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Differential expression of five prosomatostatin genes in the central nervous system of the catshark *Scyliorhinus canicula*

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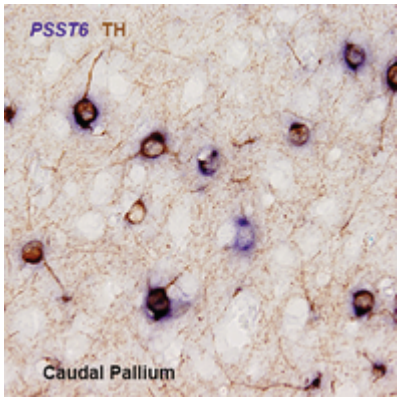
Abstract

Five prosomatostatin genes (*PSST1*, *PSST2*, *PSST3*, *PSST5*, and *PSST6*) have been recently identified in elasmobranchs (Tostivint et al., General and Comparative Endocrinology, 2019, 279, 139–147). In order to gain insight into the contribution of each somatostatin to specific nervous systems circuits and behaviors in this important jawed vertebrate group, we studied the distribution of neurons expressing *PSST* mRNAs in the central nervous system (CNS) of *Scyliorhinus canicula* using in situ hybridization. Additionally, we combined in situ hybridization with tyrosine hydroxylase (TH) immunochemistry for better characterization of *PSST1* and *PSST6* expressing populations. We observed differential expression of *PSST1* and *PSST6*, which are the most widely expressed *PSST* transcripts, in cell populations of many CNS regions, including the pallium, subpallium, hypothalamus, diencephalon, optic tectum, midbrain tegmentum, and rhombencephalon. Interestingly, numerous small pallial neurons express *PSST1* and *PSST6*, although in different populations judging from the colocalization of TH immunoreactivity and *PSST6* expression but not with *PSST1*. We observed expression of *PSST1* in cerebrospinal fluid-contacting (CSF-c) neurons of the hypothalamic paraventricular organ and the central canal of the spinal cord. Unlike *PSST1* and *PSST6*, *PSST2*, and *PSST3* are only expressed in cells of the hypothalamus and in some hindbrain lateral reticular neurons, and *PSST5* in cells of the region of the entopeduncular nucleus. Comparative data of brain expression of *PSST* genes indicate that the somatostatinergic system of sharks is the most complex reported in any fish.

Graphical Abstract

We studied the distribution of neurons expressing the five prosomatostatins (*PSSTs*) transcripts in the brain of the catshark using in situ hybridization. Differential expression of *PSST1* and *PSST6* was observed in cell populations of many brain regions, including the pallium. Combination of in situ hybridization with tyrosine hydroxylase (TH) immunochemistry showed wide colocalization in *PSST6* cells of the pallium, but not in most other brain regions. *PSST1* cells do not TH coexpression in pallial cells, indicating that they represent different populations.



Keywords: brain; differential gene expression; elasmobranchs; shark; somatostatin precursors; spinal cord

Supplementary Table 1 Expression of *PSST* transcripts in cells of the major brain components and rostral spinal cord of juvenile catshark.

The correspondence of juvenile territories according the current prosomeric model and the nomenclature of Smeets, Nieuwenhuys, and Roberts (1983) is depicted. For abbreviations, see list. –, no expression; +, some expression; ++, abundant expression.

*Ramón Anadón and Antón Barreiro-Iglesias should be considered joint senior authors.

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1 INTRODUCTION

Somatostatin (SST; also known as somatotropin release inhibiting factor; Brazeau, Rivier, Vale, & Guillemin, 1974) is a small peptide of 14 amino acids expressed in many brain neuronal populations, as shown in immunohistochemical studies in rats (Vincent, McIntosh, Buchan, & Brown, 1985). Several isoforms of SST of either 14 amino acids (SST-14) or 28 amino acids (SST-28) have been purified and sequenced from different vertebrates and used for physiological studies showing that SSTs play a key role in the regulation of growth, development and metabolism (see Günther et al., 2018). More recently, different genes encoding for SST-related peptides have been cloned from various vertebrate species revealing an unexpected diversity of this family. The evolution of the *SST* precursor (*PSST*) gene family has been strongly shaped by the different rounds of whole-genome duplications that took place in vertebrate history, especially the two basal rounds of vertebrate genome doubling (2R) (Liu et al., 2010; Tostivint et al., 2014; Tostivint, Gaillard, Mazan, & Pézeron, 2019; Tostivint, Joly, Lihrmann, Ekker, & Vaudry, 2004; Tostivint, Quan, Bougerol, Kenigfest, & Lihrmann, 2013). The current model of evolution of the PSST family proposes that the gnathostome ancestor already possessed five *PSST* genes, namely *PSST1*, *PSST2*, *PSST3*, *PSST5*, and *PSST6* (Tostivint et al., 2019). According to this model, *PSST1*, *PSST2*, and *PSST5* arose from 2R while *PSST3* and PSST6 arose subsequently by local duplication of *PSST1* and *PSST2* respectively. However, the view that *PSST6* emerged through 2R

and *PSST2* appeared later cannot be excluded because, between *PSST2* and *PSST6*, it is not yet known which of the two genes is ancestral. The origin of *PSST4*, which is only known in actinopterygians, is still unclear, even though this gene is generally thought to arose from the teleost fish-specific genome doubling (3R) (Liu et al., 2010). The distribution in the brain of the different *PSST* mRNAs has been investigated in various jawed vertebrates including rat (De Lecea et al., 1997; Fitzpatrick-McElligott, Card, Lewis, & Baldino Jr., 1988), chicken (Trabucchi et al., 2003), frog (Tostivint et al., 1996), lungfish (Trabucchi et al., 1999), goldfish (Canosa, Cerdá-Reverter, & Peter, 2004), and sturgeon (Trabucchi et al., 2002) (see Table 1).

Table 1 Main studies on *PSST* mRNA distribution in the brain of vertebrates

	PSST1	***PSST2***	***PSST3***	***PSST4***	***PSST5***	***PSST6***
Rattus norvegicus (mammals)	Somatostatin Fitzpatrick-McElligott et al., 1988	Cortistatin De Lecea et al., 1997	o	o	o	o
Gallus gallus (birds)	SS1 Trabucchi et al., 2003	o	o	o	o	SS2 Trabucchi et al., 2003
*Pelophylax ridibundus** (amphibians)	SS1 Tostivint et al., 1996	SS2 Tostivint et al., 1996	?	o	?	?
Protopterus annectens (lungfishes)	SS1 Trabucchi et al., 1999	?	?	o	?	SS2 Trabucchi et al., 1999
Cyprinus auratus (teleosts)	SS-I Canosa et al., 2004	SS-III Canosa et al., 2004	SS-II Canosa et al., 2004	NS	NS	NS
Acipenser transmontanus (chondrosteans)	SS1 Trabucchi et al., 2002	?	?	?	?	SS2 Trabucchi et al., 2002

Note: For each study the original name of the *PSST* paralog used by the authors of the study is given. o, absent; NS, no study;?, undetermined; *Previously called *Rana ridibunda*.

Elasmobranchs (sharks, rays, and skates) are cartilaginous fishes with articulated jaws that diverged over 400 million years ago from the Holocephali (chimaeras), the chondrichthyan group with nonarticulated jaws. Because of their phylogenetic position as a sister group of Osteichthyes (which contain all established vertebrate model organisms), cartilaginous fishes are a key group for understanding the changes that occurred at the agnathan-gnathostome transition. In sharks, the distribution of SST-like-immunoreactive (-ir) structures has been described in the brain of the leopard shark *Triakis scyllia* (Nozaki, Tsukahara, & Kobayashi, 1984) and the gummy shark *Mustelus manazo* (Chiba, Honma, Ito, & Homma, 1989). Other studies in elasmobranches have also reported the presence of SST-like-ir fibers in the spinal cord (Anadón, Molist, Pombal, Rodríguez-Moldes, & Rodicio, 1995; Cameron, Plenderleith, & Snow, 1990) and cerebellum (Alvarez-Otero, Perez, Rodriguez, & Anadón, 1996), and cells and fibers in the hypothalamus (Meurling & Rodríguez, 1990; Molist, Rodriguez-Moldes, & Anadon, 1992). Moreover, in *Squalus acanthias*, SST-like immunoreactivity occurred in peripheral nerve fibers and endocrine cells of the gut (El-Salhy, 1984; Holmgren & Nilsson, 1983). Most physiological studies on SST in elasmobranchs were centered on its action in the regulation of salt secretion by the rectal gland (Stoff, Rosa, Hallac, Silva, & Epstein, 1979). More recently, studies on the somatostatinergic system in elasmobranchs have been mainly directed to the identification and sequencing of *PSST* cDNAs in different species, including the catshark (also called lesser-spotted dogfish) *Scyliorhinus canicula* (Quan, Kenigfest, Mazan, & Tostivint, 2013; Tostivint et al., 2019). The catshark is considered as a model of choice in evolutionary developmental biology mainly because it is relatively easy to maintain in captivity and to have access to its eggs and embryos, which are reasonably easy to manipulate for functional experiments, and because its protracted development makes it advantageous for studying all developmental events in detail (reviewed in Coolen et al., 2009). The catshark has also been the subject of numerous neurochemical and neuroanatomical studies (reviewed in Rodríguez-Moldes, Santos-Durán, Pose-Méndez, Quintana-Urzainqui, & Candal, 2017). The three cDNAs firstly identified in *S. canicula* were named *ScPSSa*, *ScPSSb*, and *ScPSSc* (Quan et al., 2013). More recently, Tostivint et al. (2019) identified two new *PSST* cDNAs in this species and clarified the phylogeny of the five *PSSTs* showing the presence of *PSST1* (previously known in catshark as *ScPSSTa*), *PSST2*, *PSST3*, *PSST5* (previously known in catshark as *ScPSSb*) and *PSST6* (previously known in catshark as *ScPSSTc*) in cartilaginous fishes. The putative mature SST1 and SST5 peptides share the amino acid sequence (AGCKNFFWKTFTSC) with the mammalian SST-14. The putative amino acid sequence of the SST6 mature peptide (APCKNFFWKTFTSC) differs in Position 2 from the mammalian SST (Quan et al., 2013; Tostivint et al., 2019); whereas, the putative amino acid sequences of the catshark SST2 (TPCKLFFWKTFSHC) and SST3 (NCKNFFWKTYTLC) mature peptides show more differences in their sequence.

The contribution of specific SSTs to central nervous system (CNS) circuits and behaviors of sharks is completely unknown. To gain insight into the expression of the somatostatinergic system of cartilaginous fishes, we studied the transcript distribution of the *PSST* paralogs in the CNS of juvenile catshark using in situ hybridization. Our results reveal that different *PSST* transcripts are expressed differently in various neuronal populations in this species. These results indicate that the five *PSSTs* are involved in different neuronal circuits and that they have different roles and effects in the CNS. Our study provides a neuroanatomical basis for future functional work on the roles of different SSTs in the CNS of sharks.

2 MATERIAL AND METHODS

2.1 Animals

Juveniles (n = 7; between 12 and 19.5 cm in total length) of the catshark *S. canicula* were used for cDNA cloning and in situ hybridization experiments. Animals were kindly provided by the Aquarium of *O Grove* (Pontevedra, Spain). Before experimental procedures, fishes were maintained in fresh seawater tanks in standard conditions of temperature (16–18°C), pH (7.5–8.5), salinity (3.5%), and 12:12 hr day/night cycle. Adequate measures were taken to minimize animal pain or discomfort. All animal experiments were approved by the Bioethics Committee of the University of Santiago de Compostela and the *Xunta de Galicia* and

conformed to the guidelines of ~~the European Communities Council Directive of September 22, 2010 (2010/63/UE) and~~ the Spanish Royal Decree 1386/2018 for the care and handling of animals in research.

2.2 Cloning of the *S. canicula PSST2* cDNA

A juvenile of the catshark *S. canicula* was anesthetized by immersion in 0.1% ethyl 3-aminobenzoate methanesulfonate salt (MS-222; Sigma, St. Louis, MO) and the brain and spinal cord were dissected out under sterile conditions. Total RNA was isolated from these tissues using TriPure (Roche, Mannhein, Germany). The first-strand cDNA synthesis reaction from total RNA was catalyzed with Superscript III reverse transcriptase (Invitrogen, Waltham, MA) using random primers (hexamers; Invitrogen). For polymerase chain reaction (PCR) cloning, specific oligonucleotide primers (sense 5′-TGGCTGGCTTGTTGGAGACT-3′ and antisense 5′-TGTGGGAAGAGAGGGGGCTA-3′) were designed based on the *catshark PSST2* sequence (Tostivint et al., 2019). The amplified fragments were cloned into pGEM-T vectors (Promega, Madison, WI) and used for riboprobe synthesis (see below).

