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Differential expression of somatostatin genes in the central nervous system of the sea lamprey

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Abstract

The identification of three somatostatin (*SST*) genes (*SSTa*, *SSTb* and *SSTc*) in lampreys (Tostivint et al. 2016) prompted us to study their expression in the brain and spinal cord of the sea lamprey by *in situ* hybridization. These 3 genes were only expressed in equivalent neuronal populations in the hypothalamus. In other regions, *SST* transcripts showed clear differential expression. In the telencephalon, *SSTc* positive cells were observed in the medial pallium, ventral part of the lateral pallium, striatum, subhippocampal lobe and preoptic region. In the diencephalon, *SSTa* positive cells were observed in the thalamus and *SSTc* positive cells in the prethalamus, posterior tubercle, pretectal area and nucleus of the medial longitudinal fascicle. In the midbrain, *SSTc* positive cells were observed in the torus semicircularis, lateral reticular area and perioloculomotor tegmentum. Different *SSTa* and *SSTc* positive populations were observed in the isthmus. *SSTc* neurons were also observed in the rostral octavolateralis area and caudal rhombencephalon. In the spinal cord, *SSTa* was expressed in cerebrospinal fluid-contacting (CSF-c) neurons and *SSTc* in non-CSF-c interneurons. Comparison with previous immunohistochemical studies using anti-SST-14 antibodies strongly suggests that SST-14-like neurons correspond with the *SSTa* populations. Thus, the *SSTc* populations were not reported previously in immunohistochemical studies. Cluster-based analyses and alignments of mature peptides suggested that *SSTa* is an ortholog of *SST1* and that *SSTb* is closely related to *SST2* and *SST6*. Our study provides important new insights in the evolution of the somatostatinergic system in vertebrates.

Keywords:

Somatostatin, lampreys, hypothalamus, differential expression, spinal cord, neuropeptides.

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Conflicts of interest/Competing interests

None.

Ethics approval

All animal experiments were approved by the Bioethics Committee of the University of Santiago de Compostela and the *Xunta de Galicia* government and conformed to the European Union and Spanish regulations for the care and handling of animals in research.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

Histological material and raw images are available from the authors upon reasonable request. Sequences have been deposited in GenBank (NCBI).

Code availability

Not applicable.

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Introduction

A peptide originally isolated from the ovine hypothalamus was named somatostatin (SST) because its capability to inhibit growth hormone secretion from rat pituitary cells (Brazeau et al. 1973). Since its discovery, SST has been implicated in many patho-physiological functions, which include being an inhibitor of endocrine and exocrine secretions and nervous system modulation (see Gahete et al. 2010). In vertebrates, SST peptides are products of at least 6 different prosomatostatin genes named *SST1* to *SST6* (Liu et al. 2010, Tostivint et al. 2019). Different vertebrates contain different numbers of *SST* genes. *SST1*, which generates the most common isoform of SST peptide (SST-14), and *SST2* genes (also called cortistatin in mammals) have been found in most gnathostome species. On the other hand, *SST3* has been only found in chondrichthyes and actinopterygians, *SST4* in teleosts, *SST5* in chondrichthyes, actinopterygians and actinistia and *SST6* in chondrichthyes, actinopterygians and birds (Tostivint et al. 2019). The current model suggests that the 2 rounds of whole-genome duplication (WGD) in vertebrates led to the *SST1*, *SST2* and *SST5* genes, while *SST4* was duplicated from *SST1* during the third round of genome duplication in teleosts. *SST3* and *SST6* arose by duplication of the *SST1* and *SST2* genes, respectively (Liu et al. 2010, Tostivint et al. 2019) in the gnathostome predecessor. The view that *SST6* came from the second round of genome duplication and *SST2* appeared later cannot be excluded. Thus, during vertebrate evolution, fishes have maintained a larger repertoire of *SST* genes as compared to tetrapods. Studies in jawed fishes have shown that different *SST* genes are expressed in different regions of the central nervous system (CNS) (lungfish: Trabucchi et al. 1999; rainbow trout: Alexander et al. 2001; sturgeon: Trabucchi et al. 2002; goldfish: Canosa et al. 2004; orange-spotted grouper: Xing et al. 2005; common carp: Feng et al. 2015; catshark, Sobrido-Cameán et al., 2020a), which suggests that different *SSTs* play specific roles in different neuronal circuits. It is not known whether this is the case in the oldest group of extant vertebrates (i.e. jawless vertebrates or agnathans).

So far, neuroanatomical studies of the somatostatinergic system in the CNS of lampreys have been based on immunohistochemistry using antisera raised against mammalian SST-14. In lampreys, SST-like immunoreactivity has been reported in amacrine cells of the retina (Negishi et al. 1986), in neurons of the hypothalamus, thalamus, rostral rhombencephalon and in cerebrospinal-fluid contacting cells (CSF-c neurons) of the spinal cord (Hoheisel et al. 1986; Buchanan et al. 1987; Wright, 1986;

Cheung et al. 1990, 1991; Yáñez et al. 1992; Jalalvand et al. 2014, 2018). Cheung and coworkers (1990, 1991) also reported the presence of weak SST-34-like immunoreactivity in cells of the olfactory bulbs, thalamus and hypothalamus of the sea lamprey.

