Cell Tissue Res (1997) 288:497–507

Cell & Tissue Research

The neuronal system of the saccus vasculosus of trout (*Salmo trutta fario* and *Oncorhynchus mykiss*): an immunocytochemical and nerve tracing study

J. Yáñez[1], M. Rodríguez[2], S. Pérez[1], F. Adrio[2], I. Rodríguez-Moldes[2], M.J. Manso[1], R. Anadón[2]

[1] Departamento de Biología Celular y Molecular, Facultad de Ciencias, Universidad de La Coruña, E-15071 La Coruña, Spain
[2] Departamento de Biología Fundamental, Facultad de Biología, Universidad de Santiago de Compostela, E-15706 Santiago de Compostela, Spain

Received: 5 August 1996 / Accepted: 4 January 1997

Abstract. The neuronal system of the saccus vasculosus of two species of trout was studied with immunocytochemical methods and carboindocyanine-dye (DiI) tract-tracing. The cerebrospinal-fluid-contacting neurons of the saccus were immunoreactive for gamma-aminobutyric acid (GABA), glutamic acid decarboxylase (GAD), and neuropeptide Y (NPY). Immunostaining of alternate sections of the saccus vasculosus of fry with anti-GAD and anti-NPY indicated that these substances were colocalized. The tractus sacci vasculosi and the neuropil of the nucleus sacci vasculosi were also immunoreactive to these substances. The GABA, GAD, and neuropeptide Y immunoreactivity of the saccus vasculosus system appeared early in trout ontogeny. After applying DiI to various levels of the tractus sacci vasculosi of adult trout, we observed massive bilateral saccular projections to the nucleus sacci vasculosi and could follow the course of the sacco-thalamic tract. This tract extended in the subependymal region of the thalamus rostral to the nucleus sacci vasculosi and split into two small tracts that reached the subhabenular-preoptic region. Sacco-thalamic fibers formed extensive periependymal plexuses along their trajectory. Interestingly, no clear evidence of the existence of a saccopetal system was obtained. On the basis of these results, we postulate that the saccus vasculosus system modulates the function of centers of the posterior tubercle and periventricular thalamus.

Key words: Hypothalamus – GABA – Neuropeptide Y – Immunocytochemistry – Development, ontogenetic – *Oncorhynchus mykiss* (Teleostei) – *Salmo trutta fario* (Teleostei)

This work was supported by grants from the Spanish DGICYT (93–0527C0201) and the Xunta de Galicia (XUGA 20001B93).

Correspondence to: R. Anadón (Tel.: +34–81–563100, ext. 13296; Fax: +34–81–596904; E-mail: bfanadon@usc.es)

Introduction

The saccus vasculosus (SV) is a specialized ependymovascular diverticle of the caudal hypothalamus of elasmobranchs and most bony fishes. Putative functions include pressure reception, chemoreception, glucose loading, and ionic regulation of the cerebrospinal fluid (CSF; Altner and Zimmermann 1970). In the classic study of Dammerman (1910), the SV is reported to comprise coronet cells and supporting cells. According to this author, the coronet cells (named in reference to their apical crown of globule-tipped cilia) give rise to saccofugal projections to the posterior tubercle and possibly to the thalamus. Electron-microscopic studies, however, have demonstrated a third cell type in the SV, namely CSF-contacting (pseudocoronet) cells (Murakami and Yoshida 1967; Jansen and Flight 1969; Zimmermann and Altner 1970; Vigh et al. 1972; Zimmermann 1972; Rodríguez-Moldes and Anadón 1988; Corujo et al. 1990). According to these studies, the CSF-contacting cells give rise to the saccofugal axons of the tract of the SV. Histochemical studies in a few teleosts (Vigh-Teichmann et al. 1970; Zimmermann and Altner 1970; Jansen and West 1971; Vigh et al. 1972) have indicated that these cells and/or the SV tract are acetylcholinesterase (AChE)-positive. These studies have also confirmed the existence of saccular projections to the nucleus sacci vasculosi (NSV) in the posterior tubercle and to the thalamus, although the possible targets in the thalamus are still to be characterized. Since many non-cholinergic neurons show AChE activity (see Butcher and Woolf 1984), it remains to be determined whether CSF-contacting cells indeed use acetylcholine.

Electron-microscopic studies have also revealed synaptic (or synaptoid) contacts in the SV, most commonly on CSF-contacting neurons (Murakami and Yoshida 1967; Jansen and Flight 1969; Zimmermann and Altner 1970; Vigh et al. 1972; Zimmermann 1972; Rodríguez-Moldes and Anadón 1988; Corujo et al. 1990). A current hypothesis is that these presynaptic boutons come from fibers of saccopetal neurons (Altner and Zimmermann

1970; Zimmermann and Altner 1970), but the location and nature of the neurons giving rise to the boutons are not known.

The main aim of the present study was to identify the neuroactive substances (neurotransmitters and neuropeptides) of the SV neuronal system of trout and the possible targets of these cells. A second aim was to try to locate saccopetal neurons. Our results may shed light on the function(s) of the SV of fishes.

Materials and methods

Adults of rainbow trout (*Oncorhynchus mykiss*, 24 specimens) and of brown trout (*Salmo trutta fario*, 6 specimens) were used. In addition, embryos (10–12 mm body length; 36–40 days), alevins (14–22 mm body length; 45–72 days), and fry (23–45 mm; 76–185 days) of brown trout were used. Prior to fixation, fish were deeply anesthetized with tricaine methane sulfonate (Sigma). For light-microscope histochemistry (rainbow trout only), we used the AChE method of Karnovsky and Roots (1964). In preliminary trials for immunocytochemistry, antisera against serotonin (Incstar), dopamine (Affiniti), tyrosine hydroxylase (TH; Chemicon), calcitonin-gene-related peptide (CGRP; Genossys), galanin (Affiniti), somatostatin (Chemicon), thyrotropin-releasing hormone (Euro Diagnostica), α-melanocyte-stimulating hormone (Incstar), neuropeptide Y (NPY; Sigma), substance P (Chemicon, ICN Biochemicals), α-aminobutyric acid (GABA; Sigma, Affiniti), glutamic acid decarboxylase (GAD; Chemicon), and glutamate (Sigma) were assayed. Of these, the antisera (all polyclonal antisera generated in rabbit) against GABA, GAD (recombinant feline 67-kDa GAD), and NPY (porcine synthetic NPY) were found to label the SV neuronal system and were thus used for immunocytochemistry.

Adult brains were fixed by transcardial perfusion with 5% glutaraldehyde and 1% sodium metabisulfite in 0.05 M cacodylate buffer at pH 7.6 (GABA), with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4 (NPY, GAD), or with Bouin's fluid (NPY, GAD). Heads of developing brown trout were fixed by immersion in the same fixatives. The immunoreaction was revealed by the peroxidase-antiperoxidase and the streptavidin-biotin methods. No labeling was observed after preabsorption of the primary antisera with the corresponding antigen (GABA, NPY; Sigma) or after omission of the primary antiserum. The immunoreactivity of SV neurons to both anti-GABA and anti-GAD also indicated that GABA and GAD were the substances labeled by these antibodies (hereinafter, we refer to immunoreactive structures as GABAir, GADir, or NPYir). In order to investigate whether GAD and NPY could co-occur in the same neuron, alternate cryostat sections of the SV system of fry (3–4 μm thick) were incubated with anti-GAD and anti-NPY.

For tracing SV connections in rainbow trout, we used the carboindocyanine dye DiI (Eugene, Oregon), which diffuses along cell membranes in fixed tissues (Godement et al. 1987). The brains of 12 trouts were fixed in 4% paraformaldehyde in PB at 4ºC, embedded in agar blocks, and transverse-sectioned at the level required. DiI was applied with a fine needle or a 30-μm-thick micropipette (Yáñez et al. 1996) to the SV or to the tractus sacci vasculosi, (1) at its entrance to the posterior hypothalamic lobe, (2) between this entrance point and the NSV, or (3) in the decussation of the tract. DiI application to the tract gave highly reproducible results, whereas application to the saccus generally failed. After a diffusion time of 1–3 months, brains were sectioned (50 μm thick) on a Vibration microtome (Vibroslice, Campdem), mounted in PB, and photographed with a Nikon fluorescence microscope equipped with a rhodamine-type (G-2A) filter set.

Results

Immunocytochemistry

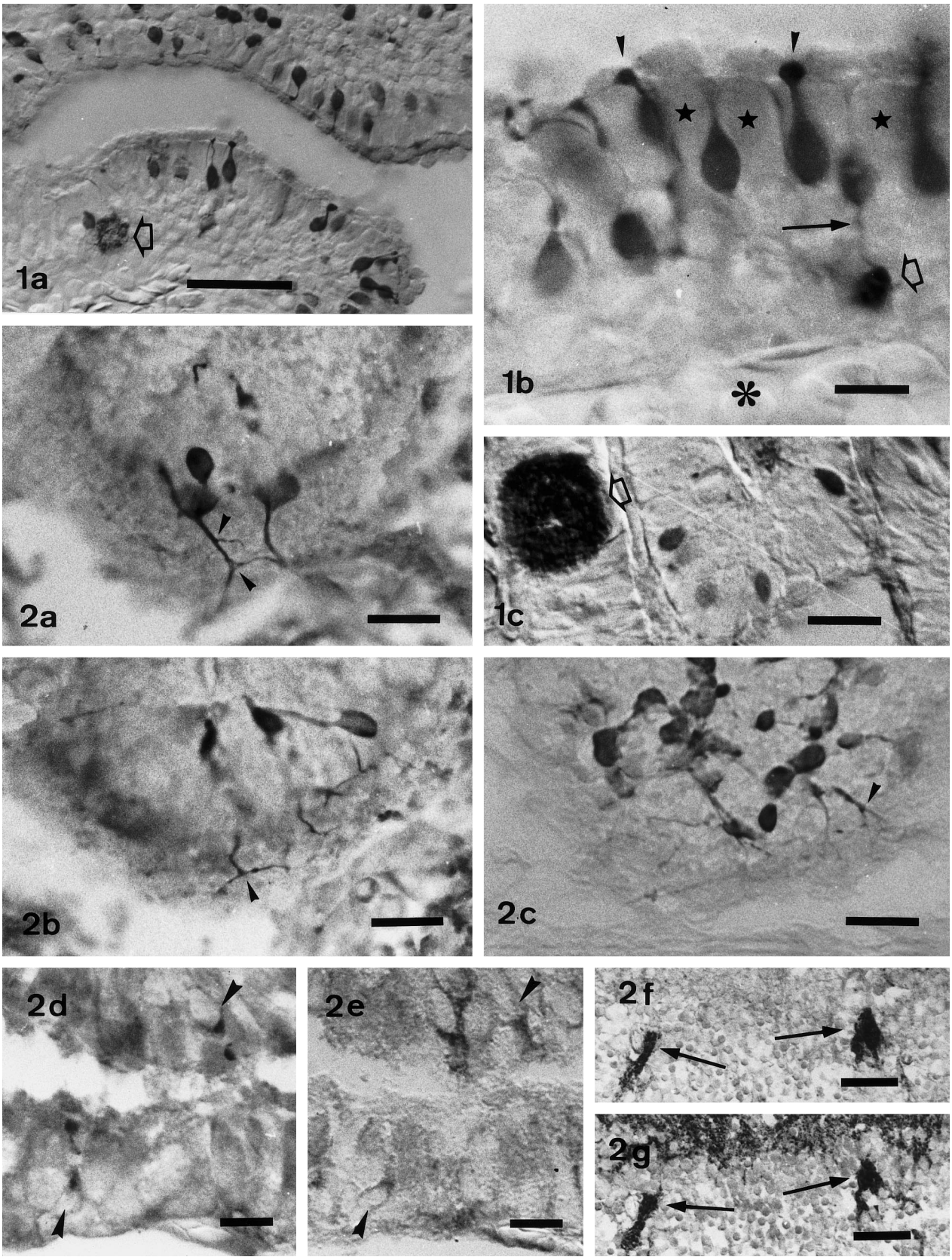
The CSF-contacting cells (but not the coronet cells or the supporting cells) of the SV of trout were immunoreactive to GABA, GAD, and NPY. These cells were bipolar, with slender apical processes ending in a terminal bulb (Fig. 1a,b). Some cells also showed a thicker process extending over the apical surface of the saccus from the dendrite. The thin basal process of CSF-contacting cells was well stained with the GABA method (Fig. 1b), and bundles of axons within the ependymal wall were clearly revealed by all procedures (Fig. 1a–c). These bundles coursed within the saccus wall toward their point of entrance in the posterior tubercle, where they joined to form thick paired SV tracts.

GABA, GAD, and NPY immunocytochemistry also revealed immunoreactive CSF-contacting neurons in the SV of trout embryos, alevins, and fry (Fig. 2a–c), although they were less numerous than in adults. Only a few neurons were observed in the saccus anlage of 10-mm embryos (Fig. 2a,b). Some basal processes immunoreactive to GABA were observed to branch in the saccus wall of embryos and alevins; some of these processes appeared to give rise to beaded collaterals in the basal region of the ependymal wall (Fig. 2a–c). A comparison of consecutive sections of the SV system of fry immunostained for GAD or NPY demonstrated CSF-contacting cells and SV tracts that were immunoreactive to both substances (Fig. 2d–g).

With the AChE method, CSF-contacting cells of the SV and SV tracts were faintly to moderately stained in the adult trout, in agreement with the results of other authors. In adult trout, the SV tracts could be followed with GABA, GAD, or NPY immunocytochemistry in the middle of the dorsal wall of the posterior hypothalamic lobe. After decussation in the caudal posterior tubercle, they gave rise to a paired field of terminals around a medial group of unstained cells and then converged in the rostralmost region of the posterior tubercle (Fig. 3a–c). Some fibers appeared to continue toward the medial thalamus (Fig. 3c,d) and were visible as two small paired tracts running close to the ventricular surface. Immunoreactive fibers of these tracts were observed among ependymal cells in some areas of the medial thalamus. How-

Fig. 1a–c. GABA-immunoreactive CSF-contacting neurons and nerve bundles (*open arrows*) in the epithelium of the saccus vasculosus of an adult trout. Note in **b** the terminal bulbs of apical dendrites (*arrowheads*) and a thin basal process (*thin arrow*). The level of the figures corresponds to that of Fig. 7 g. *Asterisk*, Blood sinusoid; *stars*, coronet cells with apical crowns of globules. **a** ×360, *Bar*: 50 μm; **b** ×1250, *Bar*: 10 μm; **c** ×625, *Bar*: 20 μm

Fig. 2. GABA-immunoreactive CSF-contacting neurons in the SV of 10 mm (**a, b**) and 12 mm trout embryos (**c**) showing the branching of basal processes (*arrowheads*). Note bouton-like enlargements of collateral branches. **d–g** Pairs of alternate thin sections showing CSF-contacting neurons (*arrowheads*) and tracts (*arrows*) of the SV system of a 28-mm fry immunoreactive to NPY (**d**, **f**) and GAD (**e**, **g**). **a–c** ×625, *Bar*: 20 μm; **d, e** ×810, *Bar*: 10 μm; **f, g** x280, *Bar*: 30 μm


1a
1b
2a
1c
2b
2c
2d
2e
2f
2g

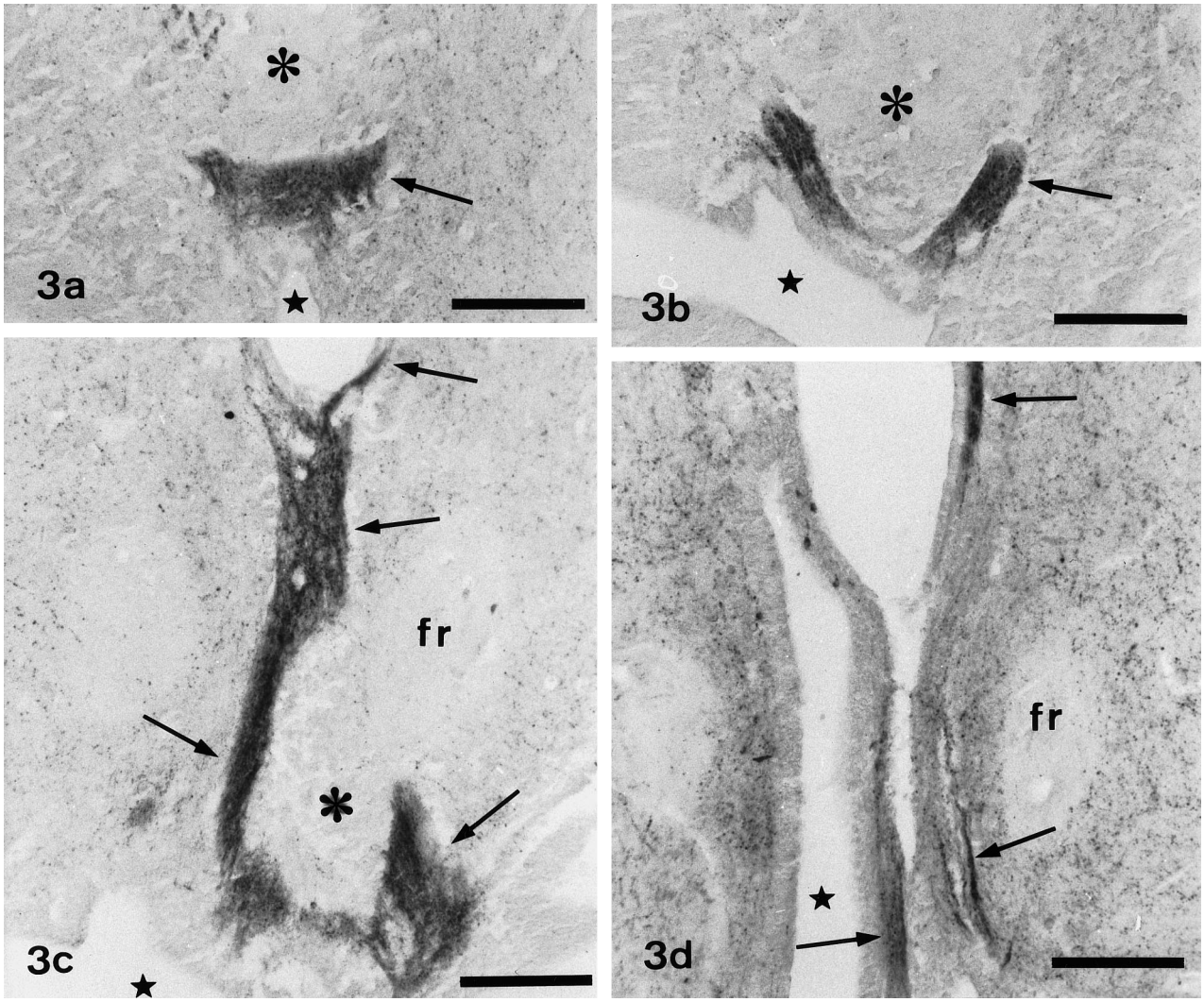


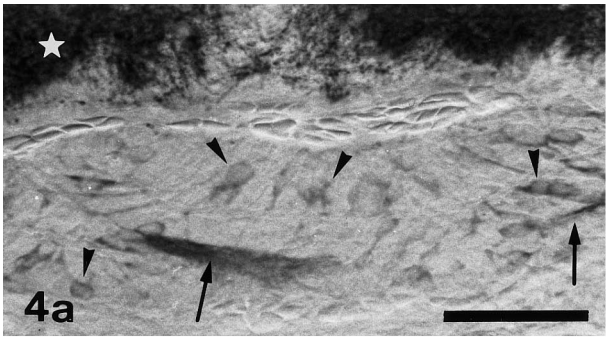
Fig. 3. Transverse sections of the posterior tubercle (**a–c**) and thalamus (**d**) showing intense NPY-immunoreactive fibers in the SV tract (*arrows*). The levels of **a, b** correspond approximately to that of Fig. 7d, those of **c, d** to that of Fig. 7c. *Asterisks*, Nucleus of the SV; *stars*, third ventricle recesses; *fr*, fasciculus retroflexus. Adult trout. ×180, *Bars*: 100 μm

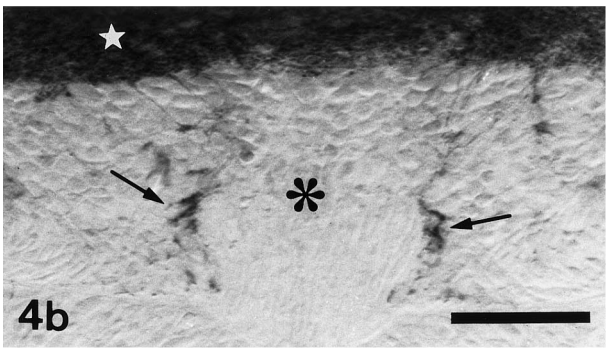
ever, the origin of these fibers could not be confirmed by these procedures, since fibers immunoreactive to GABA, GAD, or NPY were also present in neighboring regions.

In alevins, the tract of the SV could be followed to the posterior tubercle with GAD (Fig. 4a,b), because the solitary trajectory of this tract lay among the cells of the thick ependyma, which was clearly separated from those of GABAergic fibers of other origins coursing in the caudal hypothalamus. This solitary trajectory was even more clearly stained by NPY immunocytochemistry, because NPYir fibers from other origins were scarce in the posterior tubercle. In fry, the course of the SV tract to the posterior tubercle and thalamus (as revealed by immunocytochemistry) was similar to that of adults (Fig. 5a–e).

DiI nerve tracing

Application of DiI to the SV tract of rainbow trout, whether at its entrance to the posterior hypothalamic lobe or along its trajectory, produced massive labeling of the tract, which could thus be readily followed to its targets. The tract coursed to the posterior tubercle (as revealed by immunocytochemistry) and partially crossed in the decussation of the SV tract: the two sides were similarly labeled after DiI application to one or other SV tract caudal to the decussation (Fig. 6a–c). Most tract fibers ended in the neuropil of the NSV, mainly in its rostral and lateral portions, as conspicuous fields of terminals. No projections to other parts of the posterior tubercle were observed. In addition to these extensive terminal fields, a small tract of labeled fibers coursed dorsally in the walls of both sides of the third ventricle (Fig. 6b), rostral to the posterior tubercle, and close to the ventricle. Each of these fascicles gave rise to plexuses of fibers close to the ventromedial surface of the caudal thalamus, and to two small fascicles (dorsal and ventral) that could be followed to the subhabenular-preoptic region, where they were closely associated with two ventricular ridges (Figs. 6c, 7). Fibers from





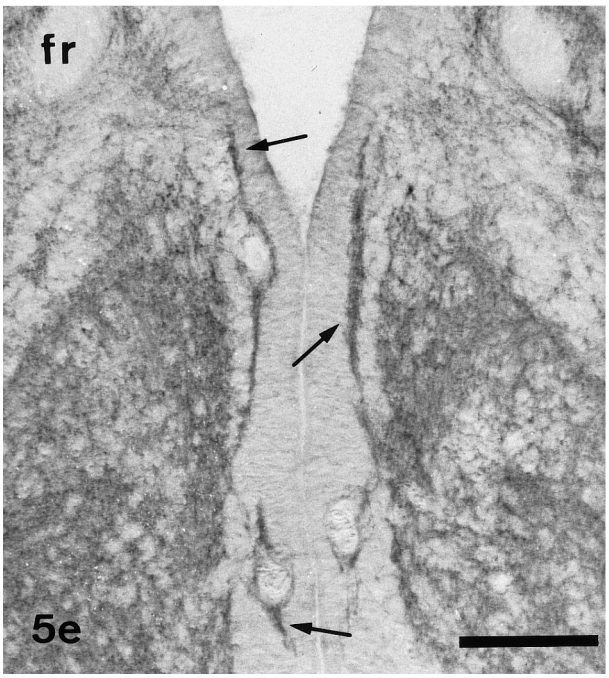
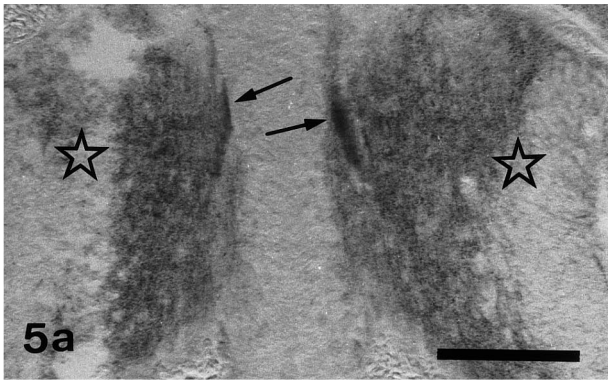
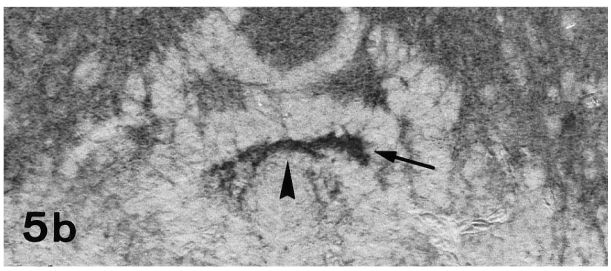
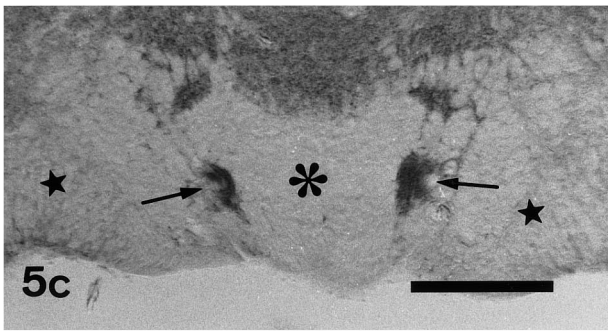


Fig. 4. Transverse sections of a post-hatching alevin (14 mm) showing GAD-immunoreactive fibers of the SV (*arrows*) in the saccus (**a**) and posterior tubercle (**b**). Note also GAD immunoreactivity of the CSF-contacting neurons of the SV (*arrowheads*). *Asterisk*, Nucleus of the SV; *white star*, GABAergic fiber-rich regions in the mesencephalic tegmentum (**a**) and caudal hypothalamus (**b**). ×360, *Bars*: 50 μm







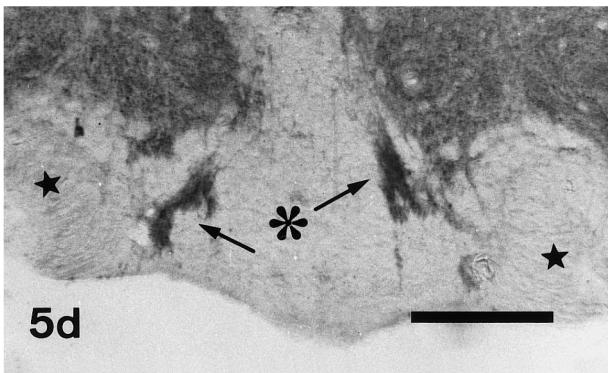


Fig. 5. Transverse sections through the junction of the SV with the posterior hypothalamic lobes (*outlined stars*) (**a**), posterior tubercle (**b–d**), and thalamus (**e**) showing the GAD-immunoreactive fibers of the SV tract (*arrows*). Note the decussation of the SV tract (*arrowhead*) in **b**. *Asterisk*, Nucleus of the SV; *stars*, nucleus recessus lateralis hypothalami; *fr*, fasciculus retroflexus. 38-mm juvenile. ×180, *Bars*: 100 μm

these small sacculo-thalamic fascicles formed two small neuropils closely associated with areas of thick ependyma in the thalamus (Fig. 6d,e). These fibers were observed among ependymal cells, even near the apical surface. A few fibers of the dorsal fascicle reached the subhabenular region, and a few fibers from the ventral fascicle extended to the preoptic region and appeared to end just medial and rostral to the magnocellular preoptic nucleus, always in periependymal plexuses (Fig. 7).

Application of DiI to the SV tract occasionally led to labeling of a few small cells in the posterior tubercle, al-

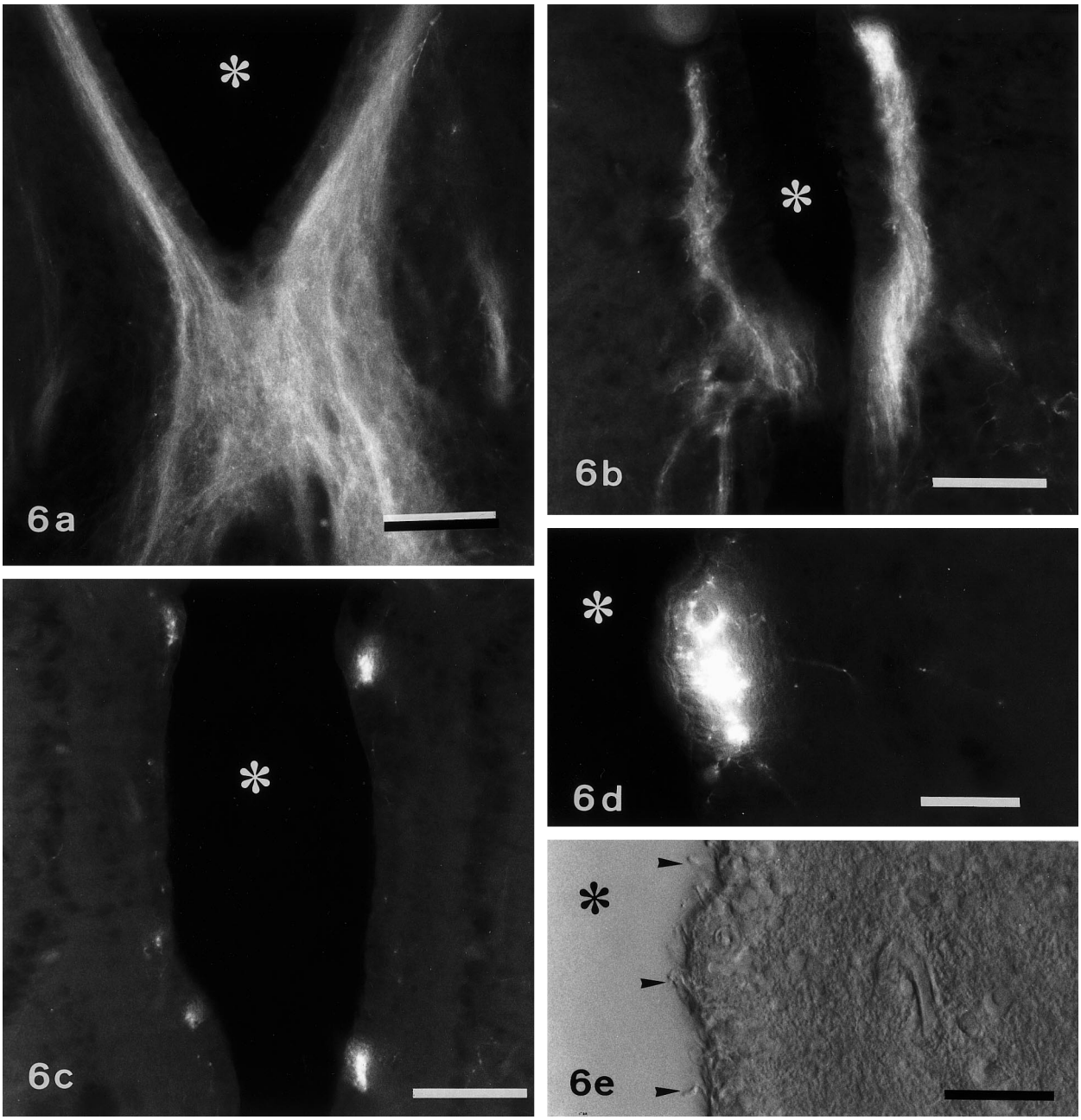


Fig. 6. Transverse sections showing bilaterally labeled tracts in the posterior tubercle (**a**) and medial thalamus (**b–d**) after unilateral application of DiI to the tract of the SV at its entrance in the hypothalamus. Note the close relationship of labeled fibers to the ependyma in the thalamus. Fluorescence microscopy. **e** Photomicrograph with differential interference contrast of the region shown in **d**. *Arrowheads*, Cilia of ependymal cells; *asterisk*, third ventricle. **a, b** Sections from a level slightly rostral to that of Fig. 7c; **c** corresponds to that of Fig. 7b. Adult trout. **a–c** ×170, *Bars*: 100 μm; **d, e** ×300, *Bars*: 50 μm

though results in this regard were inconsistent between experiments. Some of these cells were located close to the ventricle in the NSV and showed tanycyte-like morphology; others were located in the adjacent lateral recess nucleus. A few neurons were labeled in the posterior recess nucleus adjacent to the application point; this labeling was clearly artifactual. No other cells were labeled with these DiI applications, indicating that the procedure used specifically labeled the neuronal system of the SV.

Discussion

Neuroactive substances of the SV neuronal system

The present results show that the CSF-contacting cells of the trout SV are specifically revealed by GABA, GAD, and NPY immunocytochemistry, providing evidence that GABA and NPY are the neuroactive substances of the SV neuronal system. The immunoreactivity of the SV neurons, both with GABA and its synthesizing enzyme

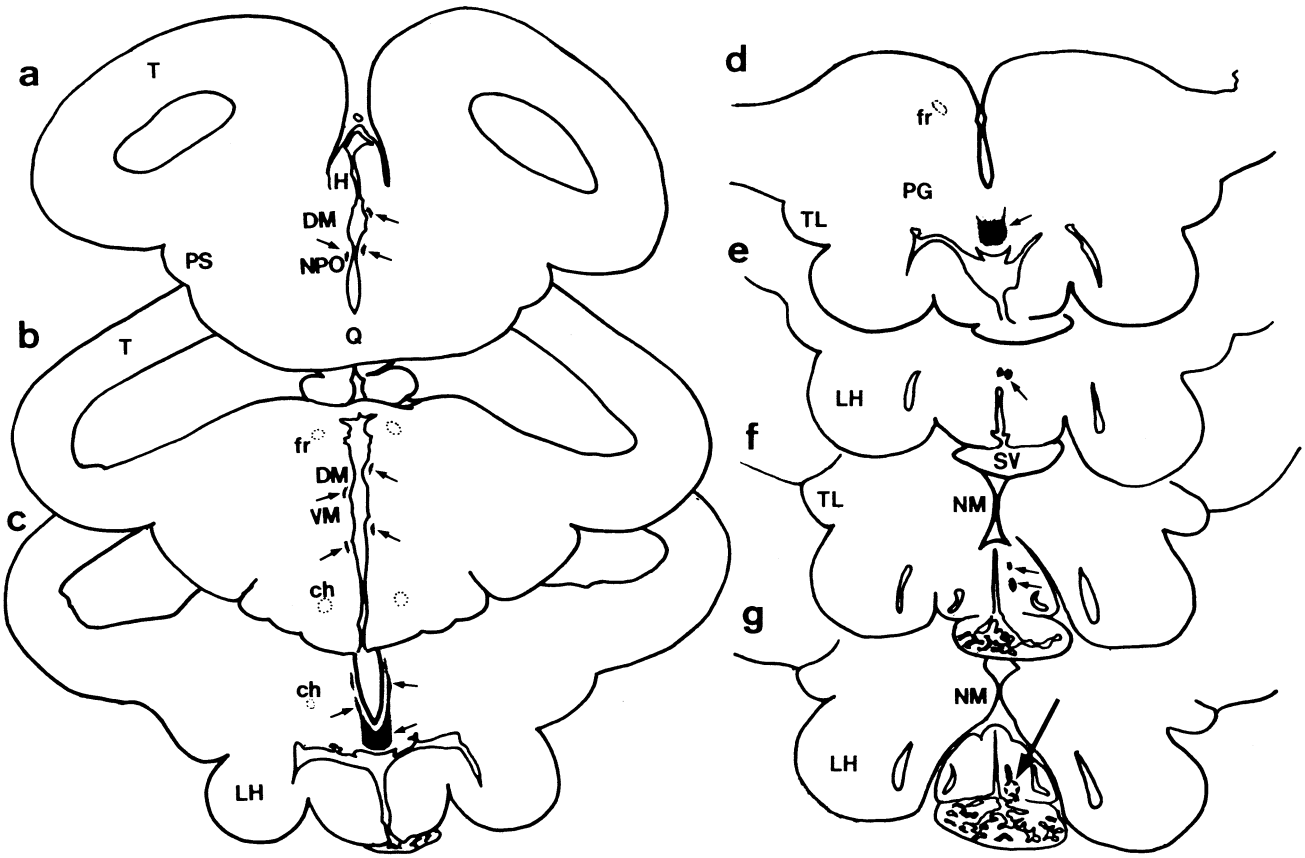


Fig. 7a–g. Schematic drawings of transverse sections through the trout diencephalon to show the course of the labeled fiber tracts (*small arrows*) of the neuronal system of the saccus vasculosus. **a**, Rostral; **g**, caudal. *Large arrow*, Point of DiI application; *ch*, horizontal commissure; *DM*, dorsomedial thalamus; *fr*, fasciculus retroflexus; *H*, habenula; *LH*, lateral hypothalamic lobes; *NM*, nucleus mammillaris; *NPO*, magnocellular preoptic nucleus; *PG*, preglomerular complex; *PS*, pretectal superficial nucleus; *Q*, optic chiasma; *SV*, saccus vasculosus; *T*, optic tectum; *TL*, torus lateralis hypothalami; *VM*, ventromedial thalamus

GAD, strongly suggests that these cells are GABAergic. The characteristics of the most abundant type of synaptic bouton observed by electron microscopy in the NSV of trout (García and Alvarez-Uría 1981), i.e., clear synaptic vesicles and the symmetrical appearance of active areas, are consistent with their being GABAergic (and thus putatively inhibitory) boutons. Similar boutons are also present in the elasmobranch NSV (Molist et al. 1992).

GABA and GAD immunocytochemistry also revealed CSF-contacting cells in 9- to 12-mm trout embryos, in good agreement with ultrastructural evidence regarding the time of appearance of these cells (Corujo et al. 1990). This suggests that these substances are present at early stages of neuron differentiation and is in agreement with the early appearance of GABA in Kolmer-Agduhr cells of the spinal cord in amphibian embryos (Dale et al. 1987). Our immunocytochemical observations also indicate that the number of CSF-contacting neurons increases considerably from embryo to adulthood, as reported previously (Corujo et al. 1990).

Early studies have indicated that CSF-contacting cells are AChE-positive in *Perca flavescens* (Zimmermann and Altner 1970), carp, eel, and *Amiurus nebulosus* (Vigh-Teichmann et al. 1970; Vigh et al. 1972). Jansen and West (1971) have reported that the CSF-contacting neurons and SV tracts of trout are faintly AChE-positive. The present histochemical results confirm these findings; in trout, the SV neurons and their tracts are faintly to moderately AChE-positive, the reaction being much less intense than in AChE-positive neurons of the neighboring hypothalamic lobes. Studies in rat brain with choline acetyltransferase (ChAT) immunocytochemistry have demonstrated that most AChE-positive cells (i.e. faintly or moderately AChE-positive neurons) are in fact non-cholinergic (Butcher and Woolf 1984). Thus, AChE histochemistry alone is insufficient for demonstrating that CSF-contacting cells are cholinergic. To the best of our knowledge, the only immunocytochemical evidence that is indicative of a cholinergic system in the SV is the presence of a ChAT-positive tract (the SV tract?) in the medial thalamus of the eel (Molist et al. 1993). In an attempt to approach this question directly, we have carried out studies in the trout brain with three different antibodies against rat brain and human placental ChAT (Chemicon). However, none of the antisera reacts with saccus cells, or with well-known cholinergic structures (such as the motor nuclei) of trout. This indicates that trout ChAT is not recognized by these antisera. Whether this protein is expressed in neurons of the trout SV system thus requires further investigation.

Our results have revealed the presence of NPY immunoreactivity in both neurons and fibers of the SV system of trout. The similar distribution of GABAir, GADir and NPYir cells and fibers suggests that these substances are colocalized in SV neurons, as has been confirmed by observations of cells stained in adjacent sections with GAD and NPY antisera. In the caudal hypothalamus, the colocalization of these substances appears specific for the saccus system, since other hypothalamic GADir neurons do not express NPY (unpublished observations). Colocalization of NPY and GABA immunoreactivity is frequently seen in neurons of most neocortical areas in cats and monkeys (Jones and Hendry 1986) but has not been mentioned in previous studies of GABA distribution in teleosts (see Martinoli et al. 1990; Medina et al. 1994). Although NPY has been found in some CSF-contacting neurons of the hypothalamus of elasmobranchs and bony fishes (Batten et al. 1990; Pickavance et al. 1992; Vecino and Ekström 1992; Chiba and Honma 1992, 1994), it has not been reported to date in the CSF-contacting neurons of the SV; however, Chiba and Honma (1994) have demonstrated NPY immunoreactivity in fibers associated with the SV of sturgeon. Colocalization of NPY and somatostatin (Batten et al. 1990) and of NPY and FMRF-amide peptide (Vecino and Ekström 1992) has been reported for some nuclei of teleosts, but neither somatostatin nor FMRF-amide peptide has been found in the SV. NPY is thought to be involved in the neuroendocrine regulation of certain pituitary functions, such as the inhibition of melanotropin release in frogs (Danger et al. 1987; De Rijk et al. 1990), the regulation of luteinizing and growth hormone release in rodents (Kerkerian et al. 1985; McDonald et al. 1985), and the modulation of the release of gonadotropin, melanotropin, and somatotropin in teleosts (Pontet et al. 1989). Projections, probably catecholaminergic, from the NSV to the hypophysis have recently been reported in the Atlantic salmon (Holmqvist and Ekström 1995). If the NSV is involved in pigmentation and reproduction, GABAir and NPYir saccofugal fibers may neutralize the inhibitory effects of this nucleus on hormone release. However, it is far from clear whether the NSV is related to pituitary regulation.

None of the other antisera against neuroactive substances or neurotransmitter-synthesizing enzymes, namely dopamine, serotonin, CGRP, somatostatin, substance P, and TH (Manso et al. 1993; Becerra et al. 1995; unpublished observations), reacts with SV cells or fibers of trout. Thus, although the presence of other neuroactive substances in the SV cannot be ruled out, these negative results clearly reduce the field of candidate substances.

With both immunocytochemical and nerve-tracing methods, we have found that the SV system of trout projects to only two regions: the NSV in the medial part of the posterior tubercle and the periventricular thalamus. This confirms reports in trout and other teleosts of projections to the posterior tubercle dorsal to the infundibulum, i.e., to the NSV (Dammerman 1910; Jansen and West 1971; Vigh et al. 1972; Zimmermann 1972), but also shows that the decussation of saccofugal fibers caudal to this nucleus is only partial, each SV tract projecting bilaterally to this nucleus. Moreover, our results confirm the presence of a small saccofugal projection to the thalamus in trout.

The NSV of trout appears to occupy a medial region of the posterior tubercle, which has been shown to receive secondary olfactory projections in *Salmo* (Northcutt and Davis 1983), as in other teleosts (Bass 1981; Davis et al. 1981; von Bartheld et al. 1984; Prasada Rao and Finger 1984; Levine and Dethier 1985; Sas et al. 1993). Thus, this nucleus may be modulated by the activity of both the olfactory and the SV nerve system. Our trout NSV probably corresponds in part to both the nucleus posterior tuberis and the NSV of himé salmon of Shiga et al. (1985). These nuclei project to the medial preoptic area and supracommissural telencephalon, regions involved in the regulation of sexual behavior (Shiga et al. 1985).

A direct saccothalamic projection was first suggested by silver staining in trout (Dammerman 1910) and has been confirmed by AChE histochemistry in other teleosts (Vigh et al. 1972). However, these studies have not identified the precise targets of this projection. The present experimental results confirm the direct saccothalamic projection and indicate the existence of a sacco-periventricular relationship similar to that observed between the caudal primary olfactory projection and the periventricular region of the supracommissural ventral telencephalon and preoptic area of trout (Manso et al. 1994; Anadón et al. 1995). The target cells of these sacco- and olfacto-periventricular projections are not known, but our observations have shown numerous beaded fibers coursing among ependymal cells, close to the ventricular surface. The close sacco-ependymal topographical relationship is even clearer during development, when a solitary saccofugal tract courses through the thick ependyma of the posterior tubercle. Neuroglial contacts have been reported on glial cells of highly specialized ependymal organs, such as the tanycytes of the median eminence of several vertebrates (for a review, see Leonhardt 1980) and the subcommissural organ of frog (Jiménez et al. 1993). If the SV and caudal primary olfactory fibers of trout contact ependymal cells, this would be a case of general ependymal regions receiving projections from identified sets of neurons and would raise new questions about neuro-glial synaptoid relationships in vertebrates. The next step is to investigate, by electron microscopy, whether saccofugal and/or caudal primary olfactory fibers contact ependymal cells or plexuses of periventricular dendrites.

Does the SV tract contain saccopetal fibers?

The presence of saccopetal fibers in the SV tract was reported early this century (see Dammerman 1910). The saccopetal system as described by Dammerman (1910) is a tract entering the SV in the area in which the pituitary joins the saccus wall (tractus thalamo-saccularis). The existence of this tract appeared less certain when Bargmann (1954) pointed out that, in elasmobranchs, the pre-

optico-hypophysial tract, which is unrelated to the saccus, passes through this area. More recently, a number of ultrastructural studies have noted synaptic boutons contacting SV neurons in both teleosts (Zimmermann and Altner 1970; Zimmermann 1972; Vigh et al. 1972; Corujo et al. 1990) and elasmobranchs (Rodríguez-Moldes and Anadón 1988). In some teleosts, such contacts have been observed on coronet cells (Vigh et al. 1972). On the basis of neuron counts in the SV and axon counts in the SV nerve of *Perca fluviatilis*, Zimmermann and Altner (1970) have suggested that about half or more of the approximately 50 000 axons of the SV tract are saccopetal, always assuming that the saccopetal axons pass into the saccus via the SV tract.

In the present study, our initial aim was to investigate experimentally the distribution of neurons that give rise to putative saccopetal fibers. To our surprise, DiI application to the SV tract of trout labeled only a few cells in the posterior tubercle, whereas labeled cells were not observed in any other brain region. The small number of labeled cells and the tanycyte appearance of a proportion of them are not readily explained by the hypothesis of Zimmermann and Altner (1970). Our results instead suggest that trout have either very few saccopetal fibers or none. If so, the infrequent synapses present in the trout saccus might be collateral or en-passant contacts of saccofugal fibers within the saccus. Interestingly, some basal processes of CSF-contacting neurons of the SV are branched in developing trout, which favor the presence of such contacts. Alternative explanations, for example, that DiI fails to diffuse through saccopetal axons or that technical problems affect the application of DiI to these fibers, cannot be ruled out, although they appear improbable. Our results thus cast serious doubts on the existence of a saccopetal fiber system. However, it should be noted that, in trout, the SV is open to the infundibulum (Dammerman 1910; Corujo et al. 1990), whereas in the perch, it is united with the caudal hypothalamus by a practically closed stalk, the nervus sacci vasculosi (Zimmermann 1972). A closed SV like that of the perch may possess a saccopetal system in order to control its functions, but such control may be unnecessary when the SV is open to the third ventricle (and thus perceives changes in the CSF), as in the trout. In any case, the putative presence of a saccopetal SV system appears to be based on circumstantial arguments; further studies in species with closed sacci vasculosi are necessary in order to confirm the existence of such systems and to elucidate relationships with SV function.

In view of its GABAergic nature, the SV system is presumably involved in inhibition of its targets. However, what kind of information does the system carry? At present, we can give no firm answer to this question, although it seems likely that this system is sensory, in view of the ultrastructural features of the SV cells, the simple shape of the neurons (which have a single apical dendrite), and the scarce saccopetal innervation. A sensory role for CSF-contacting cells was first suggested by Vigh et al. (1969, 1972). In the dogfish and trout, one of the most interesting features of SV neurons is that they possess a dendrite with an apical “end knob” bearing a pair of cilia (Rodríguez-Moldes and Anadón 1988; Corujo et al. 1990), reminiscent of the ciliated type of olfactory neuron present in teleosts (Yamamoto 1982; Muller and Marc 1984) and suggesting that the SV neurons may be chemoreceptors. Specifically, SV neurons may act as sensors of CSF composition, which is thought to be modified by the secretory activity (see Altner and Zimmermann 1970) and/or transport activity (Jansen and Flight 1969; Jansen and van Dort 1978; Jansen et al. 1981, 1982) of coronet cells. Although the presence of both CSF-contacting neurons and coronet cells in the SV makes such a dual function reasonable (Altner and Zimmermann 1970), experimental evidence is lacking.

Other GABAergic CSF-contacting cells are present in the central nervous system of vertebrates: well-known examples include the Kolmer-Agduhr cells of the spinal cord of tadpoles (Dale et al. 1987) and other cells in the central canal of the spinal cord of lampreys and teleosts (Brodin et al. 1990; Uematsu et al. 1993; Roberts et al. 1995). Some of these cells, such as the Kolmer-Agduhr cells in early developmental stages of tadpoles, have been implicated in the mechanoreception of body movements. A similar function for the CSF-contacting cells of the SV is unlikely, because the SV is contained within the brain case. Conclusive identification of the function of the SV system will require direct experimental approaches. We hope that the present study of the neurotransmitters and targets of the saccofugal pathways will encourage electrophysiologists to study this enigmatic organ.

Acknowledgements. We thank Carmen Fariña for kindly providing the rainbow trout used in this study.

References

Altner H, Zimmermann H (1970) The saccus vasculosus. In: Bourne GH (ed) Structure and function of the nervous tissue, vol 5. Academic Press, New York, pp 293–328

Anadón R, Manso MJ, Rodríguez-Moldes I, Becerra M (1995) Neurons of the olfactory organ projecting to the caudal telencephalon and hypothalamus: a carbocyanine-dye labeling study in the brown trout (Teleostei). Neurosci Lett 191:157–160

Bargmann W (1954) Über Feinbau und Funktion des Saccus vasculosus. Z Zellforsch 40:49–74

Bartheld CS von, Meyer DL, Fiebig E, Ebbesson SOE (1984) Central connections of the olfactory bulb in the goldfish, *Carassius auratus*. Cell Tissue Res 238:475–487

Bass AH (1981) Olfactory bulb efferents in the channel catfish, *Ictalurus punctatus*. J Morphol 169:91–111

Batten TFC, Cambre ML, Moons L, Vandesande F (1990) Comparative distribution of neuropeptide-immunoreactive systems in the brain of the green molly, *Poecilia latipinna*. J Comp Neurol 302:893–919

Becerra M, Manso MJ, Rodríguez-Moldes I, Anadón R (1995) Ontogeny of somatostatin-immunoreactive systems in the brain of the brown trout (Teleostei). Anat Embryol 191:119–137

Brodin L, Dale N, Christenson J, Storm-Mathisen J, Hökfelt T, Grillner S (1990) Three types of GABA-immunoreactive cells in the lamprey spinal cord. Brain Res 508:172–175

Butcher LL, Woolf NJ (1984) Histochemical distribution of acetylcholinesterase in the central nervous system: clues to the lo-

calization of cholinergic neurons. In: Björklund A, Hökfelt T, Kuhar MJ (eds) Handbook of chemical neuroanatomy, vol 3. Classical transmitters and transmitter receptors in the CNS, part II. Elsevier, Amsterdam, pp 1–50

Chiba A, Honma Y (1992) Distribution of neuropeptide Y-like immunoreactivity in the brain and hypophysis of the cloudy dogfish, *Scyliorhinus torazame*. Cell Tissue Res 268:453–461

Chiba A, Honma Y (1994) Neuropeptide Y-immunoreactive structures in the telencephalon and diencephalon of the white sturgeon, *Acipenser transmontanus*, with special regard to the hypothalamo-hypophyseal system. Arch Histol Cytol 57:77–86

Corujo A, Rodríguez-Moldes I, Anadón R (1990) Light and ultrastructural study of the development of the saccus vasculosus in the rainbow trout, *Oncorhynchus mykiss*. J Morphol 206: 79–93

Dale N, Roberts A, Ottersen OP, Storm-Mathisen J (1987) The morphology and distribution of "Kolmer-Agduhr" cells, a class of cerebrospinal-fluid-contacting neurons revealed in the frog embryo spinal cord by GABA immunocytochemistry. Proc R Soc Lond [Biol] 232:193–203

Dammerman KW (1910) Der Saccus vasculosus der Fische ein Tiefeorgan. Z Wiss Zool 96:654–726

Danger JM, Tonon MC, Lamacz M, Martel JC, Saint-Pierre S, Pelletier G, Vaudry H (1987) Melanotropin release inhibiting activity of neuropeptide Y: structure-activity relationships. Life Sci 40:1875–1880

Davis RE, Chase R, Morris J, Kaufman B (1981) Telencephalon of the teleost *Macropodus*: experimental localization of secondary olfactory areas and of components of the lateral forebrain bundle. Behav Neural Biol 33:257–279

De Rijk EPCT, Jenks BG, Roubos EW (1990) GABA and neuropeptide Y co-exist in axons innervation the neurointermediate lobe of the pituitary of *Xenopus laevis* – an immunoelectron microscopic study. Neuroscience 38:495–502

García JM, Alvarez-Uría M (1981) Ultraestructura del núcleo y nervio del saco vasculoso de *Salmo irideus*, Gibb. Trab Inst Cajal 72:279–283

Godement P, Vanselow J, Thanos S, Bonhoeffer F (1987) A study in developing visual systems with a new method of staining neurons and their processes in fixed tissue. Development 101:697–713

Holmqvist BI, Ekström P (1995) Hypophysiotrophic systems in the brain of the Atlantic salmon. Neuronal innervation of the pituitary and the origin of pituitary dopamine and nonapeptides identified by means of combined carbocyanine tract tracing and immunocytochemistry. J Chem Neuroanat 8:125–145

Jansen WF, Dort JB van (1978) Further investigations on the structure and function of the saccus vasculosus of the rainbow trout, *Salmo gairdneri* Richardson. Ultrastructural cytochemistry of membrane-bound alkaline phosphatase. Cell Tissue Res 187:61–68

Jansen WF, Flight WFG (1969) Light- and electron-microscopical observations on the saccus vasculosus of the rainbow trout. Z Zellforsch 100:439–465

Jansen WF, West R (1971) A cytochemical investigation of specific and non-specific cholinesterase activity in the saccus vasculosus of the rainbow trout. Proc K Ned Akad Wet C 74: 344–351

Jansen WF, Flight WFG, Zandbergen MA (1981) Fine structural localization of adenosine triphosphatase activities in the saccus vasculosus of the rainbow trout, *Salmo gairdneri* Richardson. Cell Tissue Res 219:267–279

Jansen WF, Burger EH, Zandbergen MA (1982) Subcellular localization of calcium in the coronet cells and tanycytes of the saccus vasculosus of the rainbow trout, *Salmo gairdneri* Richardson. Cell Tissue Res 224:169–180

Jiménez AJ, Pérez-Fígares JM, Rodríguez EM, Fernández-Llebrez P, Oksche A (1993) Synapse-like contacts between axons of the pineal tract and the subcommissural organ in *Rana perezi* (Anura) and their absence in *Carassius auratus* (Teleostei): ultrastructural tracer studies. Cell Tissue Res 273:317–325

Jones EG, Hendry SHC (1986) Co-localization of GABA and neuropeptides in neocortical neurons. Trends Neurosci 9:71–76

Karnovsky MJ, Roots L (1964) A "direct-coloring" thiocholine method for cholinesterases. J Histochem Cytochem 12:219–221

Kerkerian L, Guy J, Lefevre G, Pelletier G (1985) Effects of neuropeptide Y (NPY) on the release of anterior pituitary hormones in the rat. Peptides 5:1201–1204

Leonhardt H (1980) Ependym und circumventriculäre Organe. In: Oksche A, Vollrath L (eds) Handbuch der mikroskopischen Anatomie des Menschen, vol IV, part 10. Springer, Berlin Heidelberg New York, pp 177–666

Levine RL, Dethier S (1985) The connections between the olfactory bulb and the brain in the goldfish. J Comp Neurol 237: 427–444

Manso MJ, Becerra M, Molist P, Rodríguez-Moldes I, Anadón R (1993) Distribution and development of catecholaminergic neurons in the brain of the brown trout. A tyrosine hydroxylase immunohistochemical study. J Hirnforsch 34:239–260

Manso MJ, Becerra M, Rodríguez-Moldes I, Anadón R (1994) Primary olfactory fibres project to the caudal telencephalon, organum vasculosum laminae terminalis and preoptic area in brown trout (*Salmo trutta fario*). A developmental study with immunocytochemical markers. J Comp Neurol 342:131–143

Martinoli M-G, Dubourg P, Geffard M, Calas A, Kah O (1990) Distribution of GABA-immunoreactive neurons in the forebrain of the goldfish, *Carassius auratus*. Cell Tissue Res 260: 77–84

McDonald JK, Lumpkin MD, Samson WK, McCann SM (1985) Neuropeptide Y affects secretion of luteinizing hormone and growth hormone in ovariectomized rats. Proc Natl Acad Sci USA 82:561–564

Medina M, Repérant J, Dufour S, Ward R, Le Belle N, Miceli D (1994) The distribution of GABA-immunoreactive neurons in the brain of the silver eel (*Anguilla anguilla* L.). Anat Embryol 189:25–39

Molist P, Rodríguez-Moldes I, Anadón R (1992) Immunocytochemical and electron microscopic study of the elasmobranch nucleus sacci vasculosi. Cell Tissue Res 270:395–404

Molist P, Maslam S, Velzing E, Roberts BL (1993) The organization of cholinergic neurons in the mesencephalon of the eel, *Anguilla anguilla*, as determined by choline acetyltransferase immunocytochemistry and acetylcholinesterase enzyme histochemistry. Cell Tissue Res 271:555–566

Muller JF, Marc RE (1984) Three distinct morphological classes of receptors in fish olfactory organs. J Comp Neurol *222*: 482-495

Murakami M, Yoshida T (1967) Elektronenmikroskopische Beobachtungen am Saccus vasculosus des Kugelfisches, *Spheroides niphobles*. Arch Histol Jpn 28:265–284

Northcutt RG, Davis RE (1983) Telencephalic organization in ray-finned fishes. In: Davis RE, Northcutt RG (eds) Fish neurobiology, vol 2. Higher brain areas and functions. The University of Michigan Press, Ann Arbor, pp 203–236

Pickavance LC, Staines WA, Fryer JN (1992) Distributions and colocalization of neuropeptide Y and somatostatin in the goldfish brain. J Chem Neuroanat 5:221–233

Pontet A, Danger JM, Dubourg P, Pelletier G, Vaudry H, Calas A, Kah O (1989) Distribution and characterization of neuropeptide Y-like immunoreactivity in the brain and pituitary of the goldfish. Cell Tissue Res 255:529–538

Prasada Rao PD, Finger TE (1984) Asymmetry of the olfactory system in the brain of the winter flounder, *Pseudopleuronectes americanus*. J Comp Neurol 225:492–510

Roberts BL, Maslam S, Scholten G, Smit W (1995) Dopaminergic and GABAergic cerebrospinal fluid-contacting neurons along the central canal of the spinal cord of the eel and trout. J Comp Neurol 354:423–437

Rodríguez-Moldes I, Anadón R (1988) Ultrastructural study of the evolution of globules in coronet cells of the saccus vasculosus

of an elasmobranch (*Scyliorhinus canicula* L.), with some observations on cerebrospinal fluid-contacting neurons. Acta Zool 69:217–224

Sas E, Maler L, Weld M (1993) Connections of the olfactory bulb in the gymnotiform fish, *Apteronotus leptorhynchus*. J Comp Neurol 335:486–507

Shiga T, Oka Y, Satou M, Okumoto N, Ueda K (1985) An HRP study of afferent connections of the supracommissural ventral telencephalon and the medial preoptic area in the himé salmon (landlocked red salmon, *Oncorhynchus nerka*). Brain Res 361:162–177

Uematsu K, Shirasaki M, Storm-Mathisen J (1993) GABA- and glycine-immunoreactive neurons in the spinal cord of the carp, *Cyprinus carpio*. J Comp Neurol 332:59–68

Vecino E, Ekström P (1992) Colocalization of neuropeptide Y (NPY)-like and FMRFamide-like immunoreactivities in the brain of the Atlantic salmon (*Salmo salar*). Cell Tissue Res 270:435–442

Vigh B, Vigh-Teichmann I, Aros B (1969) Das Paraventrikularorgan und das Liquorkontakt-Neuronensystem. Verh Anat Ges Erg Anat Anz 125:683–688

Vigh B, Vigh-Teichmann I, Aros B, Varjassy P (1972) Licht- und elektronenmikroskopische Untersuchungen des Saccus vasculosus und des Nervus und Tractus sacci vasculosi. Z Zellforsch 129:508–522

Vigh-Teichmann I, Vigh B, Aros B (1970) Enzymhistochemische Studien am Nervensystem. IV. Acetylcholinesteraseaktivität im Liquorkontaktneuronensytem verschiedener Vertebraten. Histochemie 21:322–337

Yamamoto M (1982) Comparative morphology of the peripheral olfactory organ in teleosts. In: Hara TJ (ed) Chemoreception in fishes. Elsevier, Amsterdam, pp 39–59

Yáñez J, Meissl H, Anadón R (1996) Central projections of the parapineal organ of the adult rainbow trout (*Oncorhynchus mykiss*). Cell Tissue Res 285:69–74

Zimmermann H (1972) Ultrastrukturelle und cytochemische Untersuchungen am Saccus vasculosus von Knochenfischen unter besonderer Berücksichtigung der Innervation. Z Zellforsch 126:240–260

Zimmermann H, Altner H (1970) Zur Charakterisierung neuronaler und gliöser Elemente im Epithel des Saccus vasculosus von Knochenfischen. Z Zellforsch 111:106–126