2.3 In situ hybridization

For the generation of the catshark *PSST1*, *PSST3*, *PSST5*, and *PSST6* riboprobes we used a *S. canicula* embryonic cDNA library available in Dr. Mazan's laboratory (see Tostivint et al., 2019). For the generation of the catshark *PSST2* riboprobe we used the *PSST2* clone generated in this study (see above). DIG-labeled riboprobes for shark *PSSTs* were synthesized by in vitro transcription. The length of the riboprobes was as follows: *PSST1*, 600 bases; *PSST2*, 546 bases; *PSST3*, 287 bases; *PSST5*, 753 bases; *PSST6*, 670 bases. The length of the riboprobes covered the full transcript for *PSST1*, *PSST5*, and *PSST6* and most of the transcript for *PSST2* and *PSST3*.

Juvenile catsharks were deeply anesthetized with 0.1% ethyl 3-aminobenzoate methanesulfonate salt (MS-222; Sigma, St. Louis, MO) in seawater before experimental procedures. In situ hybridization experiments were performed as previously described for riboprobes against the sea lamprey serotonin 1a receptor mRNA (Cornide-Petronio, Anadón, Barreiro-Iglesias, & Rodicio, 2013). Briefly, catsharks (n = 6) were perfused intracardially with elasmobranch Ringer's solution (see Ferreiro-Galve, Rodríguez-Moldes, & Candal, 2012) followed by 4% paraformaldehyde in elasmobranch's phosphate buffer (0.1 M phosphate buffer containing 1.75% urea, pH 7.4). Catshark brains and rostral spinal cords were dissected out and postfixed in the same fixative for 48 hr at 4°C. Then, they were cryoprotected with 30% sucrose and sectioned on a cryostat in the transverse plane (14 μm sections). Five parallel series of sections were obtained from each brain/spinal cord block. The sections of each series were incubated with each of the five *PSST* DIG-labeled probes, respectively, at 70°C overnight in hybridization mix and treated with RNAse A (Invitrogen, Waltham, MA) in the posthybridization washes. Then, the sections were incubated with sheep anti-DIG antibodies conjugated to alkaline phosphatase (1:2000; Roche, Mannheim, Germany) overnight. Staining was conducted in BM Purple (Roche) at 37°C until the signal was clearly visible. Finally, the sections were mounted in Mowiol (Calbiochem; Temecula, CA).

2.4 Double in situ hybridization and immunohistochemistry

In some samples, in situ hybridization for *PSST1* and *PSST6* was followed by immunohistochemistry against tyrosine hydroxylase (TH). After the in situ hybridization signal was clearly visible, sections were rinsed twice in 0.05 M Tris-buffered saline (TBS; pH 7.4) and treated with 10% H_2O_2 in TBS for 30 min. For heat-induced epitope retrieval, sections were treated with 0.01 M citrate buffer (pH 6.0) for 30 min at 90°C and allowed to cool for 20–30 min at room temperature (RT) in the same buffer. Then, the sections were incubated in primary antibody solutions overnight at RT. The primary antibody was a mouse monoclonal anti-TH antibody (a mouse monoclonal antibody raised against TH purified from PC12 cells; Millipore, CA; Cat#MAB318; RRID: AB_2201528; dilution 1:1,000; Table 2). After rinsing in TBS, the sections were incubated with the solution containing the secondary antibody (goat antimouse IgG serum HRP conjugated (Dako, Glostrup, Denmark;

Cat# G-21040, RRID: AB_2536527, dilution 1:200; Table 2) for 1 hour at RT. All antibodies were diluted in TBS containing 15% normal serum of goat and 0.2% Triton X-100 (Sigma). The immunoreaction was developed with 0.25 mg/ml diaminobenzidine tetrahydrochloride (DAB; Sigma) containing 0.00075% H_2O_2. Finally, the sections were mounted in Mowiol.

Table 2 Antibodies used in this study

Antigen	Immunogen	Manufacturer, species antibody was raised in, Catalogue #, RRID	Dilution
TH	TH purified from PC12 cells	Millipore, mouse monoclonal, Cat# MAB318, RRID:AB_2201528	1:1,000
Mouse immunoglobulin	Mouse immunoglobin	Dako, goat anti-mouse immunoglobin HRP conjugated, Cat# G-21040, RRID:AB_2536527	1:200

2.5 Primary antibody characterization

The mouse monoclonal TH antiserum was raised against denatured TH from a rat pheochromocytoma. According to the technical information supplied by the manufacturer, it recognizes an epitope on the outside of the regulatory N-terminus and in Western blots it recognizes a protein of ~59–61 kDa. In addition, the antiserum specificity was characterized by Western blot in brain extracts of the catshark, in which it stained a single protein band of about 56–60 kDa (Carrera, Anadón, & Rodríguez-Moldes, 2012). The antibody displays wide species cross-reactivity and has been used to demonstrate the catecholaminergic systems in many species, including the catshark (reviewed in Carrera et al., 2012). The antibody does not recognize TH2 in teleosts, revealing only the TH1-immunoreactive catecholaminergic neurons (Filippi, Mahler, Schweitzer, & Driever, 2010; Yamamoto, Ruuskanen, Wullimann, & Vernier, 2010).

2.6 Imaging

A photomicroscope (Provis AX-70; Olympus, Tokyo, Japan) equipped with a color digital camera (Olympus DP70, Tokyo, Japan) was used to acquire images of the brain and spinal cord sections. Contrast and brightness of photomicrographs were minimally adjusted with Adobe Photoshop CS4 (Adobe Systems, San Jose, CA). Figure plate composition and lettering were generated using Adobe Photoshop and schematic drawings were made using CorelDRAW 12 (Corel, Ottawa, Canada).

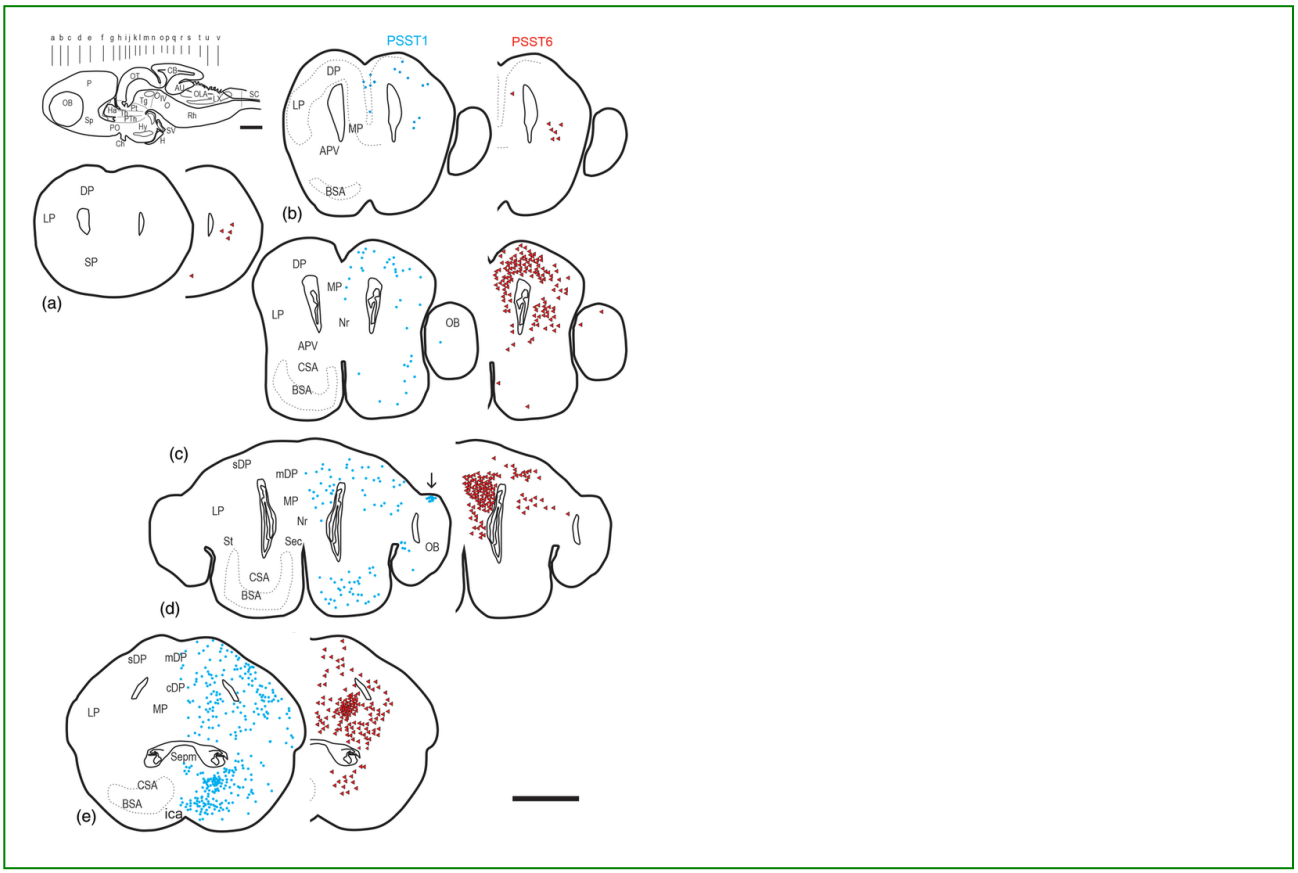
2.7 Nomenclature for brain structures

For description of brain nuclei in the juvenile catshark brain we followed in general the nomenclature of Smeets et al. (1983) adapted to new ideas on the segmental organization of the catshark brain, including changes in the limits between the major brain segments based on developmental and genoarchitectonic studies (Anadón et al., 2000; Carrera et al., 2005; Carrera, Molist, Anadón, & Rodríguez-Moldes, 2008; Carrera, Ferreiro-Galve, Sueiro, Anadón, & Rodríguez-Moldes, 2008; Carrera et al., 2012; Rodríguez-Moldes et al., 2017; see Table S1).

3 RESULTS

In situ hybridization of sections of the brain and rostral spinal cord of juvenile catshark showed differential expression of *PSST* genes. Two of these genes (*PSST1* and *PSST6*) showed wide expression in the brain (Figures 1–6, Table S1), whereas expression of *PSST2*, *PSST3*, and *PSST5* was limited to a few brain structures

(Figures 7 and 8, Table S1). In the rostral spinal cord, only *PSST1* and *PSST2* were expressed. For the description of the *PSST*-expressing neuronal populations, we follow a rostro-caudal sequence. As far as possible, the nomenclature for brain regions is updated taking into account the new studies on the regional brain organization of the catshark (e.g. Rodríguez-Moldes et al., 2017; Table S1). In currently available adult catshark brain maps there are extensive "reticular" areas that lack well-characterized nuclei and that exhibit various somatostatinergic populations. These "reticular" populations will be referred here according to their segmental position, its presumed alar or basal location, and its location relative to other topological references. In order to assess the location of some somatostatinergic populations, we also used double staining with in situ hybridization for *PSST1* or *PSST6* and immunohistochemistry for TH. TH is a general marker of catecholaminergic neuronal populations whose distribution in the brain of elasmobranchs including catshark has been well-characterized (Carrera et al., 2005; Carrera et al., 2012; Northcutt, Reiner, & Karten, 1988; Stuesse, Cruce, & Northcutt, 1994).



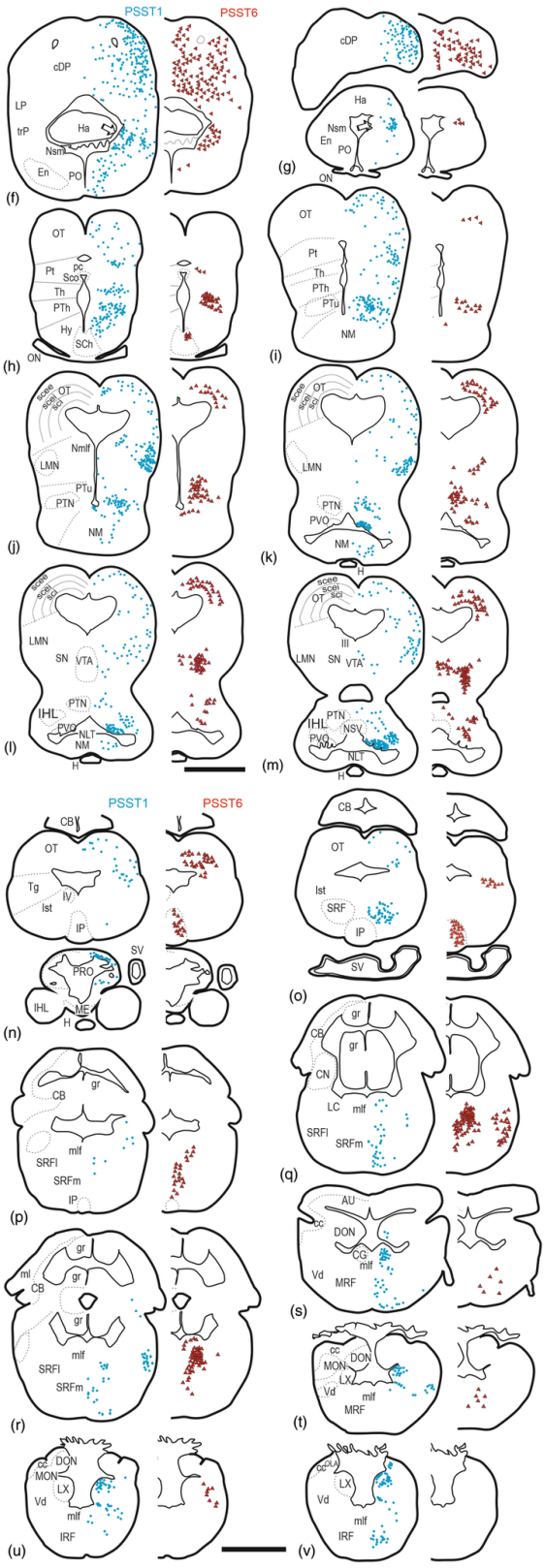


Figure 1 Schematic drawings of parallel transverse sections of a juvenile brain in situ hybridized for *PSST1* or *PSST6* showing the plotted distribution of cells expressing *PSST1* mRNA (dots in the left

figurines) and *PSST6* mRNA (~~black~~ triangles in the right figurines). Each symbol corresponds to a positive cell in a 16 µm-thick section. Anatomical structures are indicated in the left side of the left figurines. For abbreviations, see the list. The arrow in d points to a compact group of *PSST1*+ cells in the olfactory bulb. The level of sections is indicated at the upper left corner in a schematic drawing of a juvenile brain. Scale bars: 1 mm

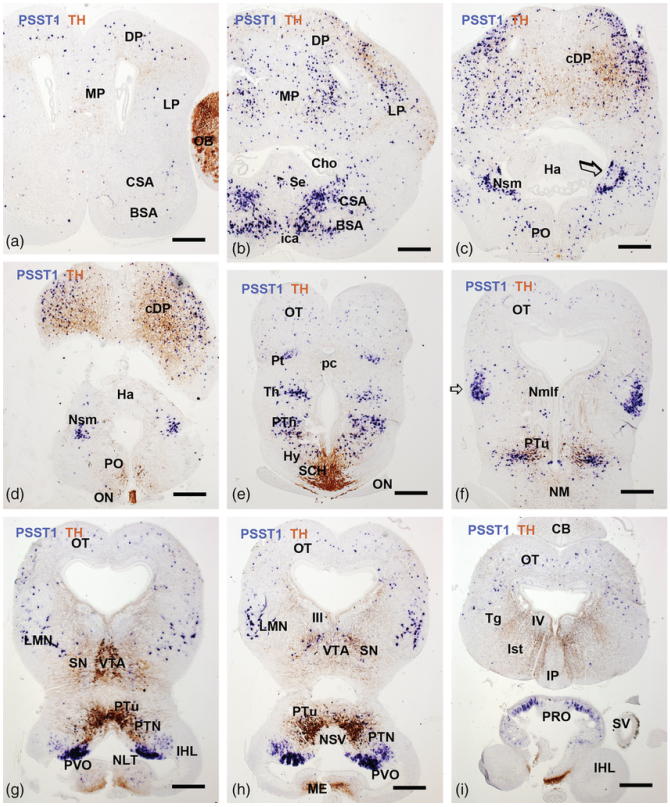


Figure 2 Low magnification photomicrographs of double stained transverse brain sections of a juvenile catshark showing in blue *PSST1* positivity and in brown TH immunoreactivity. These sections correspond to the brain shown schematically at the left side in Figure 1. For abbreviations, see the list. Scale bars, 250 µm

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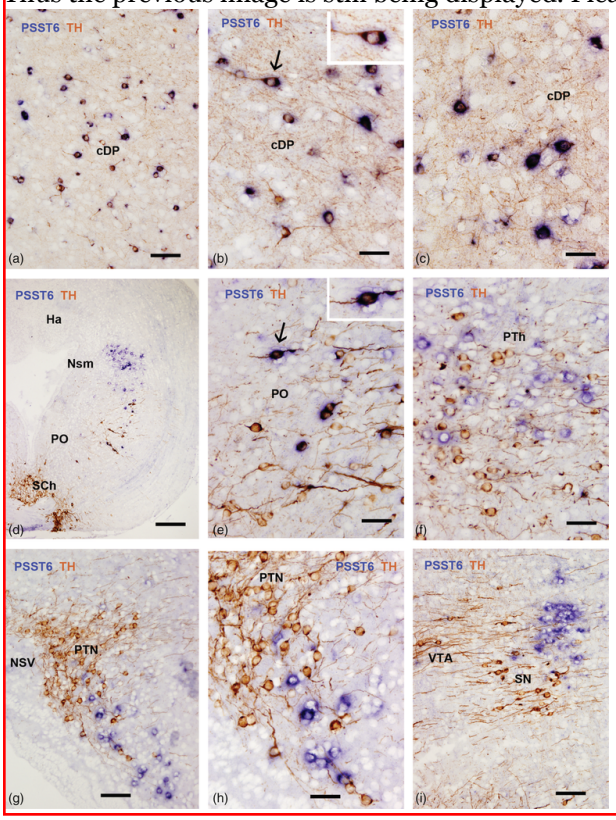

Figure 3 Photomicrographs of double stained transverse brain sections of a juvenile catshark showing *PSST1* positivity in blue and TH immunoreactivity in brown. (a–e) Low magnification photomicrographs of the hindbrain. The level of these brain sections roughly corresponds to that shown schematically at the left side in Figure 1. (f–l) Details of sections showing co-distribution of *PSST1*+ and TH-ir structures. (m,n) Sections of the spinal cord showing *PSST1* in situ hybridization alone (m) or combined with TH-ir staining (n). Inset is a detail of the *PSST1*+ cells around the central canal excepting a ventral sector that only shows TH-ir CSF-c cells (arrows). For abbreviations, see the list. Scale bars, 250 µm (a–e), 100 µm (g–n), 50 µm (f, h, l, inset in m)

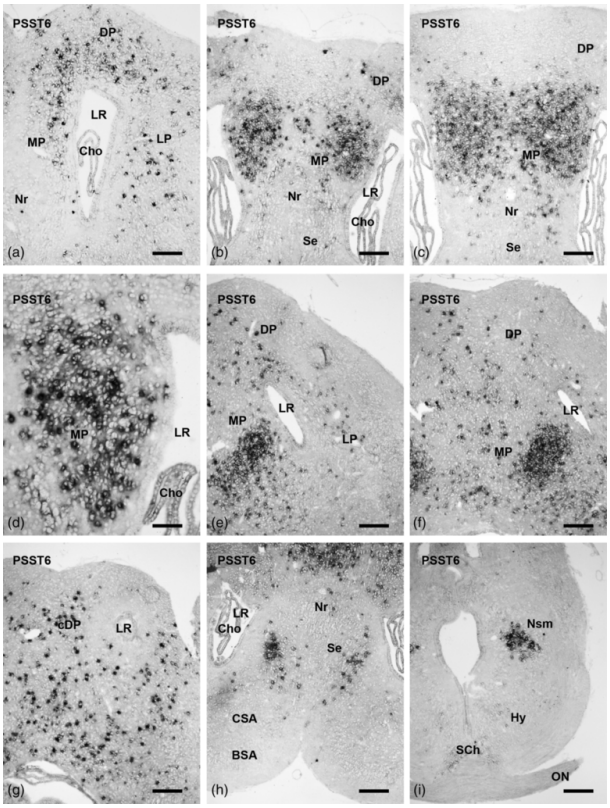

Figure 4 Low magnification photomicrographs of transverse brain sections of a juvenile catshark showing *PSST6* positive cells. These brain sections roughly correspond to the sections shown schematically at the right side in Figure 1. For abbreviations, see the list. Scale bars, 250 μm (a–c, e–i), 100 μm (d)

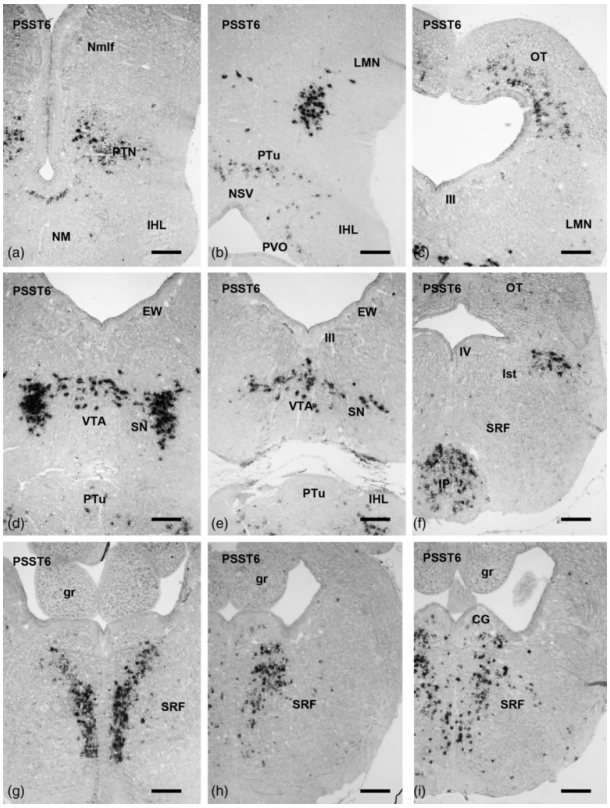

Figure 5 Continuation of Figure 4 showing low magnification photomicrographs of transverse brain sections with *PSST6* positive cells. These sections correspond to those shown schematically at the right side in Figure 1. For abbreviations, see the list. Scale bars, 250 μm

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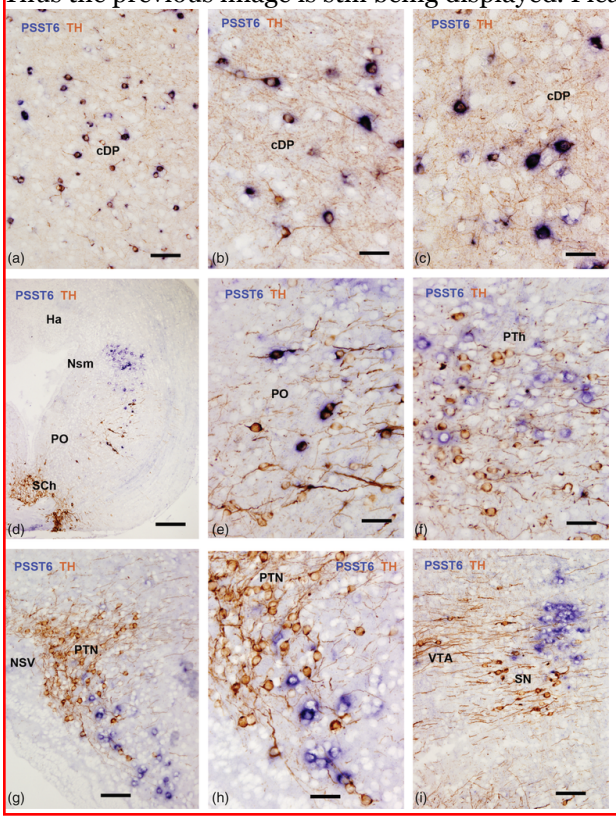

Figure 6 Photomicrographs of transverse brain sections of a juvenile catshark showing colocalization (a–c, e) or co-distribution (d, f–i) of *PSST6*+ and TH-ir structures. Arrows in b and e point to cells magnified in the insets. For abbreviations, see the list. Scale bars, 250 µm (d), 100 µm (a, g, i), 50 µm (b, c, e, f, h)

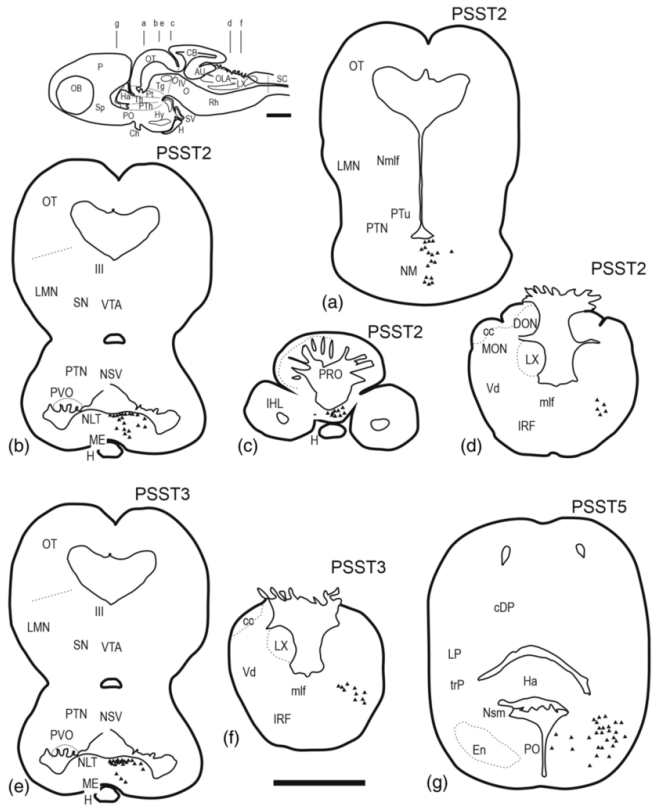

Figure 7 Schematic drawings of sections of juvenile catshark brains hybridized for *PSST2* (a–d), *PSST3* (e–f), and *PSST5* (g) showing the distribution of positive neurons (each black triangle represents a single cell in a 16 µm-thick section). Anatomical structures are indicated in the left side of the left figurines. For abbreviations, see the list. The level of sections is indicated at the upper left corner in a schematic drawing of a juvenile brain. Scale bar: 1 mm

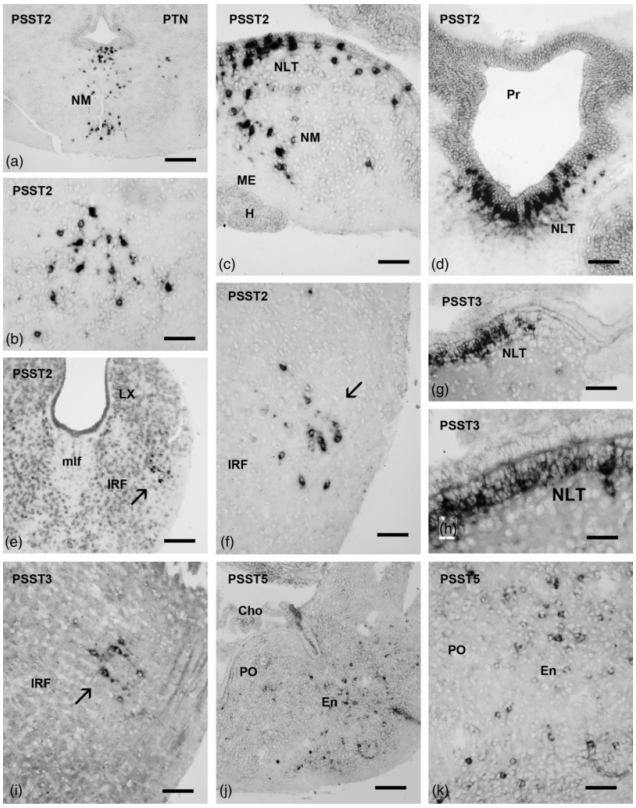

Figure 8 Photomicrographs of transverse brain sections of a juvenile catshark showing *PSST2* positive cells (a–f), *PSST3* positive cells (g–i), and *PSST5* positive cells (j–k). Arrows in e, f, and i point to groups of lateral reticular neurons. These sections correspond to the brains shown schematically in Figure 7. For abbreviations, see the list. Scale bars, 250 μm (a, e, j), 100 μm (b–d, f, g, i, k), 50 μm (h)

3.1 *PSST1* expression

3.1.1 Telencephalon

The telencephalon is considered to be an evaginated alar derivative of the secondary prosencephalon, which also includes the preoptic region and the hypothalamus (Rodríguez-Moldes et al., 2017). The large catshark telencephalon has paired hemispheres that are widely confluent in the midline producing a massive appearance in juveniles and adults (Rodríguez-Moldes et al., 2017; Smeets et al., 1983). The lateral ventricles are reduced at most levels to curved flattened canals that join caudally to the telencephalic ventricle in the impar telencephalic stalk. Big olfactory bulbs protrude laterally from the hemispheres. ~~Caudally, the impar telencephalon joins the preoptic region.~~ The hemispheres are comprised of two main histogenetic subdivisions, the pallium, and the subpallium. The catshark developing pallium may be subdivided in at least four different histogenetic territories: medial, dorsal, lateral, and ventral by using early developmental gene markers (Rodríguez-Moldes et al., 2017). In the subpallium, the putative striatal and pallidal homologs have been defined using patterns of early developmental gene expression in embryos (Quintana-Urzainqui et al., 2012; Rodríguez-Moldes et al., 2017). Based on such studies, the preoptic area was recognized as a subpallial subdomain. Equivalences have also been proposed between these embryonic primordia and adult subpallial territories, although some uncertainties exist. Since gene expression-based correspondence among the pallial and subpallial subregions of adults with those of other vertebrates was not unequivocally established, for the cell masses of juvenile catshark telencephalon we will use classical subdivisions (Manso & Anadón, 1993;

Smeets et al., 1983) based on the topography with respect the ventricles. Equivalences with developmental data will be also mentioned when appropriate (see Table S1).

The most anterior region of the catshark pallium, which borders rostrally the lateral ventricles, lacks *PSST1* positive (+) cells. Caudally, small *PSST1*+ cells appear progressively mainly in intermediate regions of the pallium (dorsal, medial, and lateral to the ventricle). In intermediate and caudal regions these cells appear distributed from periventricular to superficial layers, and *PSST1*+ cells are particularly abundant in the most caudolateral region of the pallium (dorsal pallium of Smeets et al., 1983) (Figures 1a–g, 2a–d). The pallium shows a fairly abundant population of small TH-immunoreactive (TH-ir) cells that are *PSST1* negative (Figures 2b–d, 3f).

In the rostral subpallium, at levels showing the thick cell band of the conspicuous basal superficial area (BSA), small and faint *PSST1*+ cells are located scattered among negative cells as well as in the adjacent outer and inner cell and neuropil areas. These BSA cells are scarce rostrally but their number increases notably toward caudal regions (Figures 1c–e, 2a–b). Caudally, numerous intense *PSST1*+ cells are distributed in the wide basal central area of cells and neuropil between the BSA and the ventricles (Figures 1e, 2b). These larger cells are conspicuous in caudal levels near or within the BSA. The transition of the BSA to the interstitial nucleus of the anterior commissure is marked by the appearance of a population of small TH-ir cells in this nucleus and the caudal septum, whereas the BSA is free of these cells along with its rostrocaudal extension. Scattered *PSST1*+ cells were observed in the caudal septum of Smeets et al. (1983); whereas, abundant *PSST1*+ cells were observed in the region referred as posterior medial septum by these authors (Figures 1e, 2b). Ventromedial to the caudal olfactory bulb and lateral to the BSA there is a region of scattered cells named as striatum by Smeets et al. (1983). This ill-defined striatal region shows scarce *PSST1*+ cells compared with the BSA and adjacent areas.

At the transition with the telencephalic peduncle, a dense group of small *PSST1*+ cells is observed along the region of the stria medullaris and in a small protuberance close to the insertion of the anterior medullary velum (Figures 1f–g, 2c,d, arrowed). This group ends close to the habenula and may be considered a part of the prethalamic eminence (the nucleus of the stria medullaris). The interstitial nucleus of the anterior commissure shows numerous small *PSST1*+ cells (Figures 1e, 2b). This population ends in the rostral preoptic region.

In the olfactory bulbs, a large population of small TH-ir cells is observed close and between the negative large mitral cells (Figures 2a, 3g) and processes of these TH-ir cells help to visualize the large olfactory glomeruli (not shown). In addition to these TH-ir periglomerular cells, scattered TH-ir cells are observed in the inner granular layer and periventricular regions of the olfactory bulbs (see Carrera et al., 2012). In contrast to TH-ir cells, only a few *PSST1*+ cells are observed per section in the OB, mostly in the inner granular layer and in caudal regions of the OB. Interestingly, a small compact group of *PSST1*+ neurons was observed in the dorsal outer border of the glomerular layer, which lacks TH staining (Figures 1d, 3g).

3.1.2 Preoptic region and hypothalamus

Here we consider as the preoptic region the territory of cells and fibers that extends laterally to the preoptic recess and rostral to the optic chiasm. This region joins the ~~subpallium dorsally and the~~ alar hypothalamus ventrally (in a topological view). At the level of the optic chiasm a dense population of TH-ir cells forms part of a prominent suprachiasmatic nucleus (alar hypothalamus) (Figure 2d,e). A few *PSST1*+ cells are observed in preoptic and suprachiasmatic lateral regions, whereas, more numerous TH-ir neurons are located in the periventricular regions (Figure 2d,e).

In the hypothalamus, strong *PSST1*+ expression was observed in numerous cells of the hypothalamic circumventricular (vascular) organs. These structures are located in the walls of ventral infundibular recess and extend from levels just rostral to the lateral hypothalamic recesses, invading there a band of small recesses extending laterally in its dorsal wall (hypothalamic paraventricular organ, PVO) and reaching the posterior recess walls, where they form part of the posterior recess organ (PRO) (Figures 1k–n, 2g–i, 3j). Judging from the bipolar appearance of positive cells and their location inside the ependymal layer (see Figure 3j), most of

them probably correspond to cerebrospinal fluid-contacting (CSF-c) cells. In the PRO, the distribution of these *PSST1*+ cells is sparser than in the lateral recesses. Accompanying these circumventricular organs, there is a population of faint *PSST1*+ cells, probably non-CSF-contacting, that extend laterodorsal to the diverticles of the lateral recess (Figures 1l,m, 2 g,h). In the median hypothalamic nucleus, below the lateral tuberal nucleus of Smeets et al. (1983), there is a sparse population of faint *PSST1*+ cells (Figures 1k–l, 2f).

3.1.3 Diencephalon

The catshark diencephalon is a region intermediate between the secondary prosencephalon (telencephalon plus hypothalamus) and the mesencephalon. According to the evolving prosomeric model (Puelles & Rubenstein, 2003), the embryonic vertebrate diencephalon is formed by three transverse brain segments or prosomeres p1–p3 (from caudal to rostral), whose main alar derivatives are the pretectum, the thalamus (formerly named dorsal thalamus) plus the habenula and the prethalamus (formerly the ventral thalamus), respectively. Main basal derivatives of these prosomeres include the posterior tubercle and the nucleus of the medial longitudinal fascicle.

In the prethalamus-posterior tubercle (p3), a group of *PSST1*+ cells extends in lateral regions (Figures 1h–m, 2e,f, 3h–j). This group is more compact dorsally than in the posterior tubercle. Except in most rostral (dorsal) prethalamic regions, cells of this *PSST1*+ subpopulation are intermingled with tubercular TH-ir cells, but colocalization of both substances was not observed (Figure 3h–j). Limits with dorsal hypothalamic *PSST1*+ populations are not sharp, but the diencephalo-hypothalamic external sulcus (also named sulcus thalamohypothalamicus, Smeets et al., 1983) can be used as a reference.

In the thalamus, there is an intermediate population of *PSST1*+ cells between the periventricular cell band and the meninges. This population is more condensed rostrally and becomes scattered caudally (Figures 1h,i, 2e).

In the most dorsal pretectal region, a compact group of *PSST1*+ cells is observed laterally among the fibers of the posterior commissure tract (Figures 1h, 2e). Caudally this group extends toward the lateral surface below the optic tectum and expands in a bulge of the superficial pretectum (Figure 2f, arrowed), which shows a large population of small *PSST1*+ cells. Probably this bulge corresponds to the region formerly named “nucleus geniculatus lateralis” (Smeets et al., 1983). In addition to this superficial group, small *PSST1*+ cells are observed scattered between this group and the caudal periventricular region.

3.1.4 Mesencephalon (midbrain)

The catshark midbrain is easily identifiable by the presence of a characteristically layered optic tectum dorsally (Figure 1h–o) and a wide tegmental region ventrally. The midbrain contains a ventricle, showing a heart-like appearance at middle levels. Whereas the dorsal limits with the pretectum rostrally and the anterior medullary velum caudally are clear in transverse sections, the limits of the midbrain tegmentum with the diencephalon and the isthmus and the ascription of some somatostatinergic populations are more difficult to recognize, especially in lateral regions.

The catshark optic tectum has been subdivided into six layers of neurons and fibers, named from the meningeal to the ependymal layer as stratum medullare externum (optic fiber layer), stratum cellulare externum zona externa, stratum cellulare externum zona interna, stratum medullare internum, stratum cellulare internum, and stratum periventriculare (Manso & Anadon, 1991; Smeets et al., 1983). Numerous faintly stained *PSST1*+ small cells are scattered mainly through the stratum cellulare externum zona externa, but sparse *PSST1*+ cells were also observed in the stratum cellulare externum zona interna and occasional cells in the stratum cellulare internum (Figures 1h–o, 2e–i).

In the midbrain tegmentum, the most conspicuous *PSST1*+ population was observed in the lateral mesencephalic nucleus (Figures 1j–l, 2g,h). It consists of strongly stained cells located laterally near the

meninges, and ventrally to the optic tectum. Faint *PSST1*+ cells were also observed intermingled with TH-ir neurons in the ventral tegmental area and the substantia nigra (Figures 1m, 2g,h), but they do not form a conspicuous group as that of *PSST6*+ cells (see below). Faint *PSST1*+ small cells were also observed scattered at the transition between the optic tectum and the tegmentum.

3.1.5 Rhombencephalon (hindbrain)

Just caudal to the border with the midbrain tegmental region, a conspicuous population of strongly *PSST1*+ reticular cells appears in intermedio-lateral regions of the superior reticular formation at the level of the interpeduncular nucleus, which lacks *PSST1*+ cells (Figures 1o, 3a,k). This conspicuous *PSST1*+ population disappears at intermediate rostrocaudal levels of the interpeduncular nucleus.

More caudally, at the level of the locus coeruleus (which contains TH-ir cells; Carrera et al., 2012), groups of small *PSST1*+ cells were observed extending in a vertical band near the midline between the central gray and the ventral surface, and a few cells also were present in the central gray (Figures 1q, 3b,c), probably forming part of the raphe and the superior reticular formation, which also contains serotonergic neurons (Carrera, Molist, et al., 2008). More caudally, some ventral cells of these groups extend laterally forming a sparse band parallel to the meninges (Figure 1r–s) appearing as migrating/migrated cells similarly to reticular serotoninergic neurons of the catshark superior reticular formation (Carrera, Molist, et al., 2008). Caudally, the *PSST1*+ vertical band diminishes gradually its number of cells. At the level of the trigeminal nerve entrance, a group of strongly *PSST1*+ cells appears over the medial longitudinal fasciculus. Both populations extend caudally until the level of the octaval nerve entrance.

At the level of the facial nerve entrance, *PSST1*+ cells appear around the solitary fascicle, which occupies a small longitudinal protuberance just dorsal to the sulcus limitans that is continuous with the vagal lobe. The number of *PSST1*+ cells is small before the lobe but increases notably in the vagal lobe (Figures 1t–v, 3d,e,l). In this lobe, the distribution of *PSST1*+ cells, as that of TH-ir cells observed in this region (Carrera et al., 2012; present results) is dorsal, with approximately the ventral half of the lobe free of these cells (Figure 3l). Double staining reveals that *PSST1*+ and TH-ir cells represent different populations. A conspicuous band of strong *PSST1*+ reticular cells extending ventrolaterally from the sulcus limitans region accompanies most of the extension of the solitary tract-vagal lobe (Figure 1t–v).

At caudal hindbrain levels, a conspicuous population of large *PSST1*+ reticular neurons is observed in the inferior reticular formation (Figures 1v, 3e). These cells show bipolar or triangular appearance, with one or more stained dendritic shafts. A few *PSST1*+ cells were observed in the caudal region of the octavolateralis area (OLA, Figure 1v) and a lateral group of the rostral OLA (Figure 3c).

3.1.6 Spinal cord

In the rostral spinal cord, a conspicuous population of *PSST1*+ neurons was observed around the central canal except in its ventral side (Figure 3m). The position and location is compatible with that of CSF-c neurons of the central canal. Double staining reveals that CSF-c TH-ir cells occupy the ventral region of the central canal free of *PSST1*+ neurons (Figure 3n). In addition to putative CSF-c cells, larger *PSST1*+ cells were observed scattered in the dorsal and ventral horns (Figure 3m,n).

3.2 *PSST6* expression

3.2.1 Telencephalon

As indicated above for *PSST1*+ cells, *PSST6*+ cells are occasional or lacking in the rostral pallium. More caudally, fairly abundant *PSST6*+ cells appear in medial, dorsal and lateral regions of the pallium

(Figures 1c–g, 4a–g). In the medial pallium, a dense population of intensely stained *PSST6*+ cells appears in a large area over the neuroporic recess (Figure 4b–f). Caudally this pallial population expands considerably and becomes continuous with paired condensations of intensely stained *PSST6*+ cells that are surrounded by less dense *PSST6*+ cells. No similar condensation of *PSST6*+ cells is seen in the dorsal and lateral regions of the pallium, which exhibit much lower density and fainter staining of *PSST6*+ cells. The olfactory bulbs show occasional *PSST6*+ cells (Figure 1c).

Double staining with *PSST6* in situ hybridization and TH immunohistochemistry revealed that a high proportion of caudal pallial *PSST6*+ cells were also TH-ir (Figure 6a–c). In these cells, blue *PSST6* positivity was distributed in the perikarya around the cell nucleus, whereas brown TH immunoreactivity was observed both in the perikaryon and cell processes. In a sample of 259 cells of the caudal pallium labeled by *PSST6* and/or TH, 179 cells (69.11%) were double-labeled, 9 cells (3.47%) were singly *PSST6* positive and 71 cells (27.41%) were singly TH-ir. In this sample, double-labeled cells represent 95.21% of the *PSST6*+ cells. This high degree of colocalization with TH in the caudal pallium also indicates that these *PSST6*+/TH-ir neurons are different from *PSST1*+ pallial neurons of this region, which were all TH-negative.

Scarce expression of *PSST6* was observed in the subpallium of juvenile catsharks. A small paired compact group of *PSST6*+ cells was observed in the medial septum (Figure 4h), from which sparse cells extend caudolaterally. In the BSA, only occasional *PSST6*+ cells were observed (Figure 1b–e), in sharp contrast with the distribution of *PSST1*+ cells. Subpallial *PSST6*+ cells did not show TH-ir immunoreactivity in double staining experiments. At the transition with the telencephalic peduncle, a compact ovoid group of small *PSST6*+ cells is observed forming a small protuberance close to the insertion of the anterior medullary velum (Figures 1f,g, 4i). This group is in the nucleus of the stria medullaris (Figure 6d) and ends close to the habenula. It may be considered part of the prethalamic eminence. The interstitial nucleus of the anterior commissure only shows few and pale *PSST6*+ cells (not shown).

3.2.2 Preoptic region and hypothalamus

The preoptic region of juvenile dogfish mostly lacks *PSST6*+ neurons, and only a few faintly stained neurons were observed in the lateral area at the level of the suprachiasmatic nucleus (Figure 1h). A few double stained *PSST6*+/TH-ir cells were observed in this lateral preoptic/suprachiasmatic area among abundant TH-ir cells (Figure 6e).

PSST6+ neurons are scattered in areas dorsal to the lateral and posterior hypothalamic recesses that form part of the hypothalamic PVO (Figure 1k–m), but positive cells are not present in this organ or in the median hypothalamic nucleus and lateral tuberal nucleus located over the median eminence. TH-ir cells were observed in some hypothalamic nuclei, sometimes aside or intermingled with *PSST6*+ neurons (Figure 6f–h), but no colocalization of TH with *PSST6* signal was observed.

3.2.3 Diencephalon

The main *PSST6*+ neuronal population observed in the dogfish diencephalon extends as a conspicuous cell band in intermediate mediolateral levels of the prethalamus and posterior tubercle (p3) (Figures 1h–k, 5a, 6f). In the posterior tubercle at the level of the nucleus of the saccus vasculosus (NSV) (see Molist et al., 1992), *PSST6*+ cells form conspicuous groups lateral to it (Figures 1m, 5b). Numerous prethalamic/posterior tubercular (p3) cells showed TH-ir immunoreactivity, but colocalization with *PSST6* was not observed in these cells. In the habenula, a small group of faint *PSST6*+ cells was observed near the lateral border (Figure 1g), near the nucleus of the stria medullaris, but the thalamus (p2) lacked *PSST6*+ populations. Additionally, a few *PSST6*+ cells were observed in the nucleus of the medial longitudinal fascicle (p1) in periventricular location (Figure 1j). These cells were not TH-ir.

3.2.4 Midbrain

In the catshark optic tectum, abundant *PSST6*+ neurons were observed in the stratum cellulare externum zona interna and in lateral regions of the stratum cellulare internum (Figures 1i–n, 5c), which is in contrast with the distribution of *PSST1*+ cells mainly in the stratum cellulare externum zona externa (see above). Owing the hemispheric shape of the tectum, this is best appreciable in sections passing perpendicularly through the tectal layers (Figure 1j–m).

In the mesencephalic tegmentum a paired compact *PSST6*+ reticular nucleus is located in an intermediate region lateral to the oculomotor nerve, and more scattered *PSST6*+ cells also extend between the two nuclei in the midline (Figures 1l,m, 5d,e). These reticular populations disappear at rostral isthmic levels caudally, and at the limit with the tegmental pretectal region rostrally. In double-stained sections for TH and *PSST6* (see above) these *PSST6*+ populations are co-distributed with the dopaminergic populations of the substantia nigra and ventral tegmental area, respectively (Figure 6i). However, colocalization of *PSST6* and TH was not observed in any cell, unlike in the pallium and the preoptic/prethalamic populations. A similar SST-like-ir population of the midbrain tegmentum of the gummy shark was named red nucleus by Chiba et al. (1989).

3.2.5 Rhombencephalon

The transition between the basal regions of the midbrain and the isthmus is marked by the appearance of the interpeduncular nucleus (isthmic), which is located just caudal to the oculomotor nerve exit, and the end of the substantia nigra/ventral tegmental area TH-ir populations. A rich population of *PSST6*+ neurons was observed in the dorsal and caudal regions of the interpeduncular nucleus, excepting the ventral interpeduncular neuropil (Figures 1n,o, 5f). Just caudal to this nucleus, a paired band rich in *PSST6*+ cells of the superior reticular formation ascend dorsally parallel to the raphe and the medial longitudinal fascicle and reach a more massive population of *PSST6*+ cells located below the rhombencephalic central gray (Figures 1p–r, 5g,h). Faint scattered *PSST6*+ cells are also observed ventral to the medial isthmic *PSST6*+ population. The *PSST6*+ medial reticular populations extend caudally in the trigeminal region (rhombomeres 2–3) until the level of entrance of the octaval nerve (Figure 1s). At these levels, the central gray also contains a small population of *PSST6*+ neurons (Figures 1r, 5i). A small lateral reticular population of *PSST6*+ cells that is separated from the meninges by the spinocerebellar tract is also observed (Figure 1q), but whether it is located in isthmic or trigeminal levels was not assessed. Caudally to the octaval nerve entrance, faintly stained *PSST6*+ cells were scattered in intermediate reticular areas (Figure 1t). At the level of the vagal lobe, scarce *PSST6*+ reticular cells were observed in an area between this lobe, the caudal octavolateralis nucleus, and the descending trigeminal nucleus (Figure 1u). Near the obex, reticular *PSST6*+ cells were also observed below the commissural nucleus and spinal trigeminal nucleus, extending to the transition with the spinal cord (not shown). No *PSST6*+ cells were observed in the spinal cord.

3.3 *PSST2* expression

Neurons expressing *PSST2* mRNA were observed in the median hypothalamic and lateral tuberal nuclei, the ventrolateral hindbrain (Figure 7a–d) and the dorsal horn of the rostral spinal cord. Hypothalamic *PSST2*+ cells of the median hypothalamic nucleus extend from levels rostral to the origin of the lateral recesses of the third ventricle until the transition between the NM and the neurohypophysis (Figures 7a–c, 8a–d). In rostral and intermediate levels positive cells are scattered near the midline between the third ventricle and the meninges (Figure 8a,b). At levels in which the NM exhibits the shape of a paired bulge, numerous *PSST2*+ cells appear grouped in the dense periventricular cell layer of the lateral tuberal nucleus, whereas the more ventral cells disappear gradually (Figure 8c,d). The rostro-caudal extension of the periventricular population coincides with the extension of the adenohypophysis that lies just ventral to the bed of capillaries of the median eminence in juveniles.

In the caudal hindbrain there is a population of *PSST2*+ reticular neurons that extends rostro-caudally between the levels of the entrance of the glossopharyngeal nerve and a postobecular level. These small cells form a small group located in a ventrolateral reticular area below the vagal sensory lobe (Figures 7d, 8e,f).

At rostral spinal levels, some *PSST2*+ cells are scattered in the dorsal horn over the Stieda's fasciculus medianus (direct vestibulo-spinal tract of elasmobranchs, which is located laterally to the central canal) or just below it. No positive cells located around the central canal were observed (not shown).

3.4 *PSST3* expression

Two *PSST3*-expressing neuronal populations were observed in the juvenile brain, one in the NM/lateral tuberal nucleus (NLT) and the other in the caudal hindbrain (Figures 7e–f), a pattern that reminds that reported above for the *PSST2*+ brain populations. However, no cells expressing this transcript were observed in the rostral spinal cord. Neurons of the NM/NLT are mostly located in the thick periventricular cell layer below the ependyma (Figure 8g,h), but positive cells were also seen among the cells scattered within the neuropil below the dense periventricular layer. The NM/NLT lies over the plexuses of capillaries of the median eminence (Mellinger, 1964). The second *PSST3*+ population is located near the meninges in the caudal hindbrain and consists of small reticular neurons in a ventrolateral region at the level of the glossopharyngeal nerve entrance (Figure 7f, 8i). These small reticular cells have a bipolar appearance showing radial orientation.

3.5 *PSST5* expression

In the transition between the impar telencephalon and the preoptic region there is a population with numerous *PSST5*+ neurons that are scattered in a wide lateral region corresponding to the entopeduncular nucleus (Figures 7g, 8j-k). Medially, only a few scattered *PSST5*+ cells were observed in the preoptic nucleus at the level of the preoptic recess. The magnocellular preoptic nucleus of catshark has been characterized immunohistochemically in this region (Meurling, Rodríguez, Peña, Grondona, & Pérez, 1996). The other brain regions of the juvenile catshark lack any positivity for the *PSST5* probe.

4 DISCUSSION

4.1 The five somatostatin genes exhibit different patterns of expression in the brain and spinal cord of catshark

Immunohistochemical results have revealed a widespread occurrence of SST-ir nerve cell bodies and fibers throughout the CNS of adult rats (Johansson, Hökfelt, & Elde, 1984) and other vertebrates, including sharks (Chiba et al., 1989; Nozaki et al., 1984). In situ hybridization studies reported a wide distribution of the mRNA expression of the first somatostatin gene found in mammals (Fitzpatrick-McElligott et al., 1988; Kiyama & Emson, 1990). A second somatostatin-family gene orthologous to *PSST2* was characterized in mammals and called cortistatin (*CST* or *CORT*) after its predominantly cortical expression and ability to depress cortical activity (De Lecea et al., 1997; Tostivint et al., 2004). The recent identification of five somatostatin genes in the catshark and other elasmobranchs revealed that the gene diversity of the somatostatin system in these fishes is similar to that previously reported in teleosts and clearly different to that in mammals (Tostivint et al., 2019). The study by Tostivint et al. (2019) was able to identify homologies between the five catshark *PSST* genes and those of other vertebrates and clarified the evolution of this gene family. Based on phylogenetic and gene synteny analyses the catshark *PSST* genes are now named *PSST1*, *PSST2*, *PSST3*, *PSST5*, and *PSST6* (Tostivint et al., 2019). The *PSST3* and *PSST6* genes probably arose by tandem duplication of the *PSST1* and *PSST2* genes, respectively, although other alternatives are possible (see Tostivint et al., 2019).

Comparison of patterns of expression of the five *PSST* genes revealed by in situ hybridization in the catshark brain shows large differences between ~~members of~~ these genes. Thus, the wide brain distribution of *PSST1* and *PSST6* expression is in contrast with the limited expression of *PSST2*, *PSST3*, and *PSST5*. Moreover, the distribution pattern of the two more widely expressed *PSST* genes ~~in brain neurons~~ (*PSST1* and *PSST6*) appears to be largely exclusive, although double hybridization studies appear necessary to rule out the

possibility of double expression in some neurons. The differential expressions reported here represent the first comprehensive study of the somatostatinergic systems in an elasmobranch.

Previous studies of elasmobranches using SST-like immunohistochemistry described the presence of somatostatinergic cells and/or fibers in various brain or spinal centers (Alvarez-Otero et al., 1996; Anadón et al., 1995; Cameron et al., 1990; Chiba et al., 1989; Molist et al., 1992; Nozaki et al., 1984). SST-like-ir cell distribution reported in the gummy shark (Chiba et al., 1989) is partially reminiscent of the distribution of *PSST1*+ cells observed here, although the number of reported cells was clearly lower and various populations were not noted. In these studies, the antibody used was raised against mammalian SST-14, whose amino acid sequence is identical to the putative mature SST peptides from catshark *PSST1* and *PSST5*. Future studies with antibodies specific for the other putative catshark somatostatins might also reveal differential distributions of some somatostatinergic fiber systems.

In the following we will discuss the patterns of expression of *PSSTs* in the catshark CNS in comparison with those reported in other vertebrates (see Table 1). In these comparisons, the reader should take into account that the recent study by Tostivint et al. (2019) suggested that the so-called *PSST2* gene in chicken, lungfish, sturgeons, and teleosts actually corresponds to *PSST6*. For convenience, in the text, we will mention the nomenclature used in the original studies of each species.

4.2 Wide but differential expression of *PSST1* and *PSST6* genes in pallial interneurons

The wide expression of *PSST1* and *PSST6* genes is observed in the catshark pallium, forming at least two different sets of neurons. The catshark pallium presents a poor laminar organization but exhibits numerous morphological cell types judging from studies with the Golgi method (Manso & Anadón, 1993), including areas with primitive pyramidal neurons and other large neurons. The scattered distribution and small size of *PSST1*+ and *PSST6*+ pallial neurons may suggest that they are local interneurons. The presence of SST-like-ir pallial neurons was also reported in the gummy shark using immunohistochemistry (Chiba et al., 1989). In mammals, SST and cortistatin (SST2) are expressed in subsets of GABAergic interneurons of the mammalian pallium (hippocampus, cortex) and appear to play a key role in their inhibitory circuits (De Lecea et al., 1997; Urban-Ciecko and Barth, 2016; Adler, Zhao, Shin, Yasuda, & Gan, 2019). Colocalization of SST and cortistatin expression has been reported in a portion of cortical cells (De Lecea et al., 1997). Moreover, many subpopulations of SST neurons can be distinguished by morphology, connectivity, laminar location, firing properties, and expression of molecular markers (Yavorska & Wehr, 2016). Numerous GABAergic cells are found in the catshark pallium and they appear to have a subpallial origin, migrating secondarily to the pallium (Carrera, Ferreiro-Galve, et al., 2008; Quintana-Urzainqui, Rodríguez-Moldes, Mazan, & Candal, 2015). These parallelisms with mammals are suggestive of colocalization of *PSSTs* and GABA in cells of the catshark pallium. SST-ir neurons and a large number of fibers have been also reported in the pallium (dorsal telencephalic area) of ray-finned fishes (trout: Becerra, Manso, Rodríguez-Moldes, & Anadón, 1995; sturgeon: Adrio, Anadón, & Rodríguez-Moldes, 2008). Moreover, mRNA expression of the *PSST1* gene was also reported in scattered neurons of the pallium of sturgeon (Trabucchi et al., 2002) and goldfish (Canosa et al., 2004). Unlike the catshark *PSST6*, the other *PSST* genes of these ray-finned fishes show no expression in the pallium. However, expression of both *PSST1* and *PSST2* was reported in the pallium of frog (Tostivint et al., 1996) and mammals (De Lecea et al., 1997; Fitzpatrick-McElligott et al., 1988; Kiyama & Emson, 1990). Together, past and present data indicate that the presence of *PSST1* expression in pallial neurons is a highly conserved feature in jawed vertebrates, whereas *PSST6* expression in cells also expressing TH, instead of *PSST2*, appears to be a novelty of sharks. Whether this corresponds to the ancestral condition needs to be investigated with new studies in jawless vertebrates.

A loose network of SST-like-ir fibers in the pallial neuropil was noted in the gummy shark (Chiba et al., 1989), although the origin of fibers was not established. Immunohistochemical data in the trout reveal that the pallium was the brain region most richly innervated by SST-like-ir fibers, suggesting a great importance of somatostatin in pallial circuits (Becerra et al., 1995). The presence of two types of somatostatinergic pallial

neurons adds neurochemical complexity to the cellular diversity of the catshark pallium, which also has interneurons expressing choline acetyltransferase (Anadón et al., 2000), GABA (Carrera, Ferreiro-Galve, et al., 2008), glycine (Anadón, Rodríguez-Moldes, & Adrio, 2013), ~~tyrosine hydroxylase~~TH (Carrera et al., 2005; Carrera et al., 2012), thyrotropin-releasing hormone (Teijido, Manso, & Anadón, 2002), and met-enkephalin (Quintana-Urzainqui et al., 2012). Here, colocalization between TH and *PSST6* is reported in pallial neurons (see below). These data indicate that the catshark pallium has complex interneuron circuits, but the functional significance of such interneuron diversity is currently unknown.

4.3 Expression of *PSSTs* in the catshark subpallium

The adult elasmobranch subpallium shows a cellular organization that is difficult to compare with that of lampreys and bony fishes. Following Smeets et al. (1983) the subpallium is composed of an extensive dense cellular lamina (BSA), a wide central basal area, and a thin ventral periventricular area. Medially, a septal region joins the pallium dorsally. Laterally to the BSA there is an ill-organized cellular region considered as the striatum by Smeets et al. (1983). In the telencephalic peduncle the highly developed basal region is substituted by the entopeduncular area laterally and the interstitial nucleus of the anterior commissure medially. New views of the subpallium have emerged from genoarchitectonic studies of the embryonic subpallium (reviewed in Rodríguez-Moldes et al., 2017). In catshark embryos, the subpallium primordium was characterized by the early expression of the GABAergic marker glutamic acid decarboxylase and *Dlx2* (Carrera, Ferreiro-Galve, et al., 2008; Ferreiro-Galve et al., 2008;Quintana-Urzainqui et al., 2012; Quintana-Urzainqui et al., 2015). Only the medial region of the subpallium expresses *Nkx2.1*, and sonic hedgehog (shh) is expressed just in the midline. This embryonic catshark medial subpallium is the homolog of the median ganglionic eminence of tetrapods that originates the pallidum, while most of the subpallial primordium corresponds with the mammalian lateral ganglionic eminence that originates the striatum (Quintana-Urzainqui et al., 2012; Quintana-Urzainqui et al., 2015). Unresolved questions remain about the actual correspondence between subpallial and pallial territories of the adult catshark with their amniote counterparts, but, probably, the BSA, and the associated basal central and periventricular areas form part of a subpallial subdivision derived from the homolog of the lateral ganglionic eminence, thus representing the striatum. Since the catshark BSA and basal central area receive prominent secondary olfactory projections (Yáñez, Folgueira, Köhler, Martínez, & Anadón, 2011), they may be considered as ventral striatum. Some histochemical properties of the olfactory tubercle (ventral striatum) have also been recognized in the BSA of elasmobranchs (Northcutt et al., 1988). Interestingly, there is some evidence in late catshark embryos of a cell migratory route from the medial ganglionic eminence homolog invading the prospective striatal territory (Quintana-Urzainqui et al., 2015), which suggests that BSA populations have mixed origins from both the lateral and medial ganglionic eminence homologs (for review see Quintana-Urzainqui et al., 2012). In mouse, somatostatin interneurons originated from the medial ganglionic eminence/diagonal area populate the striatum (Puelles et al., 2016), and this might be the origin for putative *PSST1* interneurons found in the catshark BSA.

The presence of SST-like-ir cells in the septum and basal area was reported in the gummy shark (Chiba et al., 1989). With regard to the expression of *PSST* genes in the catshark subpallium, *PSST1* was mainly expressed in cells of the basal and central superficial areas (ventral striatum~~?~~), whereas *PSST6* and the other *PSSTs* are not expressed there. Both *PSST1* and *PSST6* are expressed in the septum of Smeets et al. (1983), although with different patterns. *PSST1* is poorly expressed in cells of the striatum of these authors and lacks in the interstitial nucleus of the anterior commissure, which shows few *PSST6*+ cells. In sturgeon, *PSST1* and *PSST6* (as renamed by Tostivint et al., 2019; the authors of the original study named it *PSST2*) genes are expressed in the subpallium (ventral telencephalic area) with different patterns (Trabucchi et al., 2002). Two somatostatin genes are also abundantly expressed in the lateral region of the goldfish telencephalic ventral area (Canosa et al., 2004). Somatostatin-like immunochemistry reveals positive cells in the sturgeon subpallium, mainly in the lateral region of the ventral telencephalic area (Adrio et al., 2008). Since the correspondence between the various subpallial regions of ray-finned fishes and elasmobranchs has not been established, one-to-one homology of these somatostatinergic populations with those observed in the catshark subpallium is uncertain.

4.4 Olfactory bulbs

The catshark olfactory bulbs show scarce *PSST1*+ cells in the inner granular layer. An intriguing result is the small group of intensely *PSST1*+ cells found at the level of the glomerular layer. Unlike the catshark, *PSST-III* was intensely expressed in the internal cell layer of the goldfish olfactory bulb (Canosa et al., 2004), whereas in sturgeon the olfactory bulbs do not express *PSST1* (Trabucchi et al., 2002). This indicates a low degree of conservation and a high degree of specialization in the expression of *PSST* genes in the olfactory bulbs of fishes.

4.5 *PSST1* is specifically expressed by CSF-c neurons of the PVO/PRO and central canal

The paraventricular and PRO have been characterized in catshark and other elasmobranchs with histochemistry, immunohistochemistry and electron microscopy (Anadón et al., 2013; Carrera, Molist, et al., 2008; Meredith & Smeets, 1987; Meurling & Rodríguez, 1990; Molist, Rodríguez-Moldes, & Anadón, 1993; Rodríguez-Moldes, 1986; Rodríguez-Moldes et al., 1993; Rodríguez-Moldes & Anadón, 1987; Wilson & Dodd, 1973): these organs exhibit numerous dopaminergic, serotonergic and glycinergic CSF-c neurons. In *S. acanthias* and *Raja radiata* they showed numerous somatostatinergic CSF-c neurons showing colocalization with serotonin (Meurling & Rodríguez, 1990). Our in situ hybridization results reveal strong expression of *PSST1* mRNA in CSF-contacting neurons of both the hypothalamic paraventricular and PRO, and the spinal cord around the central canal. On the other hand, the other four *PSST* genes were not expressed in cells of these organs. These in situ hybridization results are in agreement with the immunohistochemical observations of distribution of SST-like expression in CSF-c cells of these organs by Chiba et al. (1989) and Meurling and Rodríguez (1990). Similar SST-like-ir CSF-c populations are also present in the hypothalamus of lampreys (Wright, 1986; Yáñez, Rodríguez-Moldes, & Anadón, 1992) and ray-finned fishes (Vigh-Teichmann, Vigh, Korf, & Oksche, 1983). Whereas monoaminergic CSF-c cell populations (dopaminergic and serotonergic) of the hypothalamus have been well characterized morphologically and biochemically in teleosts and other nonmammalian vertebrates (Vigh-Teichmann et al., 1983; Gómez-Segade, Anadón, & Gómez-Segade, 1989; Yamamoto et al., 2010; Xavier et al., 2017), their functions, as well as those of *PSST1*+ hypothalamic CSF-c cells, are largely unknown. The finding of opsins and functionally related molecules in some hypothalamic CSF-c populations of birds and lampreys (Barreiro-Iglesias, Fernández-López, Sobrido-Cameán, & Anadón, 2017; Nakane et al., 2010), suggests that some of these populations are responsive to light, but if some of these photosensitive cells express ~~somatostatins~~*PSSTs* has not yet been investigated. Recent findings in lampreys indicate that GABA/SST-like expressing hypothalamic CSF-c cells are mechano-sensitive and also detect pH changes in the cerebrospinal fluid, which suggests they form a new homeostatic mechanism of feedback control of deviations of the internal medium (Jalalvand et al., 2018).

With regard the catshark spinal cord, *PSST1*+ CSF-c cells show a horseshoe distribution around dorsal and lateral regions of the central canal, lacking in its ventral part. This distribution is complementary to that the spinal TH-ir CSF-c cells, which are located in the ventral region of the central canal (Sueiro, Carrera, Rodríguez-Moldes, Molist, & Anadón, 2003). This was confirmed here with a combination of in situ hybridization and immunostaining. Judging from the distribution of GABAergic CSF-c cells all around the catshark central canal, and the colocalization of TH and GAD in ventral CSF-c neurons (Sueiro, Carrera, Molist, Rodríguez-Moldes, & Anadón, 2004), this strongly suggests the *PSST1*+ cells are also GABAergic. Dorsal CSF-c cells, but not ventral cells, also express glycine immunoreactivity (Anadón et al., 2013), which might colocalize with *PSST1*. Presence of SST and colocalization with GABA immunoreactivity in spinal CSF-c cells was already reported in lampreys (Buchanan, Brodin, Hökfelt, Van Dongen, & Grillner, 1987; Christenson, Alford, Grillner, & Hökfelt, 1991; Jalalvand, Robertson, Wallén, Hill, & Grillner, 2014). In the catshark, these spinal cells appear to give rise to the somatostatinergic fibers innervating the glomerular fields of the spinal marginal nucleus (Anadón et al., 1995). The marginal nucleus also exhibits glycinergic neurons in catshark (Anadón et al., 2013), and glycinergic edge cells in lampreys (Villar-Cerviño, Holstein, Martinelli, Anadón, & Rodicio, 2008), revealing a conserved pattern in these basal vertebrate groups. The marginal nucleus/edge cells represent proprioceptive mechanosensory centers. In lampreys, the spinal CSF-c cells modulate the edge cell activity in response to stretching of the spinal cord during swimming (Grillner, Williams, & Lagerbäck, 1984; Jalalvand et al., 2014). Recent studies in central canal CSF-c cells of lampreys

and zebrafish reveal that these CSF-c neurons are chemo- and mechanoreceptive, sensing pH changes and modulating the locomotor activity (Böhm et al., 2016; Jalalvand et al., 2018).

Available data in ray-finned fishes reveal that both *PSST1* and *PSST6* (as renamed by Tostivint et al., 2019; the authors of the original study named it *PSST2*) genes are expressed in the hypothalamic nucleus of the periventricular organ of sturgeon (Trabucchi et al., 2002). Some somatostatinergic CSF-c neuronal populations were described in sturgeon with SST immunohistochemistry, including the preoptic recess organ, the anterior tuberal nucleus, the PRO, and the spinal central canal (Adrio et al., 2008), which is roughly similar to the distribution observed in catshark with the sum of hybridization results with the *PSST1* (PVO, CC) and *PSST2* (lateral tuberal nucleus, which possibly corresponds to the sturgeon anterior tuberal nucleus) genes.

4.6 Possible hypophysiotropic somatostatinergic hypothalamic populations

The structure of the median eminence of the catshark and its vascularization has been thoroughly described by Mellinger (1964). This eminence is located below the medial hypothalamus and receives fibers from neurosecretory cells (Knowles, 1965). Some of the axons projecting to the pituitary in teleost originate from ventromedial hypothalamic populations (Anglade, Zandbergen, & Kah, 1993; Holmqvist & Ekström, 1995; Johnston & Maler, 1992). As far as we know, the neurons afferent to the median eminence in elasmobranchs have not been identified experimentally. However, abundant SST-like-ir fibers coursed in the hypophyseal median eminence and neurointermediate lobe of the gummy shark (Chiba et al., 1989). The lateral tuberal nucleus (NLT) and the median hypothalamic nucleus (NM) of the catshark showed neurons expressing *PSST1* (~~MHIN~~NM), *PSST2* (NLT and ~~MHI~~NM), and<<Query: Can you please expand the term MHN Ans: It has to be changed for NM. I have edited the text here.>> *PSST3* (~~MHI~~NM), but not cells expressing *PSST5* or *PSST6*. The ~~MHI~~NM and ~~LTN~~NLT are located close to the median eminence, and *PSST*+ cells in this region might send fibers to it, which thus might control the secretion of growth hormone or other pituitary hormones. Further studies are needed to reveal the hypophysiotrophic populations of the catshark hypothalamus.

A population of *PSST6*+ cells was observed in the posterior tubercle lateral to the NSV, a region where SST-like-ir cells are present in catshark (Molist et al., 1992). The NSV receives a heavy neural projection from the hypothalamic saccus vasculosus, a neuro-ependymo-vascular structure characteristic of elasmobranchs and many ray-finned fishes (see Sueiro et al., 2007; Yáñez et al., 1997). However, the NSV proper only receives a few SST-like-ir fibers, probably not originated from the saccus vasculosus since no SST-like-ir neurons (Chiba et al., 1989) or *PSST* expression (present work) were observed in this structure.

4.7 Banded expression of somatostatin in the diencephalon

The main *PSST1*+ populations of the diencephalon appear to show a segmental-like distribution as dorsoventral bands in the prethalamus, thalamus, and pretectum, suggesting that there is a main band in each prosomere. The main *PSST6*+ neuronal population observed in the catshark diencephalon also extends in a band between the prethalamus and posterior tubercle. In goldfish, *PSST1*, *PSST3*, and *PSST6* (as renamed by Tostivint et al., 2019; the authors of the original study named it *PSST2*) mRNAs are expressed in nuclei known to pertain to the three diencephalic prosomeres (Canosa et al., 2004), but the pattern appears more complex than in catshark and its segmental organization was not analyzed. *PSST1* and *PSST6* (as renamed *by* Tostivint et al., 2019; the authors of the original study named it *PSST2*) expression was also noted in the prethalamus (ventral thalamus) and thalamus (dorsal thalamus) of sturgeon (Trabucchi et al., 2002).

4.8 Cells of upper and lower layers of the optic tectum differentially express *PSST1* and *PSST6*

Experimental studies in catshark reported that optic tectum receives a major retinal projection on external tectal layers (Repérant et al., 1986; Smeets, 1981), as in most vertebrates. The catshark optic tectum also

receives afferents from various brain nuclei and regions extending from the pallium to the spinal cord (Smeets, 1982), probably reaching deeper tectal layers. Notably, in the catshark optic tectum a complementary pattern of expression of *PSST1* and *PSST6* genes was observed. *PSST1*+ cells are mainly distributed through the stratum cellulare externum zona externa, whereas *PSST6*+ cells are distributed in the stratum cellulare externum zona interna and in lateral regions of the stratum cellulare internum. This may suggest specialization of outer *PSST1*+ cells for visual circuits and inner *PSST6*+ cells for other sensory and nonsensory afferents, respectively. Several types of neurons have been reported with Golgi methods in the catshark optic tectum, and some of them exhibit radial dendrites coursing through two or more layers (Manso & Anadon, 1991), but ascription of *PSST*+ cells to these cell types is not possible. The stratum cellulare externum of the catshark optic tectum is synaptically complex, exhibiting presynaptic dendrites and other unconventional synapses (Manso & Anadón, 1991). In the goldfish optic tectum, the cells expressing the *PSST1* and *PSST3* genes are both distributed in the periventricular gray zone (Canosa et al., 2004), whereas in sturgeon tectum only the *PSST6* (as renamed *by* Tostivint et al., 2019; the original study named it *PSST2*) gene is expressed (Trabucchi et al., 2002). Expression of *PSST1* was reported in the frog optic tectum (Tostivint et al., 1996). In the adult rat, *PSST* mRNA is expressed in numerous superior collicular neurons showing zonal distribution (Harvey, Heavens, Yellachich, & Sirinathsinghji, 2001). *PSST* mRNA expressing cells were rarely found in the stratum zonale (SZ) or upper stratum griseum superficiale (SGS), but formed a prominent tier in the lower third of SGS and in the upper stratum opticum (SO), a distribution that reminds the sum of that observed in catshark with two different *PSST* genes. Thus, specialization of *PSSTs* in cells of different tectal subcircuits depends on differential gene expression in sharks but not in mammals.

4.9 Tegmental expression of *PSST* genes and its topographical relation with the catecholaminergic substantia nigra/ventral tegmental area

In the midbrain tegmentum of catshark, the most conspicuous *PSST1*+ cell population was observed in the lateral reticular area whereas a paired compact *PSST6*+ reticular nucleus is located in an intermediate lateral region and more scattered *PSST6*+ cells extend in the midline. Faint *PSST1*+ cells were also observed intermingled with TH-ir neurons of the ventral tegmental area and the substantia nigra, but without forming any conspicuous group as *PSST6*+ cells. The presence of a group of large SST-like-ir neurons was reported in the tegmentum of the gummy shark and ascribed to the red nucleus (Chiba et al., 1989). By its position and cell size, it may correspond with the main *PSST6*+ population observed in the catshark midbrain tegmentum. The lateral *PSST1*+ population may be part of the region known as the lateral mesencephalic nucleus that also contains a population of glycinergic cells (Anadón et al., 2013). In the spiny dogfish, and probably also in other elasmobranchs, the lateral mesencephalic nucleus receives secondary octavolateral projections (Boord & Northcutt, 1988). This caudal midbrain *PSST1*+ population is located in a characteristic lateroventral outgrowth that contains a conspicuous precerebellar population (Pose-Méndez, Candal, Adrio, & Rodríguez-Moldes, 2014), and that may correspond to the nucleus H of guitarfish (Fiebig, 1988). Although these catshark precerebellar neurons were considered rhombencephalic, it is probable that they are mesencephalic judging from the location of *PSST1*+ cells in relation to midbrain TH-ir populations. Prominent precerebellar cell populations are found in the midbrain tegmentum of sturgeon (Huesa, Anadón, & Yáñez, 2003) and in the lateral valvular nucleus of teleosts (see Folgueira, Anadón, & Yáñez, 2006), but not in the rostral rhombencephalic tegmentum. In the frog and sturgeon midbrain tegmentum, both *PSST1* and *PSST6* (now renamed *PSST6* according to Tostivint et al., 2019; the authors of the original study named it *PSST2*) genes are expressed (Tostivint et al., 1996; Trabucchi et al., 2002), but equivalences between catshark nuclei and those of these species are not defined.

4.10 Expression of *PSSTs* in the catshark habenulo-interpeduncular system

Conspicuous expression of *PSST6*, but not of the other four *PSSTs*, is observed in numerous cells of the catshark interpeduncular nucleus (IP). The interpeduncular nucleus of the leopard shark and the gummy shark contains SST-like-ir neurons (Chiba et al., 1989; Nozaki et al., 1984), which may correspond to the *PSST6*+ group reported here. Glycinergic neurons were also reported in similar locations in the catshark IP

(Anadón et al., 2013), but if there is colocalization with *PSST6* was not investigated. In sharks, as in other vertebrates, the IP receives a heavy cholinergic habenular projection via the fasciculus retroflexus (Anadón et al., 2000; Giuliani, Minelli, Quaglia, & Villani, 2002). Two somatostatin genes are expressed in the IP of goldfish (*PSST1* and *PSST3*; Canosa et al., 2004) and frog (*PSST1* and *PSST2*; Tostivint et al., 1996). Moreover, SST-14-ir cells are found in a specific IP subnucleus in mice (Quina, Harris, Zeng, & Turner, 2017). The mammalian IP is the target of the medial habenular nucleus via the fasciculus retroflexus, and in turn projects to several brain regions, as shown in detail in mice (Quina et al., 2017). With regard to the habenula, a small group of *PSST6*+ cells was found in the catshark habenula but no similar SST-like-ir cells were reported in the gummy shark (Chiba et al., 1989). Cells expressing *PSST1* were reported in the goldfish habenula (Canosa et al., 2004). To our knowledge, no *PSST*-expressing habenular cells were reported in other vertebrates. Some afferents to the habenula of the Arabian bamboo shark (*Chiloscyllium arabicum*) have been traced experimentally (Giuliani et al., 2002); these include the BSA, the entopeduncular nucleus, the posterior tubercle, thalamus, and septum. Interestingly, the entopeduncular nucleus is the only region expressing *PSST5* in the catshark. This region also shows scarce *PSST1*+ cells. In teleosts, the habenula also receives fibers from the telencephalon (olfactory bulb, subpallium, and entopeduncular nucleus), posterior tubercle, hypothalamus and raphe, with the entopeduncular nucleus representing the main afferent nucleus (Turner et al., 2016; Yañez & Anadón, 1996). In goldfish, the entopeduncular nucleus shows cells expressing *PSST3* and *PSST1* (Canosa et al., 2004), but both genes have a wide pattern of expression in the brain, which is unlike the catshark *PSST5*. The habenula is a highly conserved nucleus that forms part of the limbic system and is involved in a number of functions (Hsu et al., 2014).

4.11 *PSSTs* and the viscerosensory lobe

In the catshark vagal lobe numerous *PSST1*+ small cells are observed in its dorsal half intermingled with TH-ir neurons, which form a separate population. In the catshark, a population of *PSST1*+ reticular cells also accompanies the solitary tract-vagal lobe at the level of the sulcus limitans, laterally to the motor nucleus. The other *PSSTs* are not expresses here. In the gummy shark (Chiba et al., 1989), the vagal lobe shows numerous SST-like-ir fibers and some cells. In the vagal lobe of sturgeon there is also expression of *PSST1* (Trabucchi et al., 2002), but distribution of SST-ir cells is not parceled as in the catshark (Adrio et al., 2008). The goldfish has a complex viscerosensory region with three lobes (facial, glossopharyngeal, and vagal), the vagal lobe being formed by several sensory and motor layers (Finger, 2008; Morita & Finger, 1985). In situ hybridization studies in goldfish (Canosa et al., 2004) indicate that *PSST1*+ cells are distributed in superficial (sensory) layers, whereas *PSST3* is expressed in cells of deep (motor) layers. In rat, SST expression was also noted in the homologous nucleus of the solitary tract using ^{32}P-labeled oligonucleotide probes (Priestley, Réthelyi, & Lund, 1991), indicating conserved patterns of expression. In mice, the solitary nucleus shows a few GABAergic that are also SST-ir (Wang & Bradley, 2010), suggesting that somatostatinergic cells of this nucleus are inhibitory interneurons, as those found in the cortex. If this also occurs in the viscerosensory lobes of catshark ~~and~~or other vertebrates needs to be investigated.

4.12 The hindbrain reticular formation and expression of *PSSTs*

The reticular region is highly developed in the catshark hindbrain (Smeets et al., 1983), occupying most of the basal plate-derived region excepting the areas occupied by defined nuclei as the motor nuclei (Anadón et al., 2000; Rodríguez-Moldes et al., 2011), or the inferior olive. The hindbrain reticular formation has been subdivided into isthmic, superior, medius, and inferior reticular nuclei mainly attending to the distribution of large reticular cells (see Smeets et al., 1983). Reticulospinal cells are the origin of major descending pathways to the spinal cord and were found in all these parts of the reticular formation (Smeets & Timerick, 1981; Timerick, Roberts, & Paul, 1992). Most of the reticulospinal neurons reported by these authors were large neurons, whereas in general somatostatinergic reticular neurons are small or medium-sized cells often distributed in lateral and/or medial groups suggesting that most of them are not descending projection neurons.

4.13 The cerebellar system

The cellular organization of the catshark cerebellum has been characterized with microscopic and immunohistochemical methods (see Alvarez-Otero, Pérez, Rodríguez, Adrio, & Anadón, 1995; Anadón et al., 2009, 2013). No positive cells for any of the five *PSSTs* were observed in the catshark cerebellum with ISH (not shown) or in the gummy shark cerebellum with SST-like immunohistochemistry (Chiba et al., 1989). Unlike in the shark cerebellum, SST-ir cells (Golgi cells, some Purkinje cells) were described in mammals with immunohistochemistry (Vincent et al., 1985) and in situ hybridization (Inagaki et al., 1989). Tracing experiments in catshark indicate that cells of the LRN and other regions containing *PSST*+ cells project to the cerebellum (Pose-Méndez et al., 2014). Moreover, SST-like-ir fibers were observed in the cerebellar nucleus of catshark (Alvarez-Otero et al., 1996; Anadón et al., 2009), but not in the cerebellar cortex of the gummy shark (Chiba et al., 1989). In mice, evidence of innervation of the cerebellar granular layer by SST-28 immunoreactive mossy fibers has been presented (Armstrong et al., 2009). If *PSSTs* are expressed in some of the precerebellar cells reported in catshark (Pose-Méndez et al., 2014) need be investigated.

4.14 Colocalization of TH immunoreactivity and *PSST6* mRNA expression in brain neurons

Initially, we decided to perform double staining with TH immunohistochemistry in catshark as a marker to clarify the topography of some *PSST1*+ or *PSST6*+ neuronal populations, because the expression of this enzyme in several brain regions containing somatostatinergic neurons is well characterized (Carrera et al., 2005; Carrera et al., 2012). Whereas most of these populations did not show colocalization of *PSSTs* and TH, to our surprise two populations in the pallium and in the lateral preoptic/suprachiasmatic area showed double-labeled cells for TH-ir and *PSST6*+. The presence of TH-ir cortical neurons has been previously reported in the pallium of mammals, elasmobranchs and some reptiles (see Smeets & González, 2000). In the rat, double immunohistochemical studies indicate that very few TH-ir cortical neurons contained immunoreactivity for SST<<Query: Asmus et al., 2007 has been changed to Asmus et al., 2008 to match reference list. please check Ans: The change is correct.>> (Asmus et al., 2008), which is similar to our observations in catshark with TH and the *PSST1* probe. However, numerous catshark pallial neurons exhibit both *PSST6* positivity and TH immunoreactivity, indicating for the first time in a vertebrate the wide coexistence of a catecholamine-synthesizing enzyme and the expression of one *PSST* gene in pallial cells. It is improbable that these cells represent an ancestral feature of jawed vertebrates that was lost in evolution, because there are no TH-ir neurons in the pallium of most non-mammalian vertebrates (Smeets & González, 2000). The presence of dopamine in elasmobranch pallial neurons was reported in a skate with dopamine antibodies (Meredith & Smeets, 1987), which suggests that the pallial TH-ir cells of catshark are also dopaminergic, and thus they may co-release dopamine and SST. The presence of dopamine as end product of TH in mammalian cortical interneurons is not known (see Asmus et al., 2008).

With regards the colocalization of TH immunoreactivity and *PSST6* expression in other brain regions, we have only found similar populations in the lateral preoptic/suprachiasmatic area. In other vertebrates, a few examples of colocalization of TH and SST immunoreactivities have been reported in urodeles and rats. Specifically, low numbers of TH-ir neurons showed colocalization of catecholamines and SST in the ventral preoptic area of a urodele (González, Moreno, Morona, & López, 2003), but not in many brain regions where catecholaminergic neurons and SST-ir cells are largely co-distributed. In the rat, double-labeled TH-ir/SST-ir cells were frequent in the preoptic periventricular nucleus and double-labeled cells were also observed in the hypothalamus (Sakanaka, Magari, & Inoue, 1990). Thus, the presence of TH/SST double-labeled neurons appears restricted to a few populations in the brain of vertebrates.

4.15 Concluding remarks

Present results reveal for the first time that the five *PSST* genes identified in the catshark *S. canicula* are expressed by different sets of brain neurons. Two of these genes (*PSST1* and *PSST6*) show wide and complex

expression throughout the brain from the telencephalon to the spinal cord, whereas the other three (*PSST2*, *PSST3*, and *PSST5*) exhibit a more restricted expression. Notably, the catshark pallium shows two sets of somatostatinergic interneurons differentiable by the coexpression of TH in *PSST6* positive neurons but not in those positive for *PSST1*. The patterns of somatostatinergic populations described in the catshark represent a great advance in our knowledge of somatostatinergic populations in elasmobranchs, known previously only from immunohistochemical studies in a few shark species. Comparison with results of these immunohistochemical studies suggests that the antibodies employed probably only revealed ~~one or two (*PSST1* and *PSST2* or *PSST3*)~~some of the somatostatins expressed in the shark brain. Although a diversity of *PSST* genes was reported in fishes and other vertebrates (Tostivint et al., 2014; Tostivint et al., 2019), comparative data of brain expression of *PSST* genes (see discussion) indicates that somatostatinergic systems of sharks ~~are~~is the most complex reported in any fish.

SSTs exert potent inhibitory actions on hormone secretion and neuronal excitability mediated by G protein-coupled receptors (Lin & Peter, 2001; Tostivint et al., 2014; Urban-Ciecko and Barth, 2016; Günther et al., 2018). The genes for six SST receptor types (SSTR1-6) have been cloned in vertebrates (Ocampo Daza, Sundström, Bergqvist, & Larhammar, 2012) and hybridization studies have shown that the transcripts of five receptors are expressed in the rat CNS (Meyerhof, Wulfsen, Schönrock, Fehr, & Richter, 1992; Señarís, Humphrey, & Emson, 1994). Pharmacological studies reveal that properties of SSTRs differ with regards affinity to ligands SST-14 and SST-28, expression patterns and regulation (Günther et al., 2018; Lin & Peter, 2001). It is expected that SSTRs will<<Query: References Urban-Ciecko and Barth, 2016; Xavier et al., 2017 have not been included in the Reference List, please supply full publication details. Ans: The references are: Urban-Ciecko J, Barth AL. Somatostatin-expressing neurons in corticalnetworks. Nat Rev Neurosci. 2016 Jul;17(7):401-9. doi: 10.1038/nrn.2016.53.

Xavier AL, Fontaine R, Bloch S, Affaticati P, Jenett A, Demarque M, Vernier P,Yamamoto K. Comparative analysis of monoaminergic cerebrospinal fluid-contacting cells in Osteichthyes (bony vertebrates). J Comp Neurol. 2017 Jun15;525(9):2265-2283. doi: 10.1002/cne.24204.>> be also expressed differentially in the brain of elasmobranchs. Future studies characterizing the affinities of the SSTRs of elasmobranchs for the different somatostatin peptides, as well as their brain distribution, may help to understand the variety of functions of their somatostatinergic system.

Abbreviations

APV	ventrolateral periventricular area
AU	cerebellar auricle
BSA	basal superficial area
CB	cerebellum
cc	cerebellar crest
CCa	central canal
cDP	caudal region of dorsal pallium
CG	central gray
Ch	optic chiasm
Cho	choroid plexus
CN	cerebellar nucleus
CSA	central subpallial area
DH	dorsal horn
DON	dorsal octavolateralis nucleus
DP	dorsal pallium
En	entopeduncular nucleus
EW	Edinger-Westphal nucleus

gl	glomeruli
gr	cerebellar granular layer
H	hypophysis
Ha	habenula
Hy	hypothalamus
ica	interstitial nucleus of the anterior commissure
IHL	inferior hypothalamic lobe
III	oculomotor nucleus
IP	interpeduncular nucleus
IRF	inferior reticular formation
Ist	isthmus
IV	trochlear nucleus
LC	locus coeruleus
LMN	lateral mesencephalic nucleus
LR	lateral recess of pallium
LX	vagal lobe
LP	lateral pallium
mDP	medial part of the dorsal pallium
ME	median eminence
mi	mitral cell region
ml	cerebellar molecular layer
mlf	medial longitudinal fascicle
MON	medial octavolateralis nucleus
MP	medial pallium
MRF	middle reticular formations
NLT	lateral tuberal nucleus
NM	median hypothalamic nucleus
Nmlf	nucleus of the medial longitudinal fascicle
Nr	neuroporic recess
Nsm	nucleus of the stria medullaris
NSV	nucleus of the saccus vasculosus
OB	olfactory bulb
OLA	octavolateralis area
ON	optic nerve
OT	optic tectum
P	pallium
pc	posterior commissure
PO	preoptic area
Pr	posterior recess
PRO	posterior recess organ
Pt	pretectum
PTh	prethalamus
PTN	posterior tubercle nucleus
PTu	posterior tubercle
PVO	paraventricular organ
Rh	rhombencephalon
SC	spinal cord
scee	stratum cellulare externum, zona externa

scei	stratum cellulare externum, zona interna
SCh	suprachiasmatic nucleus
sci	stratum cellulare internum
Sco	subcommissural organ
sDP	superficial region of dorsal pallium
Se	septal region
Sec	caudal septum
Sep	posterior medial septum
SN	substantia nigra
Sp	subpallium
SRF	superior reticular formation
SRFl	superior reticular formation, lateral part
SRFm	superior reticular formation, medial part
St	striatum
SV	saccus vasculosus
Tg	tegmentum
Th	thalamus
trP	tractus pallii
Vd	trigeminal descending root and nucleus
VH	ventral horn
VTA	ventral tegmental area
ze	zona externa

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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