At the functional level, much attention has been put on the SST-like-ir CSF-c neurons of the spinal cord of lampreys, which are located close to the central canal and colocalize with GABA (Christenson et al. 1991; Jalalvand et al. 2014). These CSF-c cells project to the spinal lateral margin where they form a GABA-ir and SST-ir plexus that contacts the dendrites of specialized mechanosensory edge cells. SST and GABA hyperpolarize edge cells in lampreys, although through different ionic mechanisms (Christenson et al. 1991). Recent work has shown that SST-ir CSF-c neurons of the spinal cord of lampreys respond to both mechanical stimulation and lowered pH (Jalalvand et al. 2016a, 2016b). Interestingly, the effects of SST on edge cells change in lampreys that recovered successfully from spinal cord lesions (Svensson et al. 2013). Similarly, hypothalamic SST-ir/GABA-ir CSF-c neurons appear to sense deviations from physiological pH (Jalalvand et al. 2018). The roles of SST on other neuronal circuits of lampreys are not known and specially those of other SSTs.

A recent study has revealed the existence of at least 3 different *SST* genes in lampreys (Tostivint et al. 2016), which appear to generate a diversity of mature SST peptides that could have important functional implications in these animals. The three *SST* transcripts cloned in the Japanese lamprey (*Lentheron japonicum*) were named *SSTa*, *SSTb* and *SSTc*, since previous phylogenetic and synteny analyses failed to clearly assign orthology relationships with those of gnathostomes (Tostivint et al. 2016). The *SSTb* and *SSTc* sequences were also cloned in *Lampetra fluviatilis* and *Petromyzon marinus*, respectively (Tostivint et al. 2016). The mature peptide coded by the *SSTa* gene (AGCKNFFWKTFTSC) is identical to mammalian SST-14 (Tostivint et al. 2016). This peptide was identified previously in brain extracts of both *Petromyzon marinus* (Sower et al. 1994) and *Lampetra fluviatilis* (Conlon et al. 1995a). On the other hand, the mature *SSTb* peptide (AGCKNFFWKTFFSSC; S substitution in position 12) has been reported in extracts of lamprey pancreas and intestine (Andrews et al. 1988; Conlon et al. 1995a, 1995b; Sower et al. 1994; Wang et al. 1999). The presence of the putative mature *SSTc* peptide of lampreys (ANCRMFYWKTMAAC) has not yet been confirmed by biochemical methods.

To advance on our knowledge of the somatostatinergic system of lampreys we decided to study the expression of the three *SST* transcripts in the CNS of larval and adult sea lampreys by means of *in situ* hybridization (ISH). Our results reveal that the three *SST* transcripts are expressed in different neuronal populations. This indicates that, as in gnathostomes, different *SSTs* are implicated in different neuronal circuits and might have differential roles in the CNS. Finally, we also attempted to clarify the phylogeny and orthology relationships of the lamprey and gnathostome *SST* genes by using cluster analyses. Our study provides an anatomical basis for future functional studies on the role of different *SSTs* in the CNS of lampreys.

Material and methods

Animals

Larvae (n = 12), and young (n = 6) and mature adults (n = 9) of the sea lamprey, *Petromyzon marinus* L., were used for the ISH experiments. Additionally, 8 sea lamprey larvae were used for RNA extraction. Sea lamprey postmetamorphic young adults and larvae were collected from the River Ulla (Galicia, Northwest Spain). Upstream migrating mature adults were obtained from a local commercial supplier.

Cloning and sequencing of the Petromyzon marinus SST cDNAs

Larvae were anesthetized by immersion in 0.1% ethyl 3-aminobenzoate methanesulfonate salt (MS-222; Sigma, St. Louis, MO, USA) and the brain and spinal cord were dissected out under sterile conditions. Total RNA was isolated from these tissues using TriPure (Roche, Mannheim, Germany). The first-strand cDNA synthesis reaction from total RNA was catalyzed with Superscript III reverse transcriptase (Invitrogen, Waltham, MA, USA) using random primers (hexamers; Invitrogen). For polymerase chain reaction (PCR) cloning, specific oligonucleotide primers (*SSTa*: F: 5'-CAACCGGAGCCCGTTACCA - 3', R: 5' - ACACGAGGTGAACGTCTTCCA - 3'; *SSTb*: F: 5'- GAGGCCACTCCACGCTAGG - 3', R: 5' - CAGCACGAAGAGAAAGTCTTCCA - 3'; *SSTc*: F: 5'-CAACCATGAAAGCGACGGC - 3', R: 5' - CATCGCCATTCCACTGCCTT - 3') were designed based on the *L. japonicum* *SSTa* and *SSTb* cDNA sequences (GenBank accession numbers: KU522235.1 and KU522234.1; please note that these sequences are annotated wrongly as b and a in GenBank; for the correct annotation follow the article by Tostivint et al. 2016) and the *P. marinus* *SSTc* cDNA sequence (GenBank accession

number: KU529940.1). The amplified fragments were cloned into pGEM-T vectors (Promega, Madison, WI, USA) and sequenced by the University of Santiago de Compostela sequencing service. The partial *P. marinus SSTa* and *SSTb* cDNA sequences generated in this study were deposited in GenBank (GenBank accession numbers: MK258196 and MK258197).

In situ hybridization

Templates for *in vitro* transcription were prepared by PCR amplification using the primers mentioned above for the *P. marinus SSTs*. In this case, the reverse primers included the sequence of the universal T7 promoter (TAAGCTTTAATACGACTCACTATAGGGAGA). For the generation of sense probes, the sequence of the T7 promoter was included in the forward primers. Digoxigenin (DIG)-labeled riboprobes were synthesized using the amplified fragments as templates and following standard protocols using T7 polymerase (Roche).

Lampreys were deeply anesthetized with MS-222 as above before experimental procedures. ISH experiments were performed as previously described for riboprobes against the sea lamprey *serotonin 1a* and *gabab* receptors (Cornide-Petronio et al. 2013; Romaus-Sanjurjo et al. 2016) or galanin and cholecystokinin neuropeptides (Sobrido-Cameán et al., 2019, 2020b). Briefly, the brains/rostral spinal cords were fixed by immersion for 12 h in 4% paraformaldehyde. Then, they were cryoprotected with sucrose 30% and sectioned in a cryostat in the transverse plane (14 µm sections). Three parallel series of sections were obtained from each brain/spinal cord. The sections of each series were incubated with each of the *P. marinus SST* digoxigenin-labelled probes, respectively, at 70°C overnight in hybridization mix and treated with RNase A (Invitrogen) in the post-hybridization washes. Then, the sections were incubated with a sheep anti-digoxigenin antibody conjugated to alkaline phosphatase (1:2000; Roche) 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, USA). Sense probes were used as controls and generated no colorimetric signal.

Imaging

An Olympus photomicroscope (AX-70; Provis) with a 20x Aplanachromic 0.75 lens and equipped with a color digital camera (Olympus DP70, Tokyo, Japan) was used to acquire images of brain and spinal cord sections from the ISH experiments. Contrast

and brightness were minimally adjusted with Adobe Photoshop CS4 (Adobe Systems, San Jose, CA, USA). Figure plates and lettering were also generated using Adobe Photoshop. Schematic drawings were generated with CorelDRAW (Corel Ottawa, Canada).

Cluster-based analysis and alignment of mature peptides

To investigate the relationship of the lamprey SSTs with the different SST precursors identified in other animals, a database of precursor sequences was obtained from Tostivint and coworkers (2019), and the recently identified echinoderm SST precursors were added as outgroups (Zandawala et al. 2017; Zhang et al. 2020). Then, an all-versus-all BLAST analysis was performed using Cluster Analysis of Sequences (CLANS; Frickey and Lupas 2004) with the scoring matrix BLOSUM62. Sequences were clustered and linkage clustering was performed with a p-value cutoff of 1e-20 to identify coherent clusters. The clustering was collapsed to 2D to enable generation of the diagram. The raw file of the clustering analysis containing the name of the sequences can be seen as Supplementary figure 1.

Alignments were performed using ClustalW, with a pairwise alignment setup in slow mode/accurate (<https://www.genome.jp/tools-bin/clustalw>). Highlighting of conserved residues was performed using BOXSHADE with 80% conservation as the minimum for highlighting (www.ch.embnet.org/software/BOX_form.html). Finally, the sequences were highlighted in phylum-specific or superphylum-specific colors: blue (agnathans), purple (mammals), orange (sauropsids), yellow (lobe-finned fishes), green (ray-finned fishes), pink (cartilaginous fishes). The accession numbers and the sequences used to build the cluster analysis and the alignment tree are shown in Supplementary file 1.

Results

Schematic drawings of transverse sections of the brain/spinal cord showing the distribution of somatostatinergic neuronal populations are presented in Figures 1 and 2. The *in situ* hybridization experiments revealed differential expression of the 3 *SST* transcripts in both adults and larvae (Figs. 1 and 2, respectively). *SSTc* expression showed a broader distribution in the brain than *SSTa*, whereas the distribution of *SSTb* was mainly restricted to the hypothalamus. Most neuronal populations were observed from larvae to adults; therefore, these will be described together. Instances in which a

neuronal population is not present in a specific developmental stage will be indicated. The terminology employed in this study for the various brain regions, nuclei and identified neurons followed those used in recent studies of our group (see Barreiro-Iglesias et al. 2017; Sobrido-Cameán et al. 2020b), which is partially adapted from that the prosencephalic model of Pombal and Puelles (1999).

SSTa expression

In the brain, *SSTa*-expressing neuronal populations were observed in the hypothalamus, diencephalon, and rhombencephalon of larvae, and young (post-metamorphic, juvenile) and mature (pre-spawning) