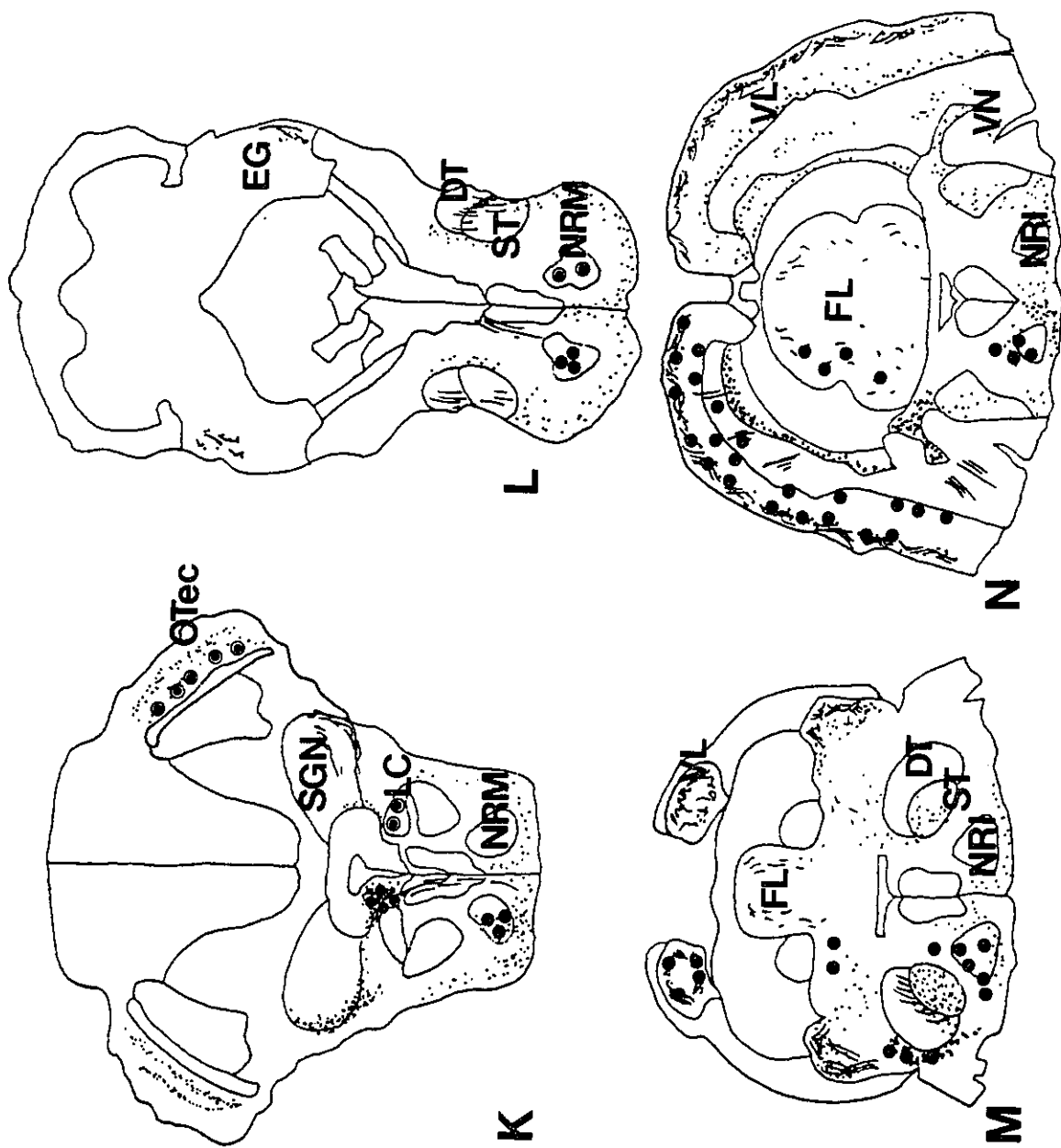
Forebrain

Fig. 2.3 is a map of the distributions of cell bodies and fibres at selected levels of the brain. Nomenclature and corresponding abbreviations are given in Appendix A. The Vl was the most anterior telencephalic nucleus displaying colocalization in its cell bodies. Almost every cell body that contained one neuropeptide also contained the other (Fig. 2.3 A and Fig. 2.4 A,B). Slightly more posteriorly, this cell body group extended down into the area of the anterior commissure and NE (Fig. 2.3 B-D). A high degree of colocalization of NPY and SRIF was also a feature of the cell groups in the Dc, although there were also a number of cells containing SRIF but no NPY (Fig. 2.3 C-D). In the telencephalon, fibres formed extensive, highly varicose networks that showed high degrees of colocalization of NPY and SRIF (Fig. 2.4 C,D). Although varicose fibers exhibiting colocalization were found throughout the rostrocaudal axis of the telencephalon, innervating a number of different cytoarchitectonic regions, they were most prominent in the lateral regions of the telencephalon. Few colocalized varicosities were found within the medial telencephalon. Superimposed upon these observations was the fact that, in general, these same regions of the telencephalon also exhibited a high degree of coincidence of single-labelled terminals. This latter phenomenon, whereby NPY- and SRIF-immunoreactive fibres and terminals were distributed in similar patterns but did not exhibit colocalization, was characteristic of many regions of the goldfish brain, and was particularly striking in the vagal lobe (Fig. 2.6). Careful observation of immunoreactivity in different regions of the telencephalon indicated that roughly 90% of immunoreactive fibers in the Dm and Dd showed colocalization. All of the the NPY-labelled varicosities in the olfactory tract also contained SRIF-immunoreactivity, but in addition, this

Fig. 2.3. The relative densities of SRIF- and NPY-immunoreactive nerve terminals (fine dots) as well as SRIF- and NPY-immunoreactive axons and cell bodies are indicated on the left and right sides, respectively, of schematic coronal sections of the goldfish brain and spinal cord (A-Q). SRIF-positive cell bodies are represented by solid dots and NPY-positive cell bodies by solid dots within circles. Cell bodies within which SRIF and NPY are colocalized are represented by asterisks on the left side. Intervals between sections are as follows: A-B=300 μm ; B-C=300 μm ; C-D=200 μm ; D-E=400 μm ; E-F=200 μm ; F-G=200 μm ; G-H=600 μm ; H-I=1000 μm ; I-J=360 μm ; J-K=120 μm ; K-L=360 μm ; L-M=840 μm ; M-N=240 μm ; N-O=700 μm ; O-P=700 μm ; P-Q= 500 μm .







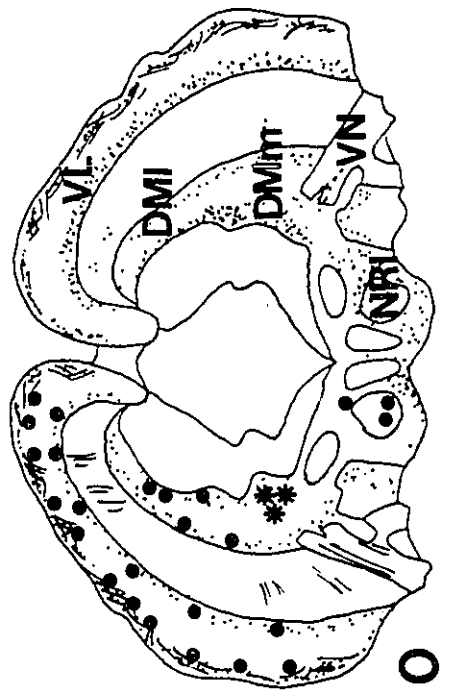
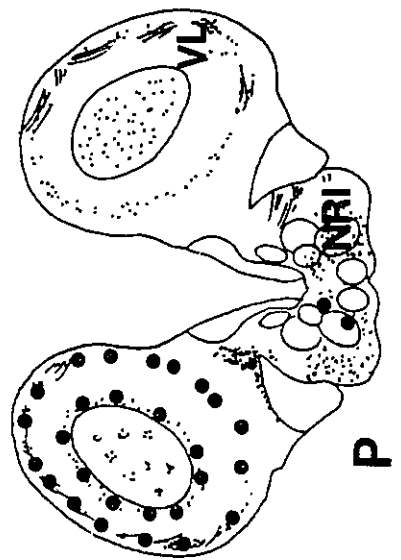
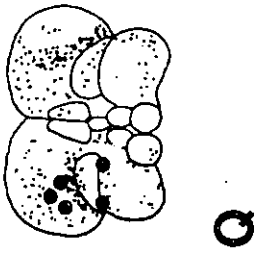
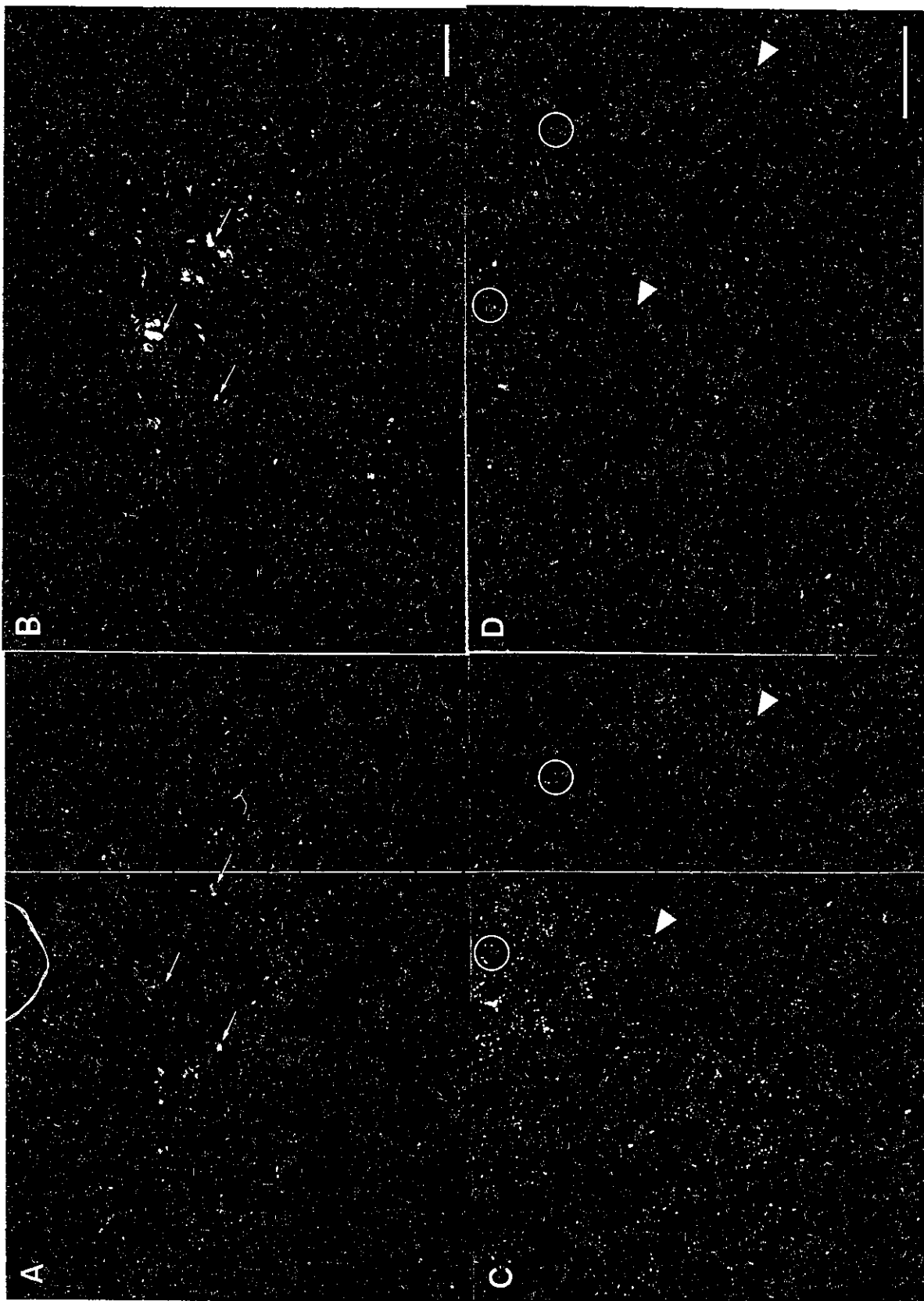


Fig. 2.4. Two-colour immunofluorescence of SRIF- and NPY-immunoreactive cell bodies, fibres and terminals in the telencephalon. **A.** NPY immunofluorescence in neurons of the VI. **B.** In the same section, SRIF immunoreactivity occurs in many of the same neurons of the VI (arrows) as does NPY in **A.** **C.** NPY-immunofluorescent fibres and varicosities in the telencephalon. **D.** In the same section, SRIF-immunofluorescent neuropil shows the same general pattern of distribution. Compared with **C**, this is an example of the general coincidence of NPY and SRIF in fibre and terminal fields of the goldfish brain. Many, but not all fibres and terminals show colocalization. Examples of fibres in which NPY and SRIF are not colocalized are indicated by arrowheads, and terminals in which the two peptides are colocalized are contained within circles. Scale bar = 50 μm .



region contained an equally large population of fibers which were SRIF-positive alone. In the DI, about 75% of both the NPY- and SRIF-positive fibers exhibited immunoreactivity for the other peptide. In addition to the colocalization and coexistence of these peptides, there were also regions of the telencephalon in which their distributions did not overlap, as is shown in Fig. 2.3 A-F.

Diencephalon and optic tectum

No diencephalic neurons showed colocalization of the two peptides examined. The parvocellular NPP/NPO contained SRIF-positive cells (Fig. 2.3 C-F). SRIF-immunoreactive cells also appeared in the NAPv and magnocellular NLTI (Fig. 2.3 E,F). Cerebrospinal fluid (CSF)-contacting cells which were SRIF-positive were seen in the NLTa, NLTp and NPPv (Fig. 2.3 E-G), with their apical dendrites extending into the ventricle. The organum vasculosum lamina terminalis also contained densely packed SRIF-positive cell bodies and varicosities (Fig. 2.3 B). SRIF-positive fibres coursed through the NLTa from the NPO in the preoptic-neurohypophyseal tract (Fig. 2.3 E), and varicose fibres from the NLTI coursed ventromedially, also toward the pituitary. In the thalamus there were two dorso-ventral arcades of SRIF-immunoreactive somata at the margin between the NDL and NDM (Fig. 2.2 and Fig. 2.3 F,G). A few SRIF-positive cell bodies were scattered in the NVM. A group of NPY-positive cell bodies were also observed in the NDL and clustered about the ventral NDM. These latter cells were continuous with larger cell bodies on the NVM border (Fig. 2.3 G,H). The NRL contained some small single-labelled SRIF-positive cells. SRIF-positive terminals were also present in the NRL but did not show NPY staining. In the habenula, SRIF-labelled

fibres were very intensely stained and had very prominent varicosities (Fig. 2.3 G).

The optic tectum contained small, unipolar immunopositive neurons uniformly distributed along the periventricular layer. SRIF- and NPY-containing tectal cells were interspersed. Various laminae of the optic tectum were characterized by SRIF- or NPY-positive varicosities of varying densities. Again, there was no colocalization in these layers (Fig. 2.3 F-K).

A new nucleus

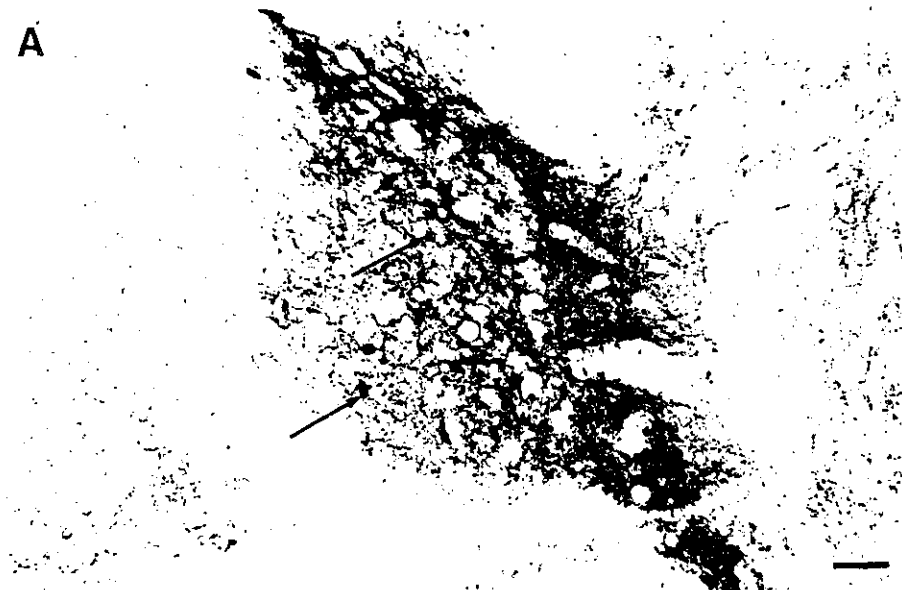
A dense parvocellular SRIF-positive cluster appeared at the interface of the NPGl and NDTL with a few scattered cells within the NDTL itself (Fig. 2.3 F and Fig. 2.5 A). The processes emanating from this group did not course in any particular direction. Adjacent sections stained for Nissl substance showed that this interface was composed of tiny cells corresponding exactly to the SRIF-defined boundaries and that it was distinctly separate from the NLTl just anterior to it. The nucleus became wider and more diffuse posteriorly, fanning out from its anterior origin. For the time being this nucleus could be named the parvocellular marginal nucleus (PM).

Midbrain

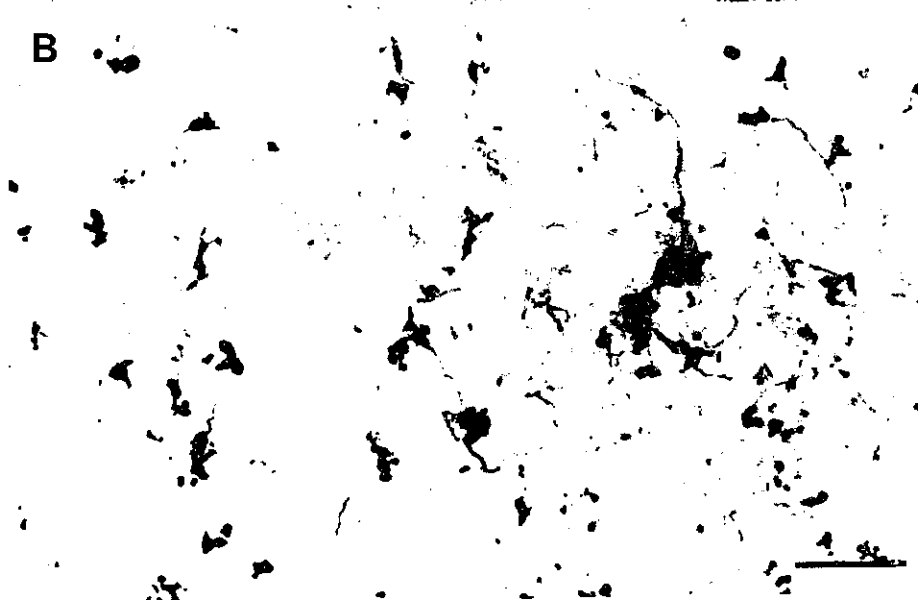
Intensely labelled NPY-positive perikarya were observed in the locus coeruleus. In the ventral midbrain, single-labelled NPY- and SRIF-positive fibres were seen descending within the tectobulbar tract. The interpeduncular nucleus was extremely SRIF-rich in the middle and ventral part. In contrast, the interpeduncular nucleus was lacking in NPY-positive fibres

Fig. 2.5. Brightfield micrographs of the parvocellular marginal nucleus and the eminentia granularis of the goldfish brain. **A.** SRIF-immunoreactive cell bodies (arrows) and fibres of the newly identified parvocellular magnocellular nucleus at the interface of the NPGI and NDTL. **B.** SRIF-positive processes with terminal boutons arranged as pericellular baskets in the eminentia granularis. Note the large size of the boutons. Immunoperoxidase method. Scale bar = 50 μ m.

A



B



except for a delicate varicose cluster on each side (data not shown).

The paralemniscal bundle, in which there were coincident NPY- and SRIF-positive fibres was most striking, coursing medially to the LL between the ventral midbrain and TS (Fig. 2.3 I). SRIF- and NPY-immunopositive processes had a coincident laminar pattern in the TS (Fig. 2.3 I,J).

In the secondary gustatory nucleus, non-varicose fibres containing NPY were distributed in a laminar pattern and SRIF-positive terminals were restricted mainly to the ventral portion of the nucleus (Fig. 2.3 J,K). Capping the lateral and laterodorsal descending trigeminal tract and secondary gustatory tract, grape-like clusters of very large SRIF-containing boutons were observed (Fig. 2.3 M). They were densely packed, apparently terminating on the large fusiform somata in this region. The terminals were so dense that it was difficult to tell if the cell bodies themselves also contained SRIF, so the designation of these cell bodies as being positively stained for SRIF is tentative. Non-varicose NPY- and SRIF-positive fibres were oriented dorsoventrally in the descending trigeminal and secondary gustatory tracts. Terminals immunoreactive for both peptides were also observed in the secondary gustatory tract but were coincident, not coexistent (Fig. 2.3 M).

In the EG, the lateral part of the caudalmost cerebellum, there were fine, dorso-ventrally directed SRIF-processes in the extreme ventrolateral part of the nucleus. NPY-labelled processes with much larger varicosities had the same orientation slightly dorsal to the SRIF group. In the dorsal EG there were grape-like clusters of large SRIF-positive varicosities, apparently forming pericellular "baskets" (Fig. 2.3 L and Fig 2.5 B).

Gustatory lobes and swim bladder

Both the facial and vagal lobes possessed cell bodies and varicose fibres immunoreactive for either NPY or SRIF. Tiny cell bodies and fibres were dispersed randomly about the facial lobe (Fig. 2.3 M,N). In the vagal lobe, lateral to the vagal nerve, there were two bands of somata, one located superficially, in the outermost lobe, and the other located at the lateral surface of the nerve (Fig. 2.3 N-P). Medial to the vagal nerve, cell bodies appeared to receive SRIF-positive axosomatic boutons. There were several layers of SRIF-positive processes in which the SRIF-somata were interspersed. Coincident NPY-positive processes were noted. The peptides were most highly coincident in the outermost varicose fibres which formed a web-like pattern (Fig. 2.6), but were not colocalized except in very few fibres (< 2%). In the caudal vagal lobe, only in DMm neurons was there colocalization of NPY and SRIF (Fig. 2.3 O and Fig. 2.7 A,B). Their axons exited with the vagal nerve (Fig. 2.3 N,O).

When swim bladder was examined for fibres containing colocalized NPY and SRIF, presumably arising from the DMm, it was found that fibres and varicosities with coexistent peptides were present, localized primarily to the ventral anterior (secretory) part of the bladder (Fig. 2.7 C,D), although some were found in the posterior (resorbent) part as well. NPY-positive varicose fibre bundles containing no SRIF were common in the secretory part of the bladder, and individual varicose SRIF-positive fibres without NPY were found in the ventral secretory part.

Fig. 2.6. Coincidence of NPY- and SRIF-immunoreactive fibres and terminals in the vagal lobe. **A.** NPY- immunoreactive varicose fibres in the superficial sensory layer of the vagal lobe. **B.** SRIF-immunoreactive varicose fibres in the same section are distributed similarly to those containing NPY in A but the two peptides are not colocalized in the same varicosities. Scale bar = 50 μm .

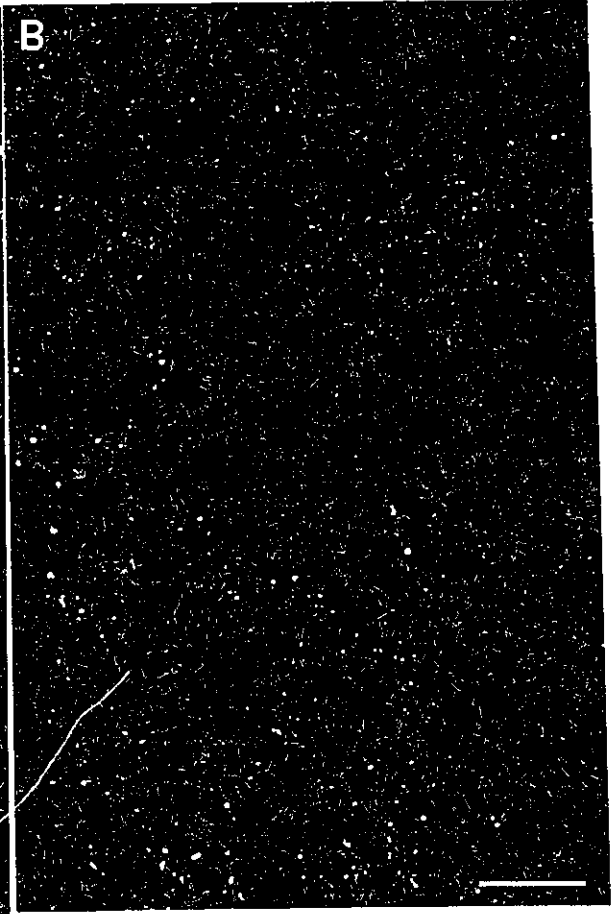
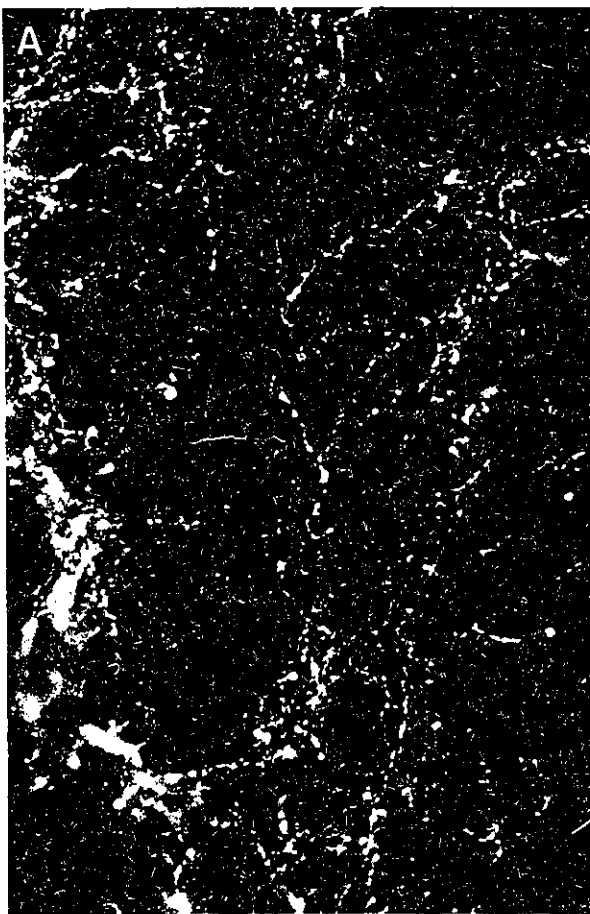
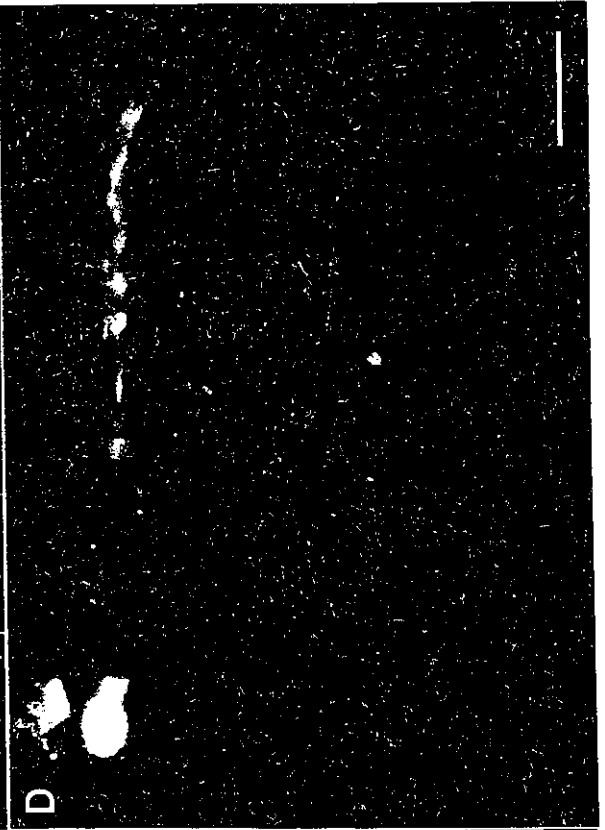
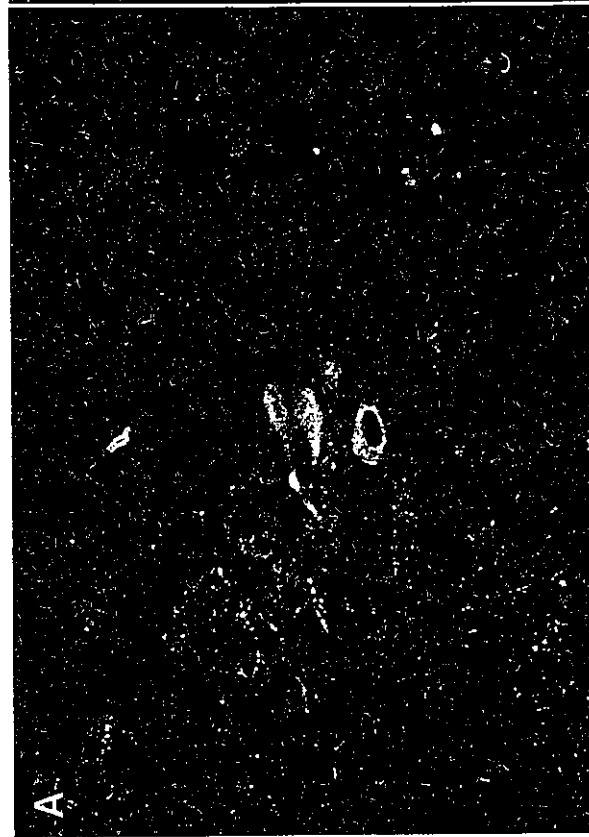


Fig. 2.7. NPY and SRIF colocalization in the DMm and swim bladder. **A.** NPY-immunofluorescent neurons in the DMm. **B.** SRIF-immunofluorescence in the same DMm neurons as those containing NPY in **A.** **C.** NPY-immunofluorescent varicose fibre in the ventral secretory chamber of the swim bladder. **D.** The same fibre that contains NPY in **C** also shows SRIF-immunoreactivity. Scale bar = 50 μm .



Reticular formation and spinal cord

A pattern in which darkly-stained SRIF-positive boutons appeared to envelop cell bodies was true of all reticular nuclei, the NRS, NRM and NRI (Fig. 2.3 J-P), and none were specifically stained for NPY, except for a few in the NRM (Fig. 2.3 L). Those cell bodies containing only SRIF had deeply stained cytoplasm, lightly stained nuclei and smoother edges. Those apparently with terminals on them had a rougher outline. Dispersed throughout the entire reticular formation, both SRIF- and NPY-immunoreactive varicosities were present although they tended to concentrate in the most medial and ventral portions (Fig. 2.3 J-P).

There were no cell bodies specifically stained for NPY in the rostral spinal cord, but small SRIF-positive somata were scattered just dorsal to the location of the motor neurons, and another cluster occurred dorsal to that one. A heavily labelled band consisting of NPY- and SRIF-positive terminals curved over and partly extended into white matter just ventral to the dorsal half of the cord (Fig. 2.3 Q).

DISCUSSION

Telencephalon

The highest densities of NPY-labelled fibres and perikarya were found in the lateral telencephalon and the lowest densities in the ventromedial telencephalon. These results are in agreement with those of Pontet et al. (1989), on the distribution of NPY-immunoreactivity in the goldfish brain. The SRIF-immunoreactive varicose fibre plexus of the telencephalon and

the SRIF-positive perikarya in the entopeduncular and ventral telencephalic regions have been reported in a range of teleosts (Batten et al., 1985; Grau et al., 1985; Oliverreau et al., 1984a; Sas and Maler, 1988). Batten et al. (1985) specified that the majority of SRIF-positive telencephalic cells in the molly were small, unipolar, lay close to the meningeal surface and then became dispersed in a transitional zone between VI and NE, exactly as they were observed in the goldfish. The high degree of colocalization in the varicosities of the dorsal telencephalon may be explained by intrinsic connections that the double-labelled VI and Dc neurons may have with other telencephalic nuclei (Murakami et al., 1983). Since these were the only double-labelled cell bodies to be found in the goldfish brain, with the exception of the medial column of the vagal motor complex (DMm), which is known to project only to the periphery (Morita and Finger, 1985), it can be suggested with some confidence that the nearby telencephalic fibres in which NPY and SRIF are colocalized are local collaterals of VI and Dc neurons. This conclusion would require modification of course if NPY-/SRIF-immunoreactive neurons were present in the olfactory bulbs. It is of note in this regard that double-labelled axons were found in the olfactory tract (data not shown).

Colocalization of NPY and SRIF in the cell bodies of the VI also suggest a possible homologue of the mammalian striatum. In the rat striatum, most if not all cells containing SRIF also contain NPY and vice versa (Vincent et al., 1983). The high proportion of colocalization in dorsal and ventral telencephalic cell bodies in this study suggests that these areas may represent a primitive striatum or other basal ganglia component in the goldfish. The high degree of colocalization of NPY and SRIF in cell bodies in Dc could mean that Dc represents the teleost dorsal, as opposed to ventral, striatum (Heimer et al., 1982), based purely on anatomical position, or that it is part of a primitive cortex, because it has been shown that some

mammalian cortical neurons contain both NPY and SRIF (Chronwall et al., 1984; Vincent et al., 1982). The ventral striatum in rat includes the olfactory tubercle which receives input from the olfactory cortex (for review, see Heimer et al., 1985). NPY-/SRIF-positive cells are also found in the olfactory cortex (Vincent et al., 1983). In goldfish, the VI, where the most striking number of cells containing both transmitters was found, also receives input from the olfactory bulbs (for review, see Northcutt and Davis, 1983). Northcutt (1981) has likened the VI to the mammalian olfactory tubercle, but it may actually be superfluous to distinguish between a ventral striatum and olfactory tubercle when, in the rat, there is a continuity between these structures in the form of cell bridges (Heimer et al., 1982). This continuity has also recently been shown in the rat by dense immunocytochemical staining for the striatal marker DARPP-32 (dopamine and cyclic AMP-regulated phosphoprotein) in neurons of the tubercle, overlying striatum, and connecting cell bridges (Walaas and Ouimet, 1989). In mammals, histochemistry has shown acetylcholinesterase and dopamine concentrations to be higher in the caudate-putamen than any other part of the telencephalon, and in amphibians, reptiles and birds, these substances are concentrated in the ventrolateral wall of the hemisphere (for review, see Sarnat and Netsky, 1981).

The mammalian striatum receives input from the dopaminergic neurons of the substantia nigra pars compacta (for review, see Heimer et al., 1985). Although the VI is a striatal candidate in terms of the colocalization of SRIF and NPY in its cell bodies, the fact that there are no dopaminergic terminals here, as implied by the absence of tyrosine hydroxylase-positive terminals (Hornby et al., 1987) and the fact that, according to some authors, there is no olfactory input to VI (Levine and Dethier, 1985), argue against this candidacy. The latter point means that VI cannot be the homologue of the olfactory tubercle and, therefore, is unlikely to

represent a homologue of the ventral striatum. No catecholaminergic basal mesencephalic group of cell bodies has been reported in the goldfish (Hornby et al., 1987), suggesting that there is no equivalent to the substantia nigra in these fish.

SRIF immunoreactivity in the diencephalon, tectum and habenula

As the distribution of NPY immunoreactivity in the diencephalon and tectum was in agreement with that observed by Pontet et al. (1989) in the goldfish brain, this part of the discussion will focus on comparing and contrasting the hypothalamic distribution of SRIF in the present work with that in the literature because, traditionally, this regional distribution of SRIF has been of more interest than any other. In a discussion emphasizing SRIF in the goldfish brain, a postulate on the exact form of SRIF observed deserves mention. In anglerfish, catfish and salmon pancreas, two forms of SRIF are expressed. These are encoded by separate genes in anglerfish and catfish and are likely to be so in salmon, since they are distributed in separate pancreatic cell populations, as they are in anglerfish (Hobart et al., 1980; Magazin et al., 1982; Nozaki et al., 1988). Since these three species are all teleosts, it would appear likely that two forms of SRIF are produced by two distinct genes in the pancreas of the goldfish also. Personal observations of the distributions of SRIF-28 and SRIF-14 in the striatum of the rat have not revealed that the two peptides are distributed in entirely separate subsets of cells, suggesting that the antibody raised against mammalian SRIF-28 may recognize both SRIF-28 and SRIF-14. Assuming that two forms are also expressed in the goldfish brain, and that their antigenic sites are similar, they may have both been recognized by the anti-mammalian SRIF-28 antibody used in this study.

The diencephalic distribution of SRIF immunoreactivity was very much like that described by Kah et al. (1982) in goldfish, by Batten et al. (1985) in the molly, by Sas and Maler (1988) in a gymnotid, and in a variety of other teleosts (Batten et al., 1985; Oliverieu et al., 1984a; 1984b). The presence of distinct positive varicosities in the circumventricular organum vasculosum, a nucleus with unusual blood brain barrier permeability, observed in the goldfish were also reported in the molly in which this region is well vascularized (Batten et al., 1985). SRIF-containing fibres have also been observed in this organ in the rat (Vincent et al., 1985). In goldfish, SRIF-immunoreactive neurons were found here as well and this observation together with the presence of NLTa cells extending processes into the ventricular wall, implicates SRIF in fluid balance. Thus, SRIF may have two basic functions in the hypothalamic area: (1) monitoring of the homeostatic state of the organism by sampling cerebrospinal fluid, and (2) neurosecretory innervation of the pituitary from the NPO and NLTl (Fryer and Maler, 1981). These authors injected HRP intravenously into the goldfish which entered the brain via retrograde transport from the pituitary, labelling the cell bodies of the NPO pars parvocellularis and NLT pars magnocellularis both of which were labelled with SRIF in the current experiment.

In addition to these consistencies with the literature, the results of the current experiment also yielded some discrepancies. They included the following. SRIF-immunoreactive processes issuing from the NPO cells and extending to the pituitary have also been reported in tilapia (Grau et al., 1985), but these follow a ventrolateral rather than ventromedial path. It is quite possible that this is a species difference or that the ventrolateral pathway is also present in the goldfish but is obscured by the dense fibre plexuses in the area. Secondly, whereas the NLTl cell bodies in the goldfish were very large, Batten et al. (1985) observed only very small

cell bodies in the NLTl of the molly. This may be the nearby newly discovered parvocellular group or it may be another species difference. The magnocellular NLTl projects to the pituitary of the goldfish (Fryer and Maler, 1981), so it may be that the parvocellular population in the molly projects elsewhere and does not perform a neuroendocrinological function through the release of SRIF.

Both the goldfish and the gymnotid, *Apteronotus leptorhynchus*, possess SRIF somata in the optic tectum (Sas and Maler, 1988). No other authors have reported this with regard to non-electroreceptive teleosts. NPY and SRIF, present in separate populations of periventricular tectal neurons, are probably involved in visual processing because the dendrites of these neurons are known to ramify in the superficial tectal layers in which retinal afferents terminate (for review, see Vanegas, 1983).

The occurrence of SRIF-positive cell bodies in the entopeduncular region of the goldfish as well as SRIF-positive fibres and terminals in the habenula may suggest a SRIF-positive projection from the entopeduncular nucleus to the habenula, homologous to that presented in the mammal in Experiment 1 and previously in the literature (Vincent and Brown, 1986). However, this projection has not been reported before in teleosts, making this argument on the basis of immunohistochemical findings somewhat tenuous. In addition, there were no NPY-positive fibers in the habenula and therefore if the SRIF-positive fibers indeed arose from entopeduncular nucleus projections they would have to have been from those very few neurons which were immunoreactive for SRIF but not NPY. The best known connection of the habenula in vertebrate systems is that projection to the interpeduncular nucleus. SRIF and NPY terminal fields have been noted in the rat interpeduncular nucleus (Chronwall et al., 1985; Vincent et al., 1985), as they were in the goldfish. SRIF-immunoreactive cell bodies have

been observed in the lateral habenula of the rat (Vincent et al., 1985), and, indeed, these may be the very neurons that project to the interpeduncular nucleus, thus explaining the presence of SRIF-positive terminal fields there. However, no cell bodies were seen in the habenula in the goldfish or in the gymnotiform (Sas and Maler, 1988), so it does not appear that SRIF or NPY characterize this projection in teleosts.

Locus coeruleus

Pontet et al. (1989) also observed NPY-positive somata in the LC of the goldfish. NPY-positive cell bodies also appear in high density in the LC of the rat (Chronwall et al., 1985). The LC also has a high noradrenaline content which is widespread across phyla, including goldfish (Kah et al., 1989; for review, see Sarnat and Netsky, 1981). In fact, tyrosine hydroxylase, the first required enzyme in the synthetic pathway of this monoamine has been localized to the LC of goldfish (Hornby et al., 1987). Therefore, NPY is colocalized with catecholamines in the locus coeruleus of goldfish as it is in other species.

Audition and mechanosensation

There were two regions of the goldfish brain typically associated with audition and mechanosensation that showed single-label NPY and SRIF immunoreactivity. These were the eminentia granularis of the cerebellum and the torus semicircularis. The paralemniscal bundle is a third anatomical structure that showed single-label immunoreactivity for both NPY and SRIF. It has not been reported previously in the literature but whether or not it plays a role in

audition and/or mechanosensation is unknown.

Eminentia granularis

The EG forms part of the vestibulolateral lobe of teleosts (Bass, 1982), homologous to the mammalian flocculi and nodulus, the archicerebellum, which is the phylogenetically oldest portion of the cerebellum (for review, see Sarnat and Netsky, 1981). The morphology of the SRIF-labelled processes in this nucleus was typical of mossy fibres, very fine with bulbous pericellular terminals. Cerebellar mossy fibres in mammals synapse on granule cells in the cerebellar cortex. This is consistent with the localization of these fibres to the EG which is, as its name states, granular. In contrast to this, it is SRIF-positive climbing fibres rather than mossy fibres which are found in the flocculus of the rat (Vincent et al., 1985). In addition, unlike goldfish, rats do not have NPY-containing terminals in the flocculus (Chronwall et al., 1985). These discrepancies could have something to do with the fact that lateral line afferents have been lost because land vertebrates no longer require sensation pertaining specifically to an aquatic environment.

Torus semicircularis and paralemniscal bundle

The laminar quality of the TS in teleosts, observed with SRIF- and NPY-labelled processes, is well known (for review, see Sarnat and Netsky, 1981) and is also characteristic of its mammalian homologue, the inferior colliculus (for review, see Webster, 1985). It is not likely that the SRIF-positive fibres in the TS arose from cells in the acoustic (octaval nuclei)

because these were not SRIF-immunoreactive. This is in contrast to the rat, in which, SRIF-terminal fields seen in the inferior colliculus are likely to have arisen from the SRIF-labelled perikarya found in the cochlear nuclei (Vincent et al., 1985). Instead, it is possible that SRIF-labelled perikarya found in the reticular formation of the goldfish contributed some of the fibres seen in the TS because, in the carp, the TS receives some of its input from the medullary reticular formation (Echteler, 1984).

The paralemniscal bundle was continuous with the toral processes but whether or not this means that an acousticolateral function can be assigned to this tract remains uncertain. These fibres are probably the NPY-positive processes observed along the LL by Pontet et al. (1989) in the goldfish. This tract is very characteristic of *Apteronotus*, in which it is immunoreactive for somatostatin, tyrosine hydroxylase, galanin (as in the goldfish, see Experiment 3) and serotonin. Serotonin-immunoreactive cell bodies are present in the dorsal raphe of *Apteronotus* and probably give rise to the paralemniscal fibres, making the paralemniscal area a possible homologue of the parabrachial nucleus of rat which is also thought to receive its dense serotonergic innervation from the dorsal raphe (L. Maler, personal communication; for review, see Törk, 1985).

Control of feeding

NPY and SRIF immunoreactivity was found in both the facial system used for food selection and the vagal system used for swallowing (Finger, 1988).

Facial lobe and secondary gustatory system

Because the facial lobe of the goldfish is somatotopically organized with respect to taste buds located about the eye, lips, operculum, trunk and pectoral fins (Puzdrowski, 1988), the fact that SRIF and NPY immunoreactivity was found throughout the facial lobe and was not restricted to any one somatotopic region suggests that these two neuropeptides are involved in taste sensation arising from all over the body. The facial lobe, or primary gustatory nucleus, is perhaps a homologue of the solitary nucleus in mammals, in which the afferent fibres of the mammalian facial nerve carrying gustatory impulses terminate. The large size of the facial lobe in goldfish as compared to other fish is in accordance with the much more extensive distribution of taste buds and better development of this sense in teleosts (for review, see Sarnat and Netsky, 1981). If the homology of the lobe with the solitary nucleus is valid, it is not surprising that NPY-positive fibres and SRIF-positive cell bodies and fibres are also located in the solitary nucleus of the rat (Chronwall et al., 1985; Vincent et al., 1985).

The secondary gustatory nucleus also contained SRIF- and NPY-positive fibres as did the secondary gustatory tract. The rat prepositus, a secondary gustatory nucleus in mammals (for review, see Sarnat and Netsky, 1981), also contains SRIF terminal fields (Vincent et al., 1985), suggesting another homologue. The SRIF-labelled fibres in the secondary gustatory nucleus may have arisen from SRIF-labelled perikarya in the facial and vagal lobes because both regions project to the secondary gustatory nucleus in catfish (Kanwal et al., 1988).

Vagal lobe

NPY- and SRIF-positive fibres and terminals were observed in the vagal lobe of the goldfish. Pontet et al. (1989) also found NPY-positive terminals in the vagal lobe of *Carassius*, and Sas and Maler (1988) found SRIF-positive terminals in the vagal lobe of *Apteronotus*. This shows consistency across species in this component of the gustatory system.

The lateral column of the vagal motor complex (DMI) upon which SRIF terminals were observed and near which NPY terminals were also observed, may mean that these neuropeptides play a role in swallowing (Finger and Morita, 1985), and, in the case of SRIF, inhibition of firing of vagal motoneurons that innervate visceral organs such as the gut, as SRIF does in mammals (Nabekura et al., 1989). The DMI is equivalent to the nucleus ambiguus of mammals (Morita and Finger, 1987). There is a very dense SRIF-positive terminal field in the nucleus ambiguus of the rat that arises from the central nucleus of the solitary tract and is thought to subserve reflex control of esophageal motility (Cunningham and Sawchenko, 1989; Vincent et al., 1985). Therefore, there is some neurochemical homology between the innervation of the nucleus ambiguus of mammals and its counterpart in teleosts.

The meshwork pattern of NPY- and SRIF-positive processes in the lateralmost sensory layer of the vagal lobe most likely arose from the fascicular organization of afferents to this layer (Morita and Finger, 1985). It is interesting that immunohistochemically identified afferents to this layer of the vagal lobe have a modular arrangement and yet adjacent sections stained with cresyl violet (data not shown) show the layer to be homogeneous and densely packed with cell bodies. The cell bodies are not compartmentalized by fibre bundles as one

would expect from a fascicular arrangement of the afferents as seen in Fig. 2.6.

DMm and swim bladder

The DMm neurons were striking because they were the only cells in the brainstem in which NPY and SRIF were colocalized. Of course, the occurrence of this colocalization in the DMm cell bodies and in the fibres in one of its targets, the swim bladder, did not rule out the possibility that other peripheral target organs of the DMm were being innervated as well. Morita and Finger (1987) discovered retrograde labelling of large and extra large cells in the medial motor column when they applied HRP to the swim bladder, gastrointestinal tract and other abdominal viscera. NPY-like immunoreactivity has already been found in gastrointestinal plexuses of the carp (Bjénning and Holmgren, 1988).

The discovery of immunoreactive fibres in both the resorbent and secretory regions of the swim bladder was not unexpected because nerve endings have been observed in both these regions. Since the DMm neurons are very large, they may be fast-conducting, releasing SRIF and NPY from terminals in the wall of the bladder to mediate immediate responses to pressure changes due to changes in depth (for review, see Steen, 1970).

In conclusion, it appears that SRIF and NPY may have roles in both the orobranchial and coelomic control exerted by the vagal nerve. All aspects of feeding, from initial determination of palatability to the consequent decision to swallow or reject food, are probably regulated by neuronal circuits within which signal transfer is mediated in part by these two neuropeptides.

A possible trigeminal nucleus

Apparent axo-somatic contact of SRIF-positive terminals on large SRIF-positive neurons were observed around the lateral border of the descending trigeminal tract. Synaptic contact could only be proven, however, through electron microscopy. The anatomical location of these neurons does not coincide with that of any nuclei previously recognized in the goldfish. Consequently, it is only based on this anatomical situation in the vicinity of the descending trigeminal tract that their identity as a trigeminal nucleus can be proposed. Perhaps these SRIF-positive neurons are a descending trigeminal nucleus, accessory to that which exists at the medullo-spinal junction, that would be involved in processing cutaneous sensation (for review, see Sarnat and Netsky, 1981). NPY- and SRIF-positive fibres were also observed to course through the descending trigeminal tract where they might mediate tactile sensation from the head (Puzdrowski, 1988).

Reticular formation and spinal cord

The rat (Vincent et al., 1985), goldfish and gymnotid (Sas and Maler, 1988) all possess SRIF-positive cells in the reticular formation and terminal fields in the spinal cord. In the rat, NPY is found in these regions also (Chronwall et al., 1985).

NPY- and SRIF-positive terminals existed in both the dorsal and ventral horns of the spinal cord of the goldfish, indicating mediation of both sensory and motor function. Terminals containing SRIF may not only arise from the SRIF-positive neurons in the reticular formation, but also from those found in the NPO and NVM (Prasada Rao et al., 1987). NPY

terminals in spinal cord were widespread throughout the grey matter, suggesting numerous functions for NPY in the spinal cord. Many of these functions may have devolved in mammals and become restricted to the modulation of pain sensation in the substantia gelatinosa where NPY terminals are found in the rat (Chronwall et al., 1985). SRIF-labelled cell bodies in the dorsal spinal cord of the goldfish might be secondary sensory neurons functioning in pain and temperature reception, based on the finding that SRIF-positive cell bodies occur in laminae II-V in the rat (Vincent et al., 1985). The more ventrally located perikarya may be interneurons, such that SRIF modulates motor reflex action in the goldfish.

CONCLUSION

In the goldfish brain, colocalization of NPY and SRIF within cell bodies is minimal, being restricted to suspected "striatal" nuclei in the forebrain and to a single vagal nucleus in the brainstem. Whatever concerted actions NPY and SRIF have in the central nervous system, they must not always necessitate existence of the peptides within the same neurons. The similarity in the general patterns of both types of fibres point out that both peptides can be present in the same systems but be segregated into distinct neuronal populations. It bears emphasis that the data on the remarkable degree of coincidence in the distribution of NPY- and SRIF-positive fibers is as suggestive of concerted action of these peptides as is evidence of their coexistence. At least in the goldfish, coincidence, not colocalization, is the rule rather than the exception. NPY- and SRIF-positive fibre distributions are somewhat coincident in the mammalian brain also, in the cortex, striatum, basal hypothalamus and amygdaloid regions (Chronwall et al., 1985; Johansson et al., 1984). It seems that it is not only the distributions

of NPY and SRIF which have altered little throughout the course of evolution, both across and within vertebrate classes, but also the principles of organization of multiple neuronal messengers.

Experiment 3

Galanin Immunocytochemistry in the Goldfish Brain

ABSTRACT

Galanin, a peptide with widespread distribution within the mammalian CNS, was found to be present in goldfish brain by immunohistochemical methods. Galanin immunoreactivity was found within neurons and nerve fibres throughout the goldfish brain, most extensively in the forebrain. A dense core of fibres and terminal fields was observed along the axis of the lateral hypothalamus-lateral forebrain bundle-septum, and major galanin-positive fibre bundles were also observed along the preoptic-neurohypophyseal and paralemniscal pathways. In the forebrain, dense terminal fields filled the area ventralis telencephali pars ventralis. In the diencephalon, perikarya were found in the nucleus preopticus periventricularis, nucleus preopticus, and nucleus lateral tuberis pars posterioris of the hypothalamus, and in the caudal nucleus posterioris periventricularis. The only cell bodies observed in the brain stem were those in the nucleus reticularis inferior, and fibres were found throughout the ventromedial extent of the reticular formation. Dense terminal staining was observed in the pars distalis of the pituitary, while minor staining was observed in the neurointermediate lobe. Galanin-positive fibres appeared in sensory structures associated with vision and taste: Varicose processes appeared in the optic tectum, facial lobe, secondary gustatory nucleus and in a fascicular pattern in the vagal lobe. Terminals were also seen in the the dorsal horn of the spinal cord. When these results were compared to those in the literature, it appeared that,

overall, galanin in the goldfish brain has a distribution similar to that found in the mammalian brain.

INTRODUCTION

Galanin (Gal) is a peptide comprising 29 amino acids, isolated from pig intestine (Tatemoto et al., 1983). Not only has the primary structure of the Gal peptide been determined, but a Gal cDNA clone has also been isolated from an estrogen-induced rat pituitary tumour cDNA library. The nucleotide sequence of this clone has also been determined and it encodes a 124-amino acid protein that is 70% identical to the precursor of porcine intestinal Gal and a 29-amino acid protein that is 88% identical to the primary structure of porcine intestinal Gal (Vrontakis et al., 1987). Its precursor includes a signal sequence, the Gal peptide and a Gal mRNA-associated peptide which has 78% homology with this region of the rat Gal precursor (Kaplan et al., 1988). Thus, there is extensive homology between the Gal found in the central nervous system (CNS) of the rat and the peripheral nervous system of the pig.

It is the C-terminal amino acid sequence varies across species and elicits intestinal motility differentially among species (Fox et al., 1988). In addition to its importance in the enteric nervous system, Gal has been characterized in other peripheral tissues including the pancreas, where it inhibits the release of insulin (Hermansen, 1988), and in autonomic ganglia of various species. In neurons of this latter system, it is found together with other peptides. Thus, Gal coexists with NPY, vasoactive intestinal polypeptide and substance P in local ganglia of the tracheal wall in chicks, with NPY and enkephalin in celiac and inferior mesenteric ganglia of the fetal guinea pig and newborn pig, and with NPY in lumbar and sacral sympathetic ganglia

of the cat (Fried et al., 1989; Lindh et al., 1989; Luts et al., 1989).

Gal and its receptors are also widely distributed throughout the mammalian CNS (rat and pig: Ch'ng et al., 1985; baboon: Flint Beal et al., 1988b; rat: Melander et al., 1986a; Melander et al., 1988; Skofitsch and Jacobowitz, 1985), but its distribution in the CNS of non-mammals has not yet been reported. Through closer examination of Gal distributions in relation to other known transmitters, it has been shown to coexist with serotonin, in the dorsal raphe, and noradrenaline in the locus coeruleus (Melander et al., 1986b). It has also been found to coexist with choline acetyltransferase (ChAT) in the neurons of the medial septum, which project to the ventral hippocampus, and in neurons of the nucleus basalis of Meynert (NbM), which project diffusely to the cortex. Specifically, Gal is thought to inhibit presynaptically the release of acetylcholine (ACh) from septal afferents to CA1 pyramidal neurons of the hippocampus (Dutar et al., 1989; Fisone et al., 1987). It is this inhibitory action of Gal on ACh release which is thought to exacerbate the progressive cortical deficiency in cholinergic afferents which characterizes Alzheimer's disease (Flint Beal et al., 1988a). Unfortunately, the rat is not a good model for this system, as no Gal is found at all in the NbM (Melander et al., 1985). The owl monkey shows colocalization in all NbM neurons (Melander and Staines, 1986), and Gal neurons form a subpopulation of NbM ChAT neurons in the human (Chan-Palay, 1988). The tuberomammillary nucleus of the hypothalamus may be involved in Alzheimer's disease because of its projections to widespread regions of the cortex (Wm. Staines, personal communication; Staines et al., 1987), and it, too, contains Gal-immunoreactive cell bodies (Staines et al., 1986).

It was an interest in the possible coexistence or coincidence of Gal and ChAT in a submammalian species which initially prompted this experiment. Preliminary work revealed

distinct regions containing ChAT immunoreactive cell bodies, such as the oculomotor nucleus and periventricular layer of the optic tectum, but no colocalization of Gal and ChAT was observed anywhere in the goldfish brain. Possible axo-somatic synaptic interaction between Gal and ChAT was, however, noted in the secondary gustatory nucleus and is discussed later.

It was then decided to present a thorough study concentrating on Gal itself in the fish brain, combining distribution of this peptide with an attempt to locate Gal receptors as well, using autoradiography. Those conditions that were optimal for a positive control using rat brain (Melander et al., 1988) did not clearly reveal receptor sites in goldfish brain. Even after changing buffer concentration and pH, results were poor. It is as though the goldfish Gal peptide that was immunohistochemically labelled is similar enough to porcine Gal to be recognized by the antibody raised against it, but that the receptor in goldfish is not similar enough to the mammalian Gal receptor to bind porcine Gal.

Although the negative results with respect to Gal receptor distribution were somewhat disappointing, mapping the distribution of the Gal peptide itself in the goldfish brain remained a valid pursuit because of comparative interests expressed in Experiment 2, including the desire to find a homologue of the tuberomammillary nucleus. There is a paucity of information on the central distribution of this neuropeptide in general in lower vertebrates. I have set out to present immunohistochemical data from a teleost, the common goldfish. This distribution will allow comparison between the goldfish and electroreceptive teleosts such as the gymnotid, in which the whole brain distribution of Gal has just been mapped (L. Maler and J. Nagy, unpublished observations). Providing this phylogenetic complement may help to confirm suspected brain homologies not only within but across vertebrate classes.

MATERIALS AND METHODS

Goldfish were perfused and brains post-fixed, stored, frozen, sectioned and mounted as described in Experiment 2.

Immunoperoxidase

Sections were processed by the single-labelling method as described for NPY in Experiment 2, with the exception that the primary antibody was rabbit anti-galanin (Peninsula) at a concentration of 1 in 2000.

Immunofluorescence

After a 15 min wash in 10 mM phosphate buffered saline (PBS), pH 7.2, sections were incubated overnight at 4° C in rabbit anti-galanin antibody (1 in 400) in 10 mM PBS, 0.3% Triton X-100. After a second 15 min wash in PBS, brain sections were incubated in FITC-conjugated donkey anti-rabbit antiserum (1 in 20; Amersham) for 30 min at 37° C. After a final 15 min wash in PBS, sections were coverslipped under glycerol with 0.1% *p*-phenylenediamine. The galanin distribution was mapped as was done for NPY and SRIF in Experiment 2. Pre-adsorption controls were conducted by adding 5 µg of peptide (Peninsula) to 2 µl of primary antibody diluted 1 in 10, allowing the mixture to stand overnight, then diluting the mixture to 1 in 200 and proceeding with primary and secondary incubations as described above. The rationale for the use of pre-adsorption controls in immunohistochemistry

is discussed in Appendix B.

RESULTS

Pre-adsorption completely abolished Gal immunoreactivity, as shown in Fig. 3.1, indicating that goldfish Gal contains a sequence either identical to or closely related to that portion of porcine Gal which is recognized by the antibody. Fig. 3.2 is a map of the distribution of Gal immunoreactivity in the goldfish brain. Abbreviations are given in Appendix A. From the anterior to the posterior telencephalon, varicosities became progressively more dense. In the olfactory tubercle just anterior to Vv, and in the Vv and ventromedial VL, Gal-positive boutons were clustered into "islands" (Fig. 3.2 A), and this feature continued caudally to the level of the rostral anterior commissure (Fig. 3.2 B). An intensely stained group of fibres and a dense terminal field appeared alongside the NPP (Fig. 3.2 C,D). This terminal field appeared to be continuous with the Vv "islands" of terminals and with a dense terminal field lateral to the NLTp (Fig. 3.2 F,G). Fibres were also observed in the region of the lateral forebrain bundle (LFB; Fig. 3.2 D). This dense group of fibres appeared to be issuing from the hypothalamus and branching off at various ventral forebrain regions in the core of the ventral telencephalon to terminate as dense fields of boutons (Fig. 3.3 A). Immunopositive perikarya appeared in the NPP (Fig. 3.2 C,D; Fig. 3.3 B) but became rarer caudally until only one or two were seen at any one level of the nucleus. The NPO contained the occasional Gal-positive perikaryon (Fig. 3.2 D) as did the mid-NAPv (data not shown). Cell bodies were also observed in the NLTa, NLTp (Fig. 3.2 F,G) and NPPv (Fig. 3.2 H). Gal-immunoreactive varicosities were present in both the rostral and proximal pars distalis

Fig. 3.1. Pre-adsorption control for Gal immunoreactivity. **A.** Gal immunoreactivity, as shown by immunofluorescence. **B.** The Gal immunoreactivity shown in **A** is completely abolished by pre-adsorption. Scale bar = 50 μm .

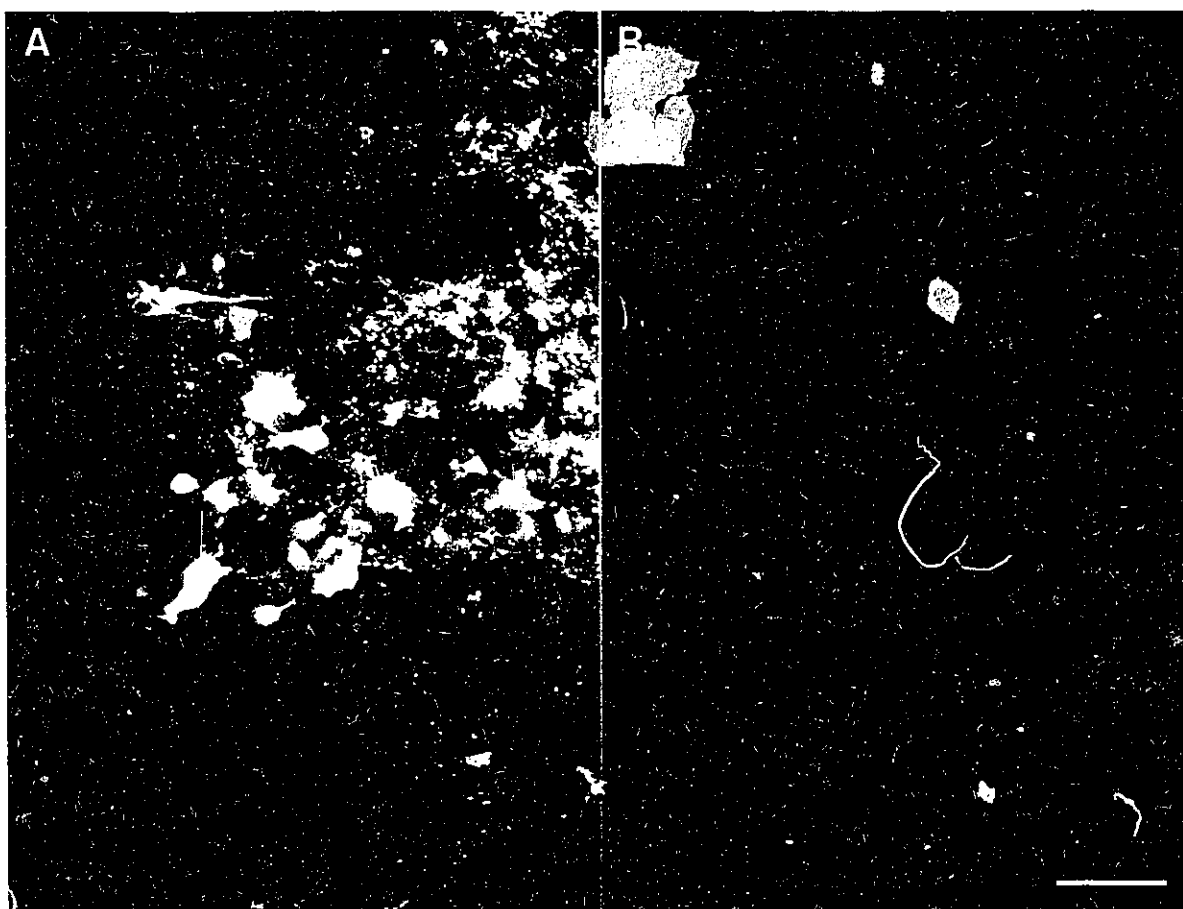
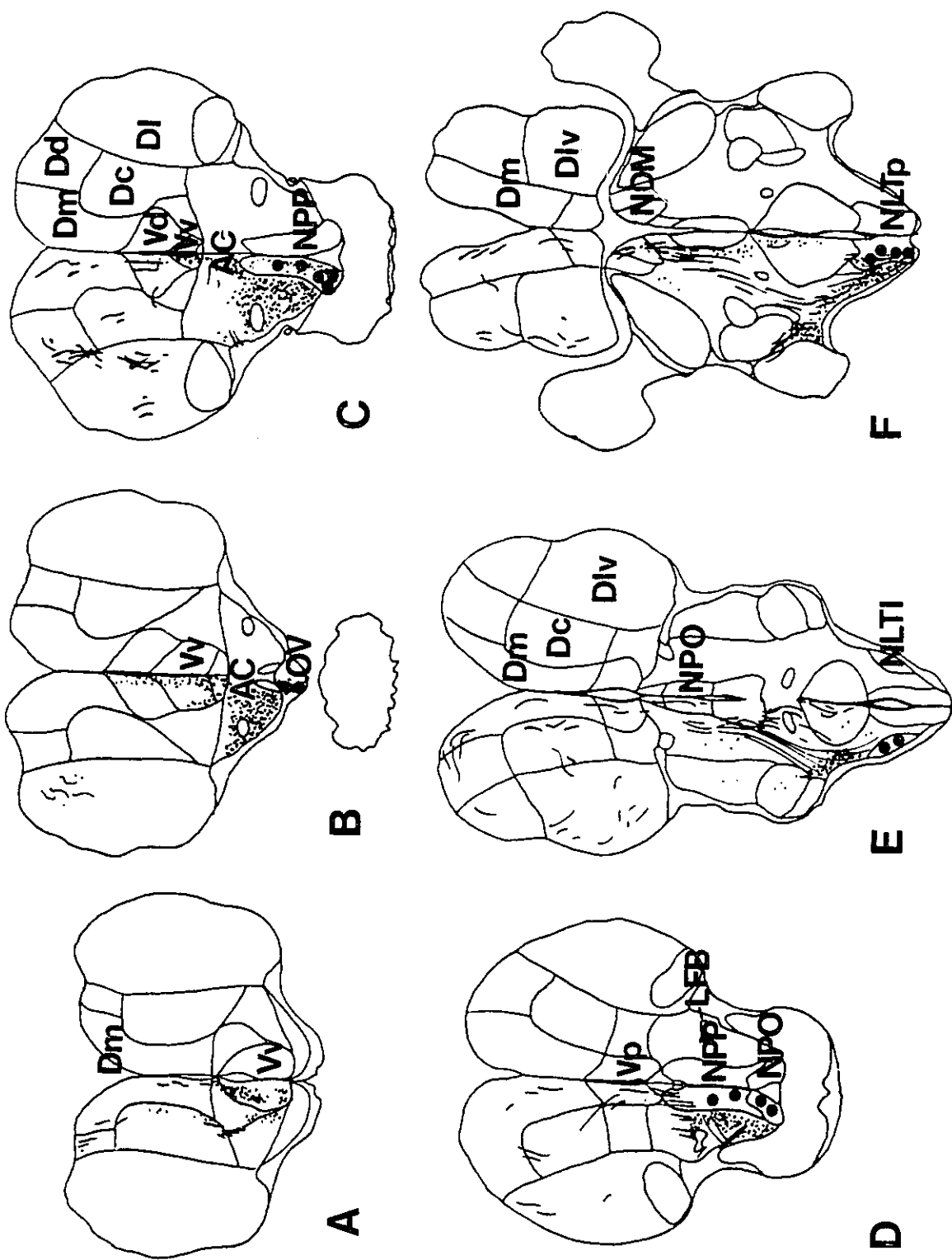
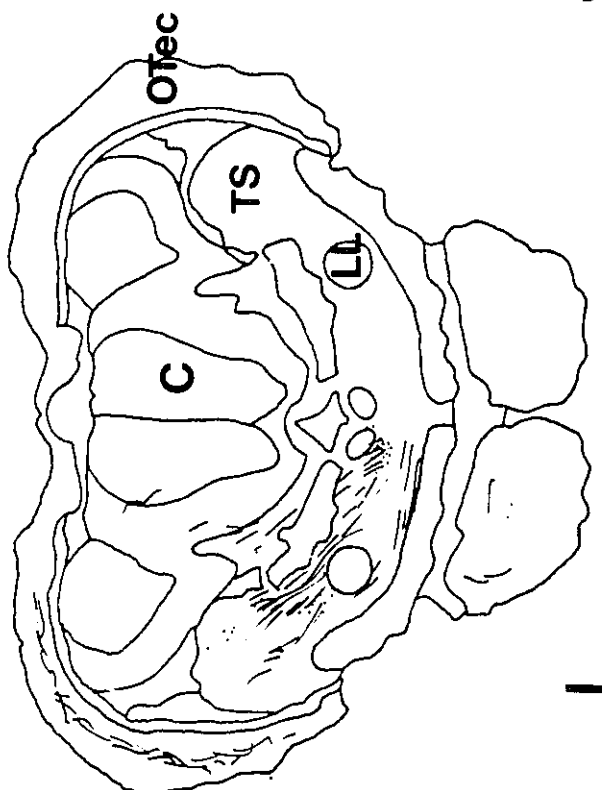
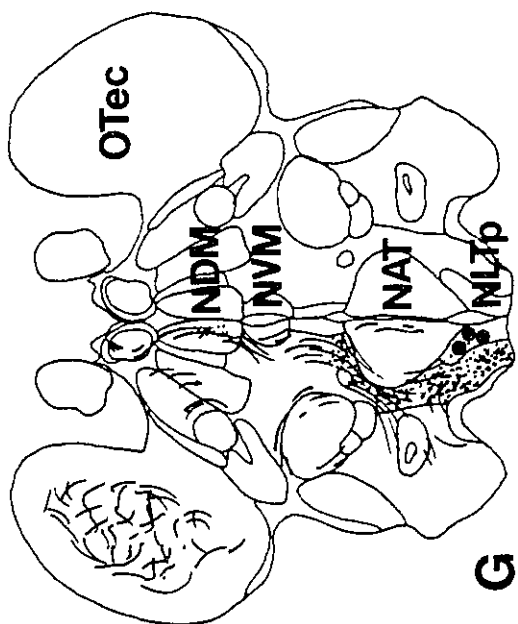
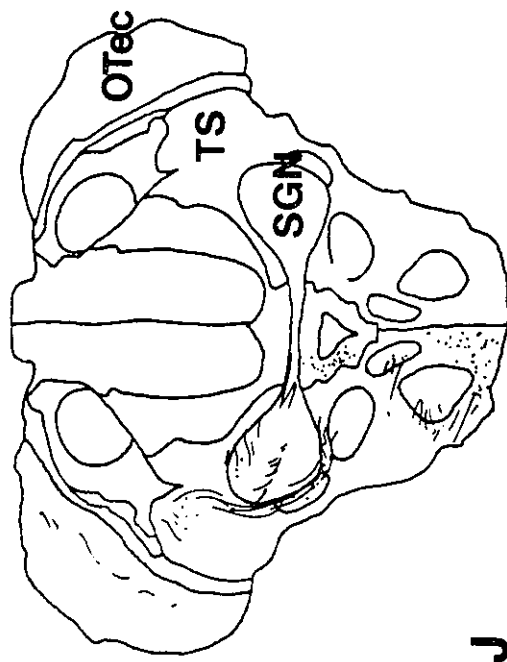
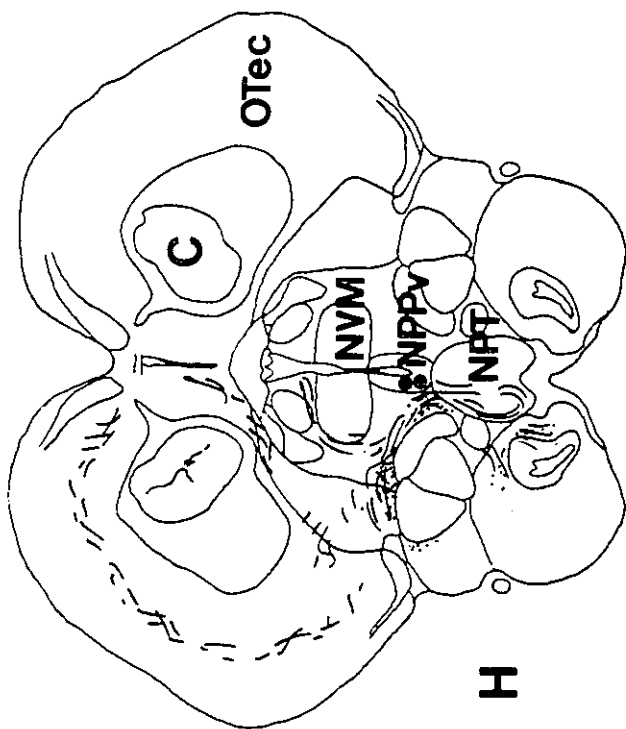
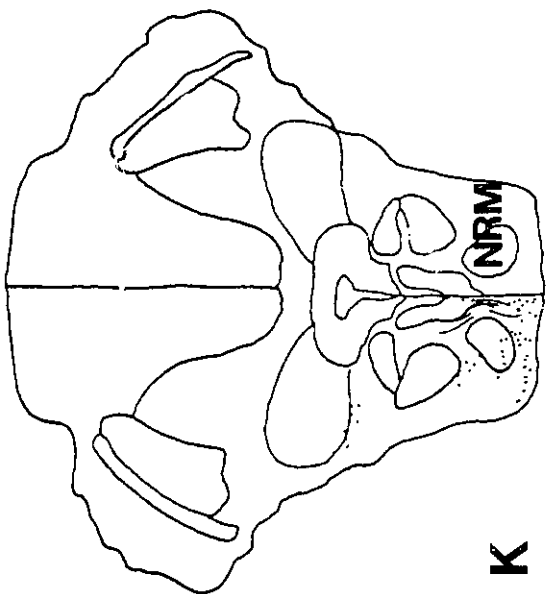
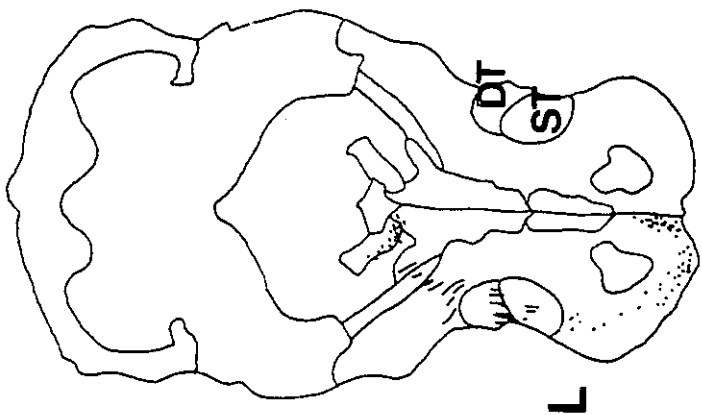
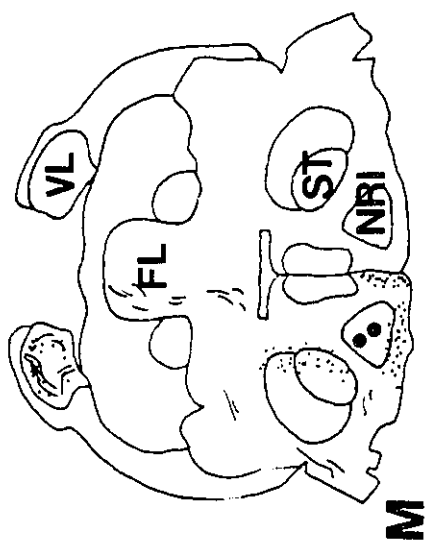
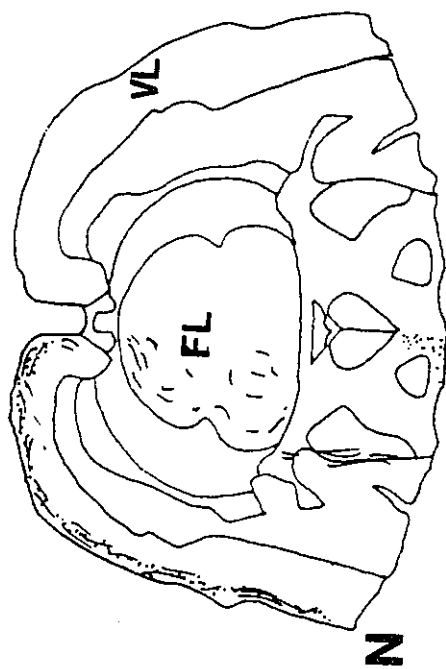
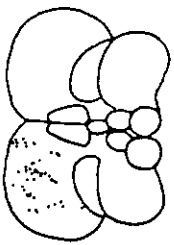


Fig. 3.2. A-Q. Map of Gal immunohistochemistry in coronal sections of the goldfish brain. Immunoreactive cell bodies (large dots), fibres and terminals (fine dots) are shown on the left and abbreviations on the right (see Appendix A). Intervals between sections are the same as those given for Fig. 2.3 in Experiment 2.

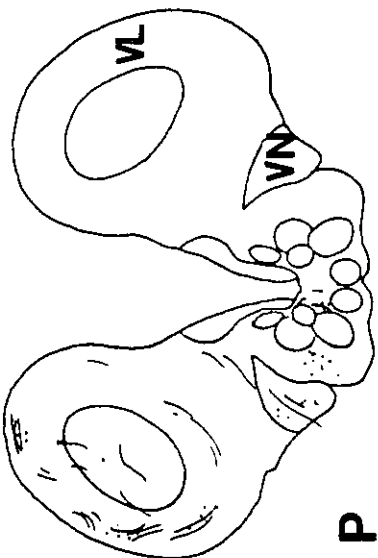




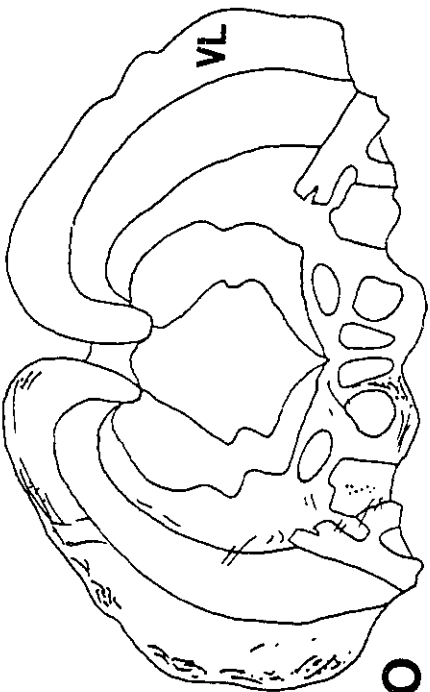




Q



P



O

(PD) of the pituitary. Varicosities were fewer in the neurointermediate lobe (NIL) than in the PD (Fig. 3.3 C).

Processes coursed in the preoptic-neurohypophyseal bundle from the NPO toward the region of the NLTI in which there were parvocellular Gal-containing neurons (Fig. 3.2 E). A dense group of relatively non-varicose fibres coursed around the circumference of the NAT, along the lateral edge of the NVM, and through the dorsal thalamic nuclei (Fig. 3.2 G) where a couple of perikarya appeared in the NDM (data not shown).

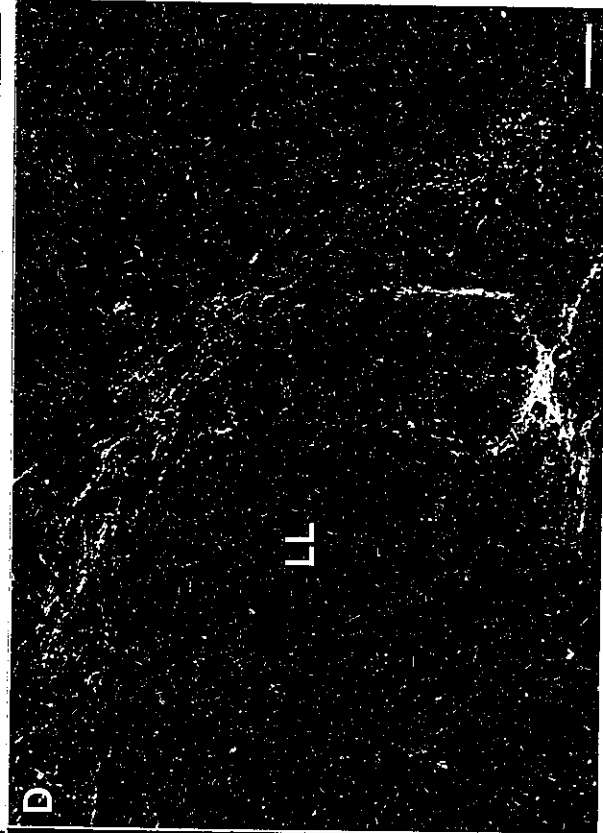
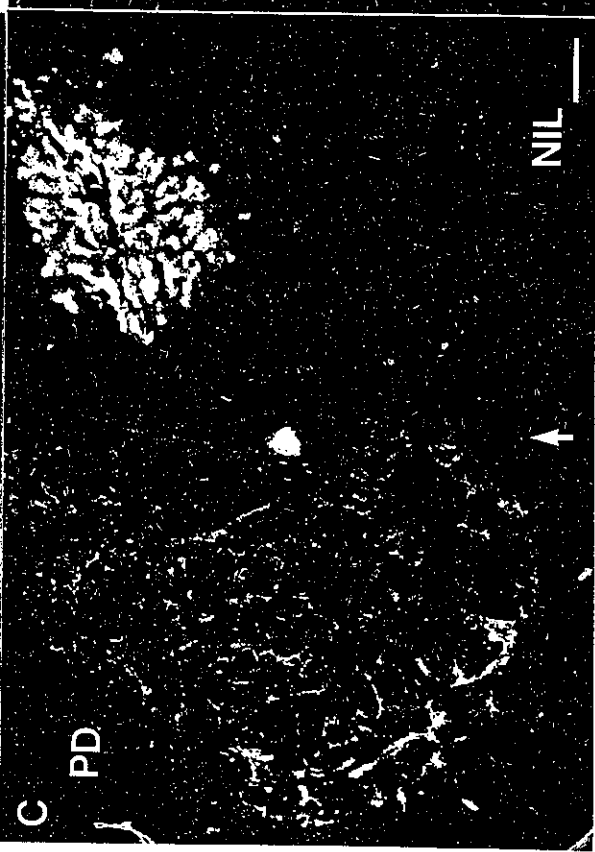
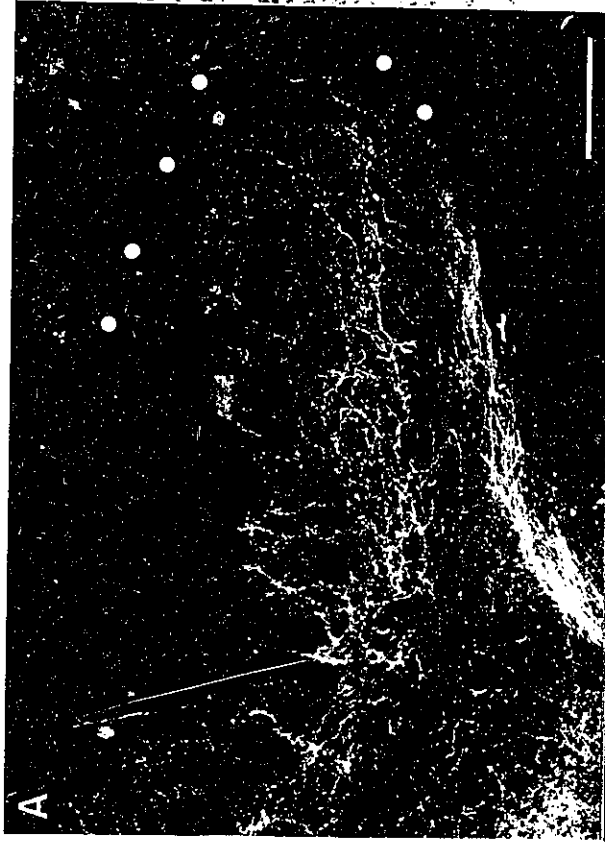
In some fish, the optic tectum showed extremely fine fibres that were not highly varicose and very few in number, while in others this structure was characterized by a distinctive cross-hatched pattern of varicose fibres in moderate density (Fig. 3.2 G-J). This inconsistency in Gal-positive fibre density in the optic tectum was suspected to be due to sexual dimorphism and is investigated more thoroughly in Experiment 4.

A dense bundle of non-varicose fibres having the same appearance as the NPY- and SRIF-immunoreactive paralemniscal bundle observed in Experiment 2, coursed around the LL to the TS (Fig. 3.2 I and Fig. 3.3 D). A varicose bundle of fibres curved around the lateral border of the secondary gustatory nucleus to the TS while others were scattered in a laminar pattern in the dorsal secondary gustatory nucleus. Some non-varicose processes coursed in another band through the middle of the nucleus and others appeared to ascend through the secondary gustatory tract (Fig. 3.2 J,L).

Throughout the extent of the reticular formation, processes and terminals were most numerous ventromedially and diminished ventrolaterally (Fig. 3.2 J-O). In the anterior NRI, there were one or two magnocellular somata stained for Gal (Fig. 3.2 M).

Very few Gal-immunoreactive fibres were observed in the cerebellum (Fig. 3.2 H, I). Very

Fig. 3.3. Gal-immunoreactivity in the goldfish brain. **A.** In sagittal section, a dense core of fibres and terminals can be seen extending from the hypothalamus (lower left) to the ventral telencephalon (right). The telencephalon is outlined by dots. **B.** Gal-positive cell bodies (arrows) and fibres in the anterior NPP. Immunoperoxidase method. **C.** Gal-immunoreactivity is more extensive in terminals of the pars distalis (PD) than in those of the neurointermediate lobe (NIL). The arrow marks the transition from high to low immunoreactivity. **D.** The fibres of the paralemniscal bundle course around the LL toward the TS (upper left). Scale bar = 100 μ m.



fine lacy fibres characterized the entire facial lobe (Fig. 3.2 M,N). Some fibres also coursed from the anterior lobe through to the ninth cranial (glossopharyngeal) nerve. The vagal nerve showed Gal-containing fibres coursing within it (Fig. 3.2 P). Throughout the vagal lobe, a network of intensely immunoreactive processes and terminals formed a web-like pattern at the superficial border of the lobe (Fig. 3.2 M-P) similar to that observed to consist of NPY- and SRIF-fibres in Experiment 2 (Fig. 2.3 M-P and Fig. 2.6). In the spinal cord, only the dorsal horn demonstrated Gal-immunoreactive staining (Fig. 3.2 Q). It contained terminals grouped into narrow mediolaterally extending bands.

DISCUSSION

The relative lack of cell bodies containing Gal in goldfish brain is most likely due to rapid transport of the peptide out of the soma toward terminal boutons, resulting in low steady state levels of Gal in the perikaryal cytoplasm. This phenomenon also characterizes Gal distributions in the brains of rats. There are two pre-treatments which have been used with success in rats and may also reveal additional Gal-positive cell body populations in goldfish brain. Colchicine inhibits microtubule formation and so prevents axonal transport, causing somal accumulation of transmitter, and it has been used successfully to visualize Gal immunoreactivity in neurons which otherwise appear to contain no Gal (Gaymann and Martin, 1989; Skofitsch and Jacobowitz, 1985). One drawback with colchicine treatment is that it lowers the intensity and density of fibre and terminal immunostaining compared to that of control animals (Skofitsch and Jacobowitz, 1985). The alternative enhancement method for Gal is based on the possibility that the peptide is not always present in cell bodies in an

immunoreactive form: Gaymann and Martin (1989) treated rat hypothalamic tissue with trypsin to expose antigenic sites of precursors of Gal prior to their post-translational cleavage into the form which is transported to and recognized by Gal antibodies in processes and varicosities.

Telencephalon

The VI of goldfish is a putative striatal homologue of mammals, based on its content of a high percentage of cell bodies colocalizing NPY and SRIF (Experiment 2), an immunohistochemical distinction in the caudate-putamen of mammals (Vincent et al., 1983). The neostriatum of rats has a sparse innervation of Gal fibres (Skofitsch and Jacobowitz, 1985), just as the VI of the goldfish showed few Gal-immunoreactive fibres. The "cortical" regions (for review, see Northcutt, 1981) showing Gal-positive processes were primarily the Dc and Dl and may be homologous to some regions of mammalian cortex, throughout which there are Gal-immunoreactive fibres (Melander et al., 1986a).

Hypothalamus and pituitary

The localization of Gal-immunoreactive perikarya to the NPP and NPO is consistent with the discovery of the peptide in the paraventricular and supraoptic nuclei, respectively, of the rat (Gaymann and Martin, 1989). The NPO/NPP are actually a continuous structure, the cells of which have progressively differentiated into the supraoptic and paraventricular nuclei, respectively (for review, see Sarnat and Netsky, 1981), of mammals and birds, the only vertebrate classes in which the two nuclei are anatomically separate (for review, see Holmes

and Ball, 1974).

Caution must be exercised, however, in establishing the homology of the supraoptic nucleus on the basis of Gal immunoreactivity because some authors have emphasized the localization of Gal to the magnocellular component of this nucleus in rat (Gaymann and Martin, 1989), whereas all the Gal-positive hypothalamic cell bodies in goldfish were parvocellular. However, both the magnocellular and parvocellular components of the NPO project to the NIL in the teleost (for review, see Holmes and Ball, 1974), so that as functional components of the hypothalamus, these neurons may indeed be homologous. In addition, it has been clearly shown by Melander et al. (1986a) that the parvocellular paraventricular nucleus in the anterior hypothalamus of the rat, like the small cell bodies of the anterior NPP in goldfish, were Gal-positive. At the very least there is immunohistochemical support for homology with the mammalian paraventricular nucleus if not the supraoptic nucleus.

Parvocellular perikarya were found to contain Gal in the goldfish NPO, and a minor group of Gal terminals were found in the NIL. This was not surprising because the parvocellular component of the NPO is known to project to the NIL in the pituitary of teleosts (Peter and Fryer, 1983). Gal may be implicated in the control of cutaneous pigmentation in fish, since the NIL is the site of melanocyte stimulating hormone secretion (for review, see Holmes and Ball, 1974). Therefore, the neuroendocrine capacity of Gal is not only peripheral, as seen in the pancreas, but also central, as suggested by these findings in the goldfish and by those in the magnocellular hypothalamo-neurohypophyseal system of mammals where Gal is colocalized with the hormones oxytocin and vasopressin (Gaymann and Martin, 1989; Skofitsch et al., 1989).

The Gal-positive terminals found in the pars distalis probably arise from the Gal-containing

cell bodies in the NLT complex, specifically, the NLTa, NLTp and NLTl. The NLT is responsible for controlling adenohypophyseal functions (for review, see Holmes and Ball, 1974), and by inference, Gal is probably involved in this control. Similarly, Gal has been found in the external layer of the median eminence of the rat, through which hypothalamic neurons course toward the anterior pituitary (Palkovits et al., 1987), and it has also been found to alter plasma levels of growth hormone secreted by the anterior pituitary when infused into the third ventricle of rats (Ottlecz et al., 1986). However, the relative densities of Gal-immunoreactive terminals in the anterior and posterior lobes of goldfish pituitary are in sharp contrast to those in the rat, in which the anterior pituitary is devoid of terminals while the posterior lobe contains a dense innervation (Skofitsch and Jacobowitz, 1985). This may result from the fact that these authors used male rats, when in fact the content of Gal in the anterior pituitary is significantly greater in females, as are the levels of Gal mRNA (Gabriel et al., 1989). The sexes of the fish used in the present experiment were undetermined, but it may be that a sex difference also exists in the goldfish pituitary.

There are a number of candidate nuclei for a goldfish homologue of the mammalian tuberomammillary nucleus based on content of Gal-immunoreactive cell bodies. One is the NLTl because its anatomical location, like that of the tuberomammillary nucleus, is at the lateral base of the brain. The NLTl projects to the pituitary (Fryer and Maler, 1981), and the tuberomammillary nucleus may also do this, as fibres positive for the enzyme adenosine deaminase, an immunohistochemical marker for tuberomammillary neurons and their projections, are found in the median eminence of the rat (Staines et al., 1987). However, caution must be exercised in suggesting this homology for the following reason. Histamine is another neurotransmitter found in the tuberomammillary nucleus and its projections in

mammals (Panula et al., 1989). Some portion of histamine immunoreactivity has been found to be due to cross-reaction of anti-histamine antibodies with leutinizing hormone releasing hormone in the median eminence of rats (Berkenbosch and Steinbusch, 1987), suggesting that fibres containing adenosine deaminase in the median eminence do not arise from the tuberomammillary nucleus but from some other source, and, therefore, the tuberomammillary nucleus does not project to the pituitary after all.

The NLTa and NLTp also contained Gal-positive cell bodies, and these nuclei are immediately anterior to the nucleus recessus posterioris, found to contain Gal-positive cell bodies in gymnotid fish (L. Maler, personal communication). Gal-positive cell bodies may continue caudally into this nucleus. The nucleus recessus posterioris was not observed in sets of sections of goldfish brain stained for Gal, probably because this nucleus is very small in the rostro-caudal dimension. It could easily have been missed in sectioning through the brain. Perhaps after sectioning through this nucleus at smaller intervals, Gal-immunoreactive cell bodies would be seen and, therefore, would support a homology between this teleost nucleus and the tuberomammillary nucleus of mammals.

Recently, evidence has been found for a tuberomammillary nucleus in amphibians. In the frog *Xenopus laevis*, histamine has been found in cell bodies of the dorsal posterolateral hypothalamus. It is unclear which nucleus in goldfish is homologous to the dorsal posterolateral nucleus of frogs, but the mere fact that a tuberomammillary homologue has been found in a lower vertebrate certainly encourages the search for such a homologue in fish. In addition, the highest density of histaminergic fibres in *Xenopus* has been observed in the septum (Airaksinen and Panula, 1989), just as the highest density of Gal-immunoreactive fibres were observed in the Vv, the putative septum of goldfish. These fibres were seen in

sagittal section to arise from the hypothalamus (Fig. 3.3 A), suggesting homologous pathways between goldfish and frogs. The vector of the Gal-positive pathway observed in the ventral forebrain of the goldfish would have to be confirmed through connectivity experiments, but it is probable that the fibre/terminal core consisted of hypothalamic efferents to the forebrain olfactory and limbic centres, as these connections are a constant feature throughout phylogeny (for review, see Sarnat and Netsky, 1981). Therefore, this pathway may be termed a hypothalamo-septal pathway. Gal-immunoreactive cell bodies were indeed observed in the hypothalamus and dense terminal fields in the olfactory tubercle and Vv, assuming the Vv is homologous to the mammalian septum (for review, see Northcutt, 1981). Certainly it has a dorso-ventrally oriented midline position in the subpallium; that is, beneath the "cortex", the Dc and Dl, as it does in mammals. In fact, in one fish there were faintly stained Gal-positive cell bodies, as there are in the septum of mammals (Chan-Palay, 1988; Melander et al., 1986; Melander and Staines, 1986; Melander et al., 1985; Skofitsch and Jacobowitz, 1985). However, other immunohistochemical markers do not support this homology. In preliminary work, I found no specific ChAT immunostaining in the goldfish telencephalon, although ChAT-labelled perikarya are found in the Vv of the midshipman (Brantley and Bass, 1988). It might be more fruitful to examine the brain of this latter teleost for Gal/ChAT coexistence in search of support for a septal homologue. Of course, the lack of an exact immunohistochemical correlate with mammals does not necessarily mean that the Vv is not homologous, because, across wide species divergence, new peptides may subserve neuronal communication that, in terms of connectivity and function, is equivalent in different species.

Furthermore, the pattern of Gal-positive fibres coursing through the ventral midline forebrain and branching off at various points in midline telencephalic and diencephalic areas

(Vv and NDM; Fig. 3.3 A and Fig. 3.2 F,G) resembles closely the projection pathways of the tuberomammillary nucleus in rat, as shown by immunohistochemical staining for histamine and adenosine deaminase: Tuberomammillary neurons project to the thalamus and along the base of the hypothalamus to branch out in the septum (Panula et al., 1989; Staines et al., 1987). Overall, it appears that there is good evidence for a homologue of the tuberomammillary nucleus in the goldfish, although precisely which set of hypothalamic neurons represents the cell bodies of origin awaits further work.

Galanin in sensory systems

Gal appears to have a role in acoustic reception, based on the presence of processes in the torus semicircularis (see discussion, Experiment 2). Small numbers of fibres have also been seen in the inferior colliculus of the rat (Skofitsch and Jacobowitz, 1985) which is the mammalian homologue of the torus semicircularis (Sarnat and Netsky, 1981).

The involvement of Gal in gustatory sensation is evident by the presence of Gal-positive fibres in the secondary gustatory nucleus and secondary gustatory tract. Nuclei mediating taste in mammals, the nucleus of the solitary tract and the nucleus ambiguus, have also been found to be innervated by Gal-immunoreactive fibres (Skofitsch and Jacobowitz, 1985). In preliminary experiments, the only location in the goldfish brain I found to exhibit any correspondence between Gal and ChAT was the secondary gustatory nucleus. Gal fibres delicately embraced the outer boundaries of the nucleus which contained a very dense grouping of ChAT-positive cell bodies. Based on findings that Gal has a G-protein-mediated inhibitory action (Nishibori et al., 1988), perhaps Gal keeps a check on the gustatory impulses mediated

by this cholinergic cell group.

The superficial layer of the vagal lobe, in which there were Gal-containing axons and terminals in the form of fascicles, is the site of termination for primary sensory fibres of the vagus nerve which innervate the taste buds located on the gill arches and gill rakers (Morita and Finger, 1985). NPY- and SRIF-positive fibres were present in exactly the same pattern in the same layer of the vagal lobe (Experiment 2). Determining if Gal coexists with either of these other neuropeptides is the next logical step in this work and will suggest whether or not Gal has a role in co-mediating taste sensation from this specific orobranchial region with NPY and SRIF. The facial lobe possessed Gal-positive processes in all its dimensions, meaning that Gal probably mediates taste sensation from all of the facial sensory rami, bringing in impulses from taste buds distributed in the skin around the eye, about the nares, in the upper and lower lips, in the palate and on the operculum (Puzdrowski, 1988).

The presence of Gal-positive terminals in the dorsal spinal cord was consistent with the findings of Skofitsch and Jacobowitz (1985) and Melander et al. (1986a) in the superficial layers of the dorsal horn in rat. To determine if the terminals observed arose from primary sensory afferents, one could examine the dorsal root ganglia for the presence of immunoreactive cell bodies, or cut the afferents and see if the Gal immunoreactivity in the dorsal horn disappeared. Gal-immunoreactive cell bodies have been found in the trigeminal and dorsal root ganglia of the rat. Moreover, capsaicin treatment results in a marked reduction of Gal immunoreactivity in the dorsal horn of the rat (Papka and Traurig, 1989). Therefore, in goldfish, Gal may have a neuromodulatory role in nociception, as has been suggested for rat (Skofitsch and Jacobowitz, 1985). The Gal-positive terminals could also have been the boutons of descending tracts, however. They could have arisen from the positively-labelled

NRI somata, as retrograde tracing has shown these neurons to project to the spinal cord in goldfish (Prasadao Rao et al., 1987).

Finally, it can be said that looking at the distribution of Gal in a relatively simple nervous system has shown that there are countless potential roles for this novel neuropeptide to play in sensory processes and integration of activity between one brain region and another. Its distribution in the goldfish brain is similar to that in the mammalian brain such that the bases for some neuroanatomical homologies can be strengthened.

Experiment 4

The Density of Galanin-Immunoreactive Fibres in the Goldfish Optic Tectum is Sexually Dimorphic

ABSTRACT

Immunohistochemistry revealed a marked degree of sexual dimorphism in the density of galanin-immunoreactive fibres in goldfish optic tectum. These processes were significantly more abundant in males than females along virtually the entire rostrocaudal axis of the optic tectum. In some regions the sex difference in galanin-positive fiber density was almost a full order of magnitude. In addition to this difference between the sexes, there was a significant gradient of galanin-immunoreactive fibre density in male fish such that values in the rostral two thirds of the tectum were much higher than those in the caudal third. In contrast, in females, a uniformly low density was found along the entire tectum. Ways in which this dimorphism may have arisen are discussed and in particular those actions taking place through steroidal mechanisms.

INTRODUCTION

Since the first whole brain distributions of galanin (Gal) were published (Melanders, 1986a; Skofitsch and Jacobowitz, 1985), its study in the central nervous system has focussed primarily on its sublocalization within a few anatomical regions. It is typical that general

mapping of neuroanatomical distributions of neurotransmitters or neuropeptides leads to subsequent concentration on one or more specific questions of anatomical distribution and colocalization.

In rat, for example, the coexistence of Gal with acetylcholine has been pursued in the basal forebrain and hippocampus (Chan-Palay, 1988; Melander et al., 1985; Melander and Staines, 1986; Vogels et al., 1989). The neurohypophysis and magnocellular hypothalamus are other regions where the coexistence of Gal with other neuropeptides and neurohormones has been of interest. In these latter nuclei, Gal is colocalized with NPY, dynorphin, oxytocin and vasopressin (Gaymann and Martin, 1989; Sawchenko and Pfeiffer, 1988; Skofitsch et al., 1989). In addition to elucidating anatomical relationships between Gal and other neuropeptides and neurohormones, work in this area has pointed to an endocrinological role for Gal. For example, Gal increases the plasma levels of leutinizing hormone (LH) in ovariectomized rats pretreated with estradiol benzoate and progesterone. Presumably, under the influence of estrogen, Gal activates the hypothalamic leutinizing hormone-releasing hormone (LHRH) neurons, the processes of which excite LH release from the anterior pituitary into the bloodstream (Sahu et al., 1987). The fact that Gal activity is regulated by a sex steroid is one example of how one may begin to think of Gal in terms of sexual dimorphism. Furthermore, estrogen induces the expression of the Gal gene in the anterior pituitary, such that estrogen administration to ovariectomized rats substantially increases the synthesis of the Gal peptide in this lobe, bringing its concentration in tissue from undetectable to detectable levels by immunohistochemical techniques (Kaplan et al., 1988; Vrontakis et al., 1987; 1989). In fact, sex differences in Gal content in the hypothalamo-hypophyseal system are most pronounced in the anterior pituitary: Female rats have higher Gal-like immunoreactivity here than do males.

This difference is correlated with more abundant Gal mRNA in the anterior pituitary of females, providing further evidence for a greater expression of the Gal gene in females (Gabriel et al., 1989).

While mapping the whole brain distribution of Gal in the goldfish (Experiment 3), it was noted anecdotally that there was an inconsistency in the density of Gal-immunoreactive processes in the optic tectum, whereby some fish had a far denser pattern of fibres than did others. The present study was carried out to provide a formal evaluation of this possibility.

MATERIALS AND METHODS

Goldfish (*Carassius auratus*) of approximately 35g (5 males and 5 females) were deeply anesthetized with tricaine methane sulfonate (MS-222; Sigma). To sex fish, an incision along the posterior lateral line was made and the gonads examined. Perfusions, post-fixations, storage of brains and subsequent processing of tissue by the immunoperoxidase method was identical to that described in Experiment 3. Sections were taken at 300 μ m intervals throughout the optic tectum, beginning at the rostral pole and continuing to the caudal pole. Microphotographs were taken under brightfield conditions using a Zeiss Axioplan Universal microscope and Kodak Pan-X 32 ASA film.

Choice of fibre samples

Five tectal levels were selected for analysis to represent the optic tectum as a whole. From each of these levels, one zone of the tectal tissue was selected to represent that section (tectal

level) as a whole. The levels and the locations of these zones are shown in Fig. 4.1. A zone of fibres was the equivalent of a microscopic field as seen using a 40x objective. Each fibre in each zone was traced along its entire length using an Olympus BH-2 microscope and a BH2-DA drawing attachment.

Statistical analysis

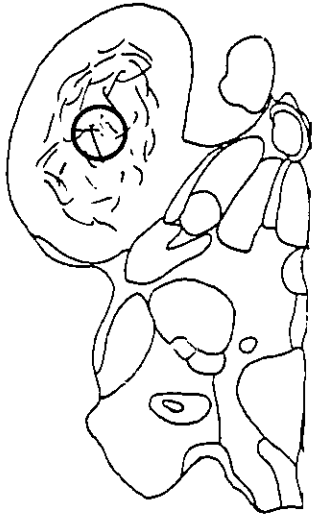
Each fibre in each field was traced on a magnetic graphics tablet and the lengths digitized and summed by an image analyzer (IBAS Interactive Image Analysis System, Kontron). As the section thickness, and therefore, tissue volume had remained constant across all fields, total fibre length reflects the density of fibres.

A factorial analysis of variance (ANOVA; $\alpha=0.05$; Statview 512) was carried out to test the significance of the differences between the density means of males and females at each tectal level. To gain a simple picture of the sex difference, the male : female ratio of grand mean fibre density (across all tectal levels) was calculated. For a general discussion of the use of statistics in neuroanatomy, refer to Appendix C.

RESULTS

A sex difference in Gal-fibre density was starkly evident upon microscopic examination of the immunoreacted tectal tissue (Fig. 4.2). The density appeared far greater in males than females, and statistical analysis confirmed this. In both sexes, Gal-immunoreactive fibres were found only in the deep layers of the optic tectum, the stratum centrale (SAC) and stratum

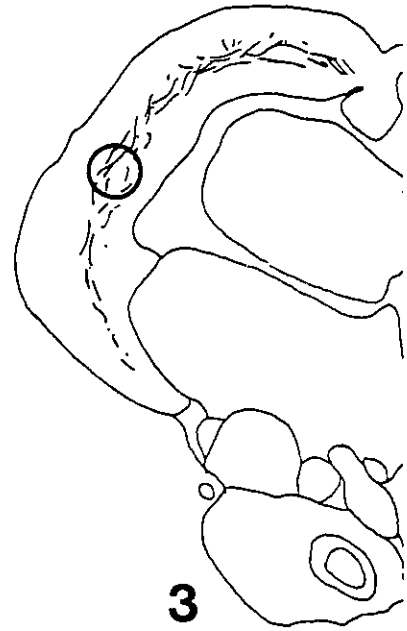
Fig. 4.1. Schematic frontal sections showing the tectal levels used for statistical analysis. Circles show the field sampled at each level (not to scale).



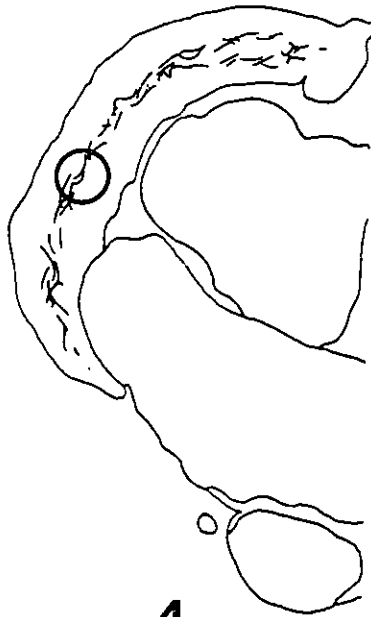
1



2



3



4



5

Fig. 4.2. Gal-immunoreactive fibres in the optic tectum of the goldfish. **A.** Gal-immunoreactive axons in the optic tectum of a male goldfish. **B.** Gal-positive axons at a comparable level of the optic tectum of a female goldfish are much finer and sparser and far less varicose. Scale bar = 50 μm .

A



B

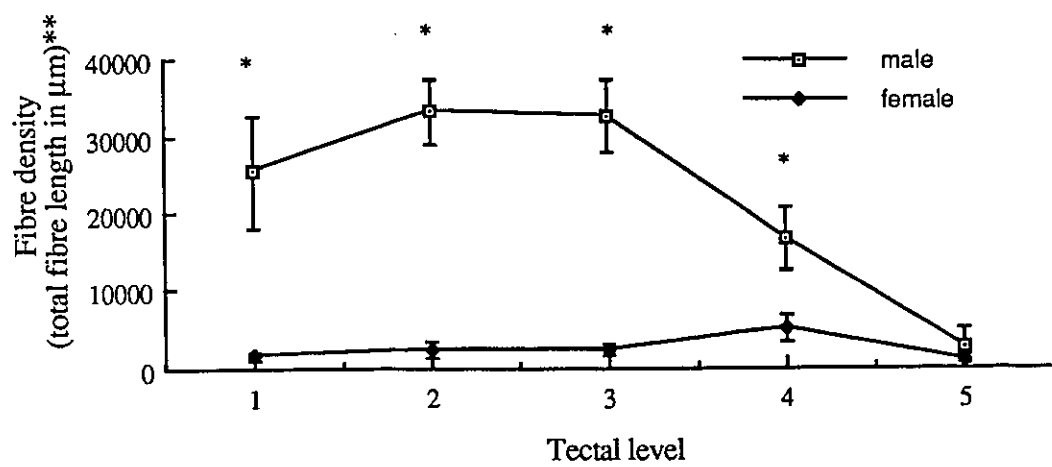


griseum centrale (SGC; for review, see Meek, 1983). The fibres in both sexes were characterized by varicosities-en-passant. Although in males these varicosities were far more numerous than in females, this appeared to be due to greater fibre density rather than a greater number of varicosities per unit fibre length (data not shown). The different orientations of the processes gave them a distinct cross hatched pattern in males, but not in females because they were few in number and more widely spaced. The fibre density in male tecta was significantly higher than that in female tecta at all but the caudalmost level (Fig. 4.3). The mid-tectum was the most dramatically dimorphic (level 2: $df=1,8$, $F=49.756$, $p<.001$; level 3: $df=1,8$, $F=38.972$, $p<.001$). The densities of Gal-positive fibres in the rostralmost and caudalmost tectum, that is at levels 1 ($df=1,8$, $F=10.102$, $p<.05$) and 4 ($df=1,8$, $F=7.081$, $p<.05$), were also significantly different between the sexes, but less so than those at mid-tectal levels. Overall, males had approximately 9x the Gal-positive fibre density of females.

DISCUSSION

By the measures used in this experiment, Gal-positive fibres were significantly more common in the optic tecta of male goldfish than in those of females. This sexual dimorphism could have arisen via a number of mechanisms. (1) Males possess a greater number of Gal-containing neurons that project to the optic tectum. (2) The number of neurons is equivalent in both sexes, but males exhibit greater fibre branching within the tectum. (3) The sex difference is not anatomical but reflects a difference in the degree of Gal expression; that is, females possess the same fibres or neurons that males do but something other than Gal is synthesized in them.

Fig.4.3. Gal-immunoreactive fibre density (mean $\pm$ standard error of the mean) along the rostrocaudal axis (level 1 to level 5) of the optic tectum of male and female goldfish. *Denotes $p < .05$ (compared with the same tectal levels in females). **Summed fibre length measured per $18.8 \times 10^3 \mu\text{m}^3$ (volume of 40x field).



The first neurotransmitter systems of the CNS found to be sexually dimorphic were the cholinergic and catecholaminergic systems (for review, see De Vries, 1984). Neuroanatomical sex differences have been found in a number of species but are particularly well studied in the rat. The sex differences in the medial preoptic area (MPOA) of the rat hypothalamus were first demonstrated to differ greatly in male and female neuronal populations by cytoarchitectonics (for review, see Gorski, 1984), and, more recently, applications of immunocytochemical techniques have shown it to be sexually dimorphic in the density of enkephalin and cholecystikinin (CCK)-positive fibres and the presence of larger CCK-positive cell bodies in males than females (Larriva-Sahd et al., 1986; Micevych et al., 1987; Simerly et al., 1988). These differences can be explained by the actions of circulating sex steroids on neuronal steroid receptors (for review, see McEwen, 1981). For example, substance P immunoreactivity is significantly reduced in the medial amygdala of castrated rats (Malsbury and McKay, 1987), suggesting that testicular steroids regulate the expression of this neuropeptide in this nucleus. Estrogen-concentrating cells are present in goldfish brain (Kim et al., 1978), implying that this substrate for neuronal steroid action, the steroid receptor, is also present. There is also testosterone uptake in the basal optic tectum of toadfish (Fine et al., 1982), suggesting that there may be a sex specialization in the tectal region that is perhaps homologous to the deep layers in which Gal-fibres were observed in the goldfish. This is a collateral confirmation of the results of the present experiment, indicating that, indeed, there does exist a basis for sexual dimorphism in this part of the optic tectum.

Steroid activity might prevent the death of Gal-synthesizing neurons that project to the tectum in male goldfish, as it is known to do in the sexually dimorphic spinal motoneurons innervating the musculature controlling the penis in rats (Sengelaub and Arnold, 1989).

Steroids might also induce the arborization of dendrites on male tectal neurons post-synaptic to the Gal-afferents, providing them with greater trophic support and, thus, allowing their greater ramification than those in females. This is suggested by the fact that testosterone increases the number of dendritic synapses in the sexually dimorphic song control pathway in the telencephalon of some birds (for review, see DeVoogd, 1986). The expression of Gal in this tectal circuitry may be inhibited by steroids acting at the genomic level to decrease peptide synthesis (for review, see McEwen, 1981). A sex difference in biosynthetic capacity has been shown in the sexually dimorphic bed nucleus of the stria terminalis (BNST) in rats: The BNST cells in males label significantly more densely with a radioactive probe for mRNA encoding vasopressin precursor than those in females (Miller et al., 1989). A mechanism by which Gal expression is regulated at the level of transcription by a steroid is the most likely to explain sex differences in Gal distribution if one is to extrapolate from the numerous demonstrations of this mechanism in the anterior pituitary of the rat. Initially, the mRNA transcript corresponding to a cDNA clone isolated from estrogen-induced prolactin-secreting pituitary tumours was extensively homologous with porcine Gal precursor (Vrontakis et al., 1987). Later, it was found that pituitary levels of mRNA encoding rat Gal fluctuated during the estrous cycle (Kaplan et al., 1988), and now correlated increases in the concentration of Gal-immunoreactivity and, by inference, increases in Gal synthesis in the anterior pituitary of female rats have been revealed (Gabriel et al., 1989; Vrontakis et al., 1989).

Gal-neurons may form the neural substrate for a sex-specific behaviour in goldfish. There is ample evidence for this in other species. Sex-specific behaviours are often studied in relation to the hypothalamus. For example, injection of CCK-8 into the ventromedial hypothalamic nucleus of the rat inhibits lordosis behaviour in females (Babcock et al., 1988). In addition,

some sex-specific behaviours are under the control of extrahypothalamic regions, of which the optic tectum is one. Male electroreceptive fish and South African clawed frogs produce mating "vocalizations" different from those of females. The characteristics of the electric organ and laryngeal musculature controlling these vocalizations are correspondingly dimorphic (Bass and Baker, 1989; Fluet and Bass, 1989; for review, see Kelley, 1986a). Similarly, in rats, the density of peptidergic connections to the cremaster motoneurons in female rats are markedly reduced compared to those of males, most likely because the cremaster muscles innervated by these neurons control scrotal thermo-regulation, something that is obviously not needed in females (Nagy and Senba, 1985; Newton and Hamill, 1988; Newton et al., 1989). The optic tectum is not just a sensory structure, and, it, too, can co-ordinate complex motor acts. The Gal-immunoreactive afferents may be synapsing on the efferent neurons in the deep layers that, via descending projections of the tectobulbar tract to the reticular formation, effect food pursuit, attack, escape and exploratory behaviours in response to the perception of form in the environment (Northcutt, 1983; Vanegas, 1983), in which different stimuli are salient to different degrees in males and females and by which stimuli are processed differently by males and females.

GENERAL DISCUSSION

From isolated examination of Experiments 1 to 4, it may not be readily apparent how they might be linked together intellectually. They did, in fact, follow a temporal sequence and ambitions and findings in one led to or inspired the next. In Experiment 1, it was found that the projection of the entopeduncular nucleus to the ventoanterior/ventrolateral thalamus of the rat does not contain SRIF and, therefore, the subdivisions of the entopeduncular nucleus (EP) with respect to its targets can be distinguished by an immunohistochemical marker. This observation is important in that one can now determine the differential striatal inputs to the limbic (habenular, SRIF-containing) and motor (VA/VL, non-SRIF-containing) components of the EP without having to prove which EP component is being observed by going to the greater technical difficulty of using a retrograde tracer. Small injections of Fluorogold could be made in the rostral and caudal components projecting to the habenula and VA/VL, respectively, using SRIF immunohistochemistry as a marker to monitor that, indeed, the injections have been made within the correct subdivisions of the EP. Alternatively, an anterograde tracer, such as PhAL, could be injected into the striatum and the component of the EP in which the striatal terminals are observed could be easily identified using a double-label immunohistochemical procedure. More than anything, the result of Experiment 1 has allowed a great simplification of procedures designed to label topographical striato-entopeduncular projections.

It was thought that the study of basal ganglia might benefit from the identification of a basal ganglia homologue in non-mammalian species. A series of immunohistochemical markers for basal ganglia in mammals were applied to goldfish brain, but only one, the high degree of coexistence of NPY and SRIF in cell bodies of the VL, yielded encouraging results. The search for this homologue was a potentially naive idea, because the discovery of extensive NPY/SRIF

colocalization observed in the VI of the goldfish brain may have been a general phenomenon. To ensure that this colocalization, characteristic of the mammalian striatum (Vincent et al., 1983), was truly unique, parts of the goldfish brain outside the forebrain had to be examined for NPY/SRIF double-immunofluorescence. Only then could the true significance of this discovery of NPY/SRIF coexistence in the VI be determined and any confidence in ascribing striatal homology to this nucleus be acquired. This was the rationale behind mapping the single-label and double-label distributions of NPY and SRIF in Experiment 2. Consultation of the literature made it appear well within the realms of possibility that the double-labelled cell bodies in VI, with their contiguity with those in the entopeduncular nucleus of the goldfish (NE), are evolutionary precursors to basal ganglia nuclei as we know them in mammals.

Comparison of the distributions of NPY and SRIF in the goldfish brain to those in the rat brain reported in the literature showed that the coexistence of neuropeptides is a conserved feature of the nervous system. Not only did the cell bodies of the VI exhibit almost 100% colocalization, but so did the terminals of the dorsal telencephalon, strongly suggesting that local collaterals of the VI project to this region. Complete colocalization was also observed in the medial column of the vagal motor complex, and the identity of these neurons confirmed by the discovery of NPY/SRIF colocalization in nerve fibres of the swim bladder, a peripheral target of this nucleus (Morita and Finger, 1987). In this way, immunohistochemical techniques reveal clues as to the connections between different nervous structures.

Neither can the coincidence of neurotransmitters be ignored in comparative studies of neuroanatomy, as this phenomenon of extensive overlap of single-labelled SRIF- and NPY-positive fibres was so strikingly evident in the goldfish brain, particularly in the telencephalon and the vagal lobe. Thus, NPY and SRIF do appear to act in the same systems. Certainly the two transmitters are tied together anatomically, sometimes by coexistence and

sometimes by coincidence. Exactly how they are functionally linked will become clear through physiological studies. The functional interaction of these neuropeptides with the inhibitory actions of GABA will be of some interest in the future in the brains of lower vertebrates. Glutamic acid decarboxylase (GAD), the enzyme which synthesizes GABA, has been found to coexist with SRIF in a number of brain regions such as the reticular nucleus of the thalamus in the cat (Oertel et al., 1983). I have begun processing goldfish brain sections for GAD immunohistochemistry and the results are interesting in terms of the extensive amount axosomatic terminal labelling present throughout the brain, and the prominent terminal labelling in some regions in which NPY- and SRIF-positive terminals were also very dense. Coexistence of SRIF and NPY was not observed as frequently as the coincidence of these two neuropeptides in the goldfish brain, suggesting that here coincidence of neurotransmitters may be a more general principle of neuronal organization than coexistence.

In the introduction, it was proposed that application of recent immunohistochemical techniques to the study of a lower vertebrate might reveal that this animal is a better model for the study of some phenomenon than a higher vertebrate. The results of Experiment 2 suggest the swim bladder of the goldfish as such a model. The swim bladder is a hydrostatic organ. Since the DMm neurons are very large, they may be fast-conducting, releasing SRIF and NPY from terminals in the wall of the bladder to mediate immediate responses to pressure changes due to changes in depth. As such, an *in vitro* preparation of the swim bladder may serve as a good lung model, a model for the actions of neuropeptides in peripheral blood flow and gas exchange (L. Maler, personal communication). There is a pulmonary component to the vagal nerve of all terrestrial vertebrates (for review, see Sarnat and Netsky, 1981). The layers in both the anterior and posterior chambers containing capillaries contain nerves as well, and there are other ultrastructural similarities to lungs (Morris and Albright, 1979). The distal chamber is

composed of resorbent epithelium, is well-vascularized, and is reminiscent of primitive lungs. Gas is removed by reabsorption into the blood circulating in the resorbent area. The mechanism is identical to that in respiratory organs (Steen, 1970), such as human lungs in which O₂ and CO₂ are exchanged by diffusion into the surrounding capillary according to differential partial pressures across the alveolar epithelial wall. The potential coexistence of SRIF and/or NPY with other transmitters such as GABA in the DMm and swim bladder should also be examined, for it may be that SRIF and NPY actually have no direct interaction with one another but rather may both modulate GABA function.

Even single-label immunohistochemical mapping studies such as that in Experiment 3 to determine the distribution of Gal in the goldfish brain can be fruitful in proposing structural homologies between lower and higher vertebrates. Experiment 3 was begun with more confidence than Experiment 2, after it was realized just how much data could be obtained from a mapping study of a non-mammalian brain. The original ambition was to find areas of Gal/ChAT coexistence, again through double-immunofluorescence, and of Gal receptors, through autoradiography, both techniques that have been used successfully in rat, particularly to designate forebrain regions lesioned in animal models of Alzheimer's neuropathology (Fisone et al., 1987; Flint Beal et al., 1988). These initial experiments did not meet with great success, and, although mapping studies are not as focussed as other types of experiments such as Experiment 1 and Experiment 4, they nonetheless form a basis for further study. Gal-immunoreactivity was found in fibres of the preoptic-neurohypophyseal bundle, in cell bodies of the NPO/NPP, supporting homology with the paraventricular and supraoptic hypothalamic nuclei of the mammal, and in fibres of the paralemniscal bundle and superficial sensory vagal lobe just as was SRIF-immunoreactivity in Experiment 2. The pars distalis and neurointermediate lobe of the pituitary contained vastly different numbers of

Gal-immunoreactive terminals, a finding consistent with that in rat (Gabriel et al., 1989), providing a first indication that levels of Gal-immunoreactivity could be sexually dimorphic in the goldfish brain, as confirmed in Experiment 4.

It would be interesting to pursue the possible coexistence of SRIF and Gal in these structures and in the mammalian brain in support of further homologies. There is a great deal of heuristic value in the observations on single-label distributions in that they prompt us to confirm our results through double-label immunohistochemistry. For instance, the presence of a Gal-immunoreactivity alone in a pathway in the goldfish brain was so strongly reminiscent of the tuberomammillary projections in the rat brain identified by adenosine deaminase and histamine immunohistochemistry. It is clear that the next step in this area is an attempt to confirm a tuberomammillary homologue by reacting goldfish brain with antibodies to histamine, as adenosine deaminase immunohistochemistry has already been attempted and failed to reveal the presence of the enzyme in the fish brain.

It is important to point out that the distribution of NPY/SRIF and Gal in the goldfish brain were not identical to any other published distribution of neuronal messengers, emphasizing that the findings reported are not simply the result of antibody cross-reactivity. We can certainly suggest that because antibodies raised against a synthetic peptide produce similar immunostaining patterns in both goldfish and rats, both the peptide and the nuclei and pathways characterized by it, if not homologous, are certainly an indication of a similar genetic response by the animals of both radiations to the same selective environmental pressures (Northcutt, 1981).

Experiment 4, a natural follow-up to an immunohistochemical distribution study, showed that biochemical neuroanatomy is by no means an end in and of itself, but an opening to fields of study tangentially related to it, such as that of sex differences. In the future, these results

may encourage other workers to extend to a lower vertebrate current findings on steroidal regulation of sexual behaviour in male humans and other mammals elicited by the injection of neuropeptides (Dornan and Malsbury, 1989). Already, these data can be generalized: We have begun collecting data suggesting that male pre-pubertal rats have more Gal-positive fibres in the superior colliculus than female littermates (data not shown), the superior colliculus being the mammalian homologue of the optic tectum. In addition, we have preliminary evidence that male rats have a greater density of Gal-immunoreactive fibres in the occipital cortex than do females. This is an interesting addition to these findings because the occipital, or visual, cortex, is not homologous to optic tectum. Its connections are not the same, nor does it stem from the same anlage as the colliculus in developing mammals. However, it is functionally similar to the tectum because it controls visual input from the retina and visual motor function through its connections to the superior colliculus. As described in the introduction, change in neuropeptide sequence and, for that matter, the basis of all change in all living organisms is genetic, and the origin of this component of tectal circuitry may be controlled by the same genes in both sexes, but some epigenetic influence, such as gonadal steroids, may determine its fate (Konishi and Akutagawa, 1987).

A sex difference in the optic tectum also has implications for change in the sense of neuronal plasticity in sensory systems. Bass (1986) reviews how sensory electroreceptors in electric fish are plastic enough in response to steroids to alter their spectral sensitivity to the discharge frequency generated by the sexually dimorphic electric organ motor system. This example inspires the view that although the tectum is typically looked upon as a sensory structure, the separation of motor and sensory components in a potentially hormone-sensitive system like the tectal Gal-immunoreactive system is a non-issue because sensory and motor systems interact.

The results of this experiment might prompt the broader kind of question that is asked in the field of sexual dimorphism: how ethological sex differences have a correlate in neuroanatomical sex differences. They also emphasize the oddness of the male state. The default developmental pattern expressed in the absence of male hormones is female (for review, see Kelley, 1986b). The existence of a far greater density of Gal-containing processes in the male optic tectum than in that of the female may allude to questions of what it means for a neuronal system to alter its fibre sprouting and/or peptide content at a certain stage of maturation. The regenerative capacity of goldfish retinal fibres is well known (Becker and Cook, 1988), and this sex difference in Gal-positive fibres may reflect plasticity in other non-retinal systems of the CNS.

Comparing distributions of neuronal messengers across the wide expanse between diverse species, as in Experiments 2 and 3, or contrasting distributions of neuronal messengers across the more narrow expanse between sexes, as in Experiment 4, is a potentially limited enterprise; there is a tendency to overlook differences and overemphasize similarities when only a couple of vertebrate groups are used. Although it was primarily the similarities between the brains of rats and goldfish that were discussed in this thesis, we must realize that differences are not to be dismissed because they can provide clues both to regression of neuronal structures that are no longer needed in the current environment of a particular species or sex and to specialization of neuronal structures to a level of complexity that helps the organism cope optimally with its environment. Certainly in a complete examination of evolutionary trends in the neurotransmitter content of the brain, invertebrates should be included for the following reasons. Some experimentors favour the view that most features of vertebrate evolution, including that of the nervous system, can be accounted for by the hypothesis that the invertebrate ancestor of all vertebrates is the nemertine worm. In addition, the catecholamine

transmitters have been studied more extensively in invertebrates than vertebrates. Acetylcholine, GABA and the catecholamines are already known to be present in the nervous system throughout phylogeny (for review, see Ramsay, 1981), and this repertoire of transmitters awaits the addition of neuropeptides to provide a more complete picture of the points during evolution at which certain brain structures, both common and unique, emerged. Provided that students of evolution remain aware of this, they can continue to approach phylogenetic comparisons through the neurochemical as well as other routes.

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APPENDIX A

ABBREVIATIONS

Technical

CCK	cholecystokinin
FITC	fluorescein isothiocyanate
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
Gal	galanin
HRP	horseradish peroxidase
NPY	neuropeptide Y
PBS	phosphate buffered saline
SRIF	somatotropin release inhibiting factor (somatostatin)

Rat Neuroanatomy

BNST	bed nucleus of the stria terminalis
CM-Pf	centromedian-parafascicular nuclei of the thalamus
CPu	caudate-putamen
EP	entopeduncular nucleus
Gpi	globus pallidus, internal segment
LHb	lateral habenula
MPOA	medial preoptic area of the hypothalamus
TPC	pedunculo-pontine tegmentum, pars compacta
VA/VL	ventroanterior/ventrolateral thalamus

Goldfish Neuroanatomy

AC	anterior commissure
C	cerebellum
Dc	area dorsalis telencephali pars centralis
Dd	area dorsalis telencephali pars dorsalis
Di	area dorsalis telencephali pars lateralis
Dlv	area dorsalis telencephali pars lateralis ventralis
Dm	area dorsalis telencephali pars medialis
DML	lateral column of the vagal motor complex
DMm	medial column of the vagal motor complex
DT	descending trigeminal tract
EG	eminentia granularis

FL	facial lobe
LC	locus coeruleus
LFB	lateral forebrain bundle
LL	lateral lemniscus
NAP _v	nucleus anterioris periventricularis
NAT	nucleus anterior tuberis
NDL	nucleus dorsolateralis thalami
NDM	nucleus dorsomedialis thalami
NDTL	nucleus diffusus tori lateralis
NE	nucleus entopeduncularis
NH	nucleus habenularis
NIL	neurointermediate lobe of the pituitary
NLTI	nucleus lateral tuberis pars lateralis
NLT _p	nucleus lateral tuberis pars posterioris
NFGl	nucleus preglomerulosus pars lateralis
NPO	nucleus preopticus
NPP	nucleus preopticus periventricularis
NPP _v	nucleus posterioris periventricularis
NPT	nucleus posterior tuberis
NRI	nucleus reticularis inferior
NRL	nucleus recessus lateralis
NRM	nucleus reticularis medialis
NRS	nucleus reticularis superior
NVM	nucleus ventromedialis thalami
OTec	optic tectum
OV	organum vasculosum
PD	pars distalis of the pituitary
PM	parvocellular marginal nucleus
SAC	stratum centrale
SGC	stratum griseum centrale
SGN	secondary gustatory nucleus
ST	secondary gustatory tract
TS	torus semicircularis
Vd	area ventralis telencephali pars dorsalis
Vi	area ventralis telencephali pars lateralis
VL	vagal lobe
VN	vagal nerve
Vp	area ventralis telencephali pars postcommissuralis
Vv	area ventralis telencephali pars ventralis

APPENDIX B

PRE-ADSORPTION CONTROLS FOR ANTIBODY SPECIFICITY IN IMMUNOHISTOCHEMISTRY

One of the main problems in immunohistochemistry is establishing the specificity of the results. This is because antibodies may cross-react with immunogens structurally related but not identical to those of interest (for review, see Hökfelt et al., 1984). Peptides may belong to families, the members of which share the same or similar amino acid sequences at the immunogenic site. For example, NPY belongs to the pancreatic polypeptide superfamily, the members of which have extensive sequence homologies across species. Porcine NPY has over 80% homology with fish pancreatic polypeptide (for review, see Tatemoto, 1989). How, then, do we know if the peptide localized immunohistochemically in goldfish brain is identical to that peptide against which the primary antibody has been raised or if it is another member of the peptide family which is specific to goldfish? Of course, antibodies may also cross-react with peptides that have not yet been discovered. It cannot actually be proven that there is absolute specificity of antibodies raised against a particular neuroactive substance, even when there is complete abolition of the immunohistochemical reaction after preadsorption. This proof could only be obtained if every peptide in existence except, of course, that one being studied, were preadsorbed to the antibodies and abolition of staining never occurred. In fact, such experiments are being carried out and it hardly ever happens that an antibody does not cross-react with a substance other than the one against which it was raised. Recently, this has been found to be true of the anti-Gal antibody used in Experiments 3 and 4. After

pre-adsorption, there always remains a non-specific stain in the pancreatic islets. The type of non-specific staining usually varies with the animal in which the antibody is raised, but in the case of porcine anti-Gal, the same staining remains in the pancreatic islets no matter what species of rabbit is used to raise the antibody. Another example of the need for pre-adsorption controls, demonstrating that these controls are not trivial, is that cited in Experiment 3, where anti-histamine antibodies have been found to cross-react with leutinizing hormone-releasing hormone in the median eminence of the rat (Berkenbosch and Steinbusch, 1987). For the above reasons, expressions such as "SRIF-like immunoreactivity" are used in immunohistochemical studies and are implied in the experiments described in this thesis.

Pre-adsorption controls are performed because they are the only control that is currently possible to test the specificity of a primary antibody. At first, this control seems intellectually weak because it seems to go without saying that an antibody raised against Gal, for example, will have its antigenic site blocked by the Gal peptide. However, such a specificity test does indeed have a strong, if not entirely satisfactory intellectual basis. It is only galanin-like immunoreactivity which can be said to be adsorbed away, not Gal immunoreactivity per se, and this will remain the case until the amino acid sequence of goldfish Gal is proven to be identical to that of porcine Gal, against which the primary antibody was raised. In other words, that the Gal control in Experiment 3 showed complete abolition of staining means that only porcine Gal immunoreactivity was adsorbed away, not necessarily that goldfish brain contains a peptide similar to mammalian Gal, although this is the most likely interpretation. To reiterate, the only true control is pre-adsorption with every other peptide in existence and to have all these controls fail in the species of interest, which, in these experiments is the goldfish, showing that goldfish NPY, SRIF or Gal are different from all other peptides.

Because the controls in Experiments 2 and 3 succeeded, we are only confident enough to say that goldfish NPY, SRIF and Gal are peptides closely related to those in mammals.

APPENDIX C

A RATIONALE FOR USING STATISTICS IN ANATOMY

Although anatomical studies necessitate a qualitative appreciation for the form and structure of organisms, they do not preclude the quantitation of these morphological features. In fact, numerical approaches in the field of neuroscience are common. When populations or groups of individuals, whether they be rats responding to injections of a drug or neuronal processes containing a particular neuropeptide, are studied, these quantities of information, as opposed to a single datum, can be dealt with by statistics. For example, these approaches may take the form of RIA measures of neuropeptide content of a particular neuroanatomical structure, or of morphometric analysis of neuronal area and length. To employ statistics in biological sciences requires a particular frame of mind that entertains the possibility, however remote, that cause and response variables in nature may occur by chance (Sokal and Rohlf, 1987).

Statistics are usually used to extract information that is not visually apparent, and, at first, it may seem that there is no point in using statistics to analyze a finding that is as obvious upon plain inspection as that in Experiment 4. It would seem that the significance of the difference between the tectal Gal-positive fibre densities of males and females need not be inferred, because it is so evident through visual assessment via the microscope (Fig. 4.2). Nevertheless, gaining experience in applying the correct statistic in biology is valuable in preparation for future situations when a difference is not so obvious and a statistic is required to bring it out. Although many regions in the female goldfish brain showed lower levels of Gal immunoreactivity than those in males (data not shown), the optic tectum was selected as the

structure of analysis because it exhibited the most dramatically obvious dimorphism and so could serve as the most striking representative of a widespread anatomical condition. These other regions did not exhibit the same degree of sexual difference as did the optic tectum, however, and so would require statistics to reveal the dimorphism.

To measure the variation in natural phenomena such as the density of tectal fibres caused by factors, perhaps genetic, that are difficult to control or identify, we must employ a tool that can determine the error of measurement and ascertain the reality of differences (Sokal and Rohlf, 1987). The inclusion of statistics with the morphological description in Experiment 4 showed that, indeed, morphology can be approached numerically and future investigators have the option of comparing their results, if they choose to expand on this data, in the form, qualitative or quantitative, that they feel is the most suited to their research. A numerical result may be more easily manipulated for the purpose of comparing future results with those obtained in Experiment 4 when other researchers study the same phenomenon under altered conditions. For example, the significance of the sex difference might change after the breeding season when hormonal surges decline. If the change were examined over the course of a year, no matter how graded or subtle, it could find expression in numbers. A strictly morphological approach does not have the power to do this. Descriptive wording is relatively limited to adjectives such as 'more' or 'less', 'greater' or 'smaller' but cannot answer very satisfactorily questions such as 'How much greater?'. We must be as objective in the evaluation of anatomical data as that of any other and statistics can help us to do this. Although figures (such as the micrographs in Experiment 4) do not lie, statistics can express our degree of belief or disbelief as a probability rather than a vague, general statement (Sokal and Rohlf, 1987). Numbers allow for a better understanding of the exact extent of an anatomical difference and the spread of these anatomical values within a group. For instance, although all female

goldfish have relatively few Gal-immunoreactive fibres, some females have more than other females (raw data not shown). This difference among females was not obvious under the microscope. Perhaps the former were perfused closer to the spawning season than the latter, such that they were under the influence of greater concentrations of circulating steroids (see Discussion, Experiment 4). These differences may also have been due to individual variation. Thus, possible reasons for anatomical differences may come to mind more readily in the face of quantitative results. In this way, statistical results may have more heuristic value than purely qualitative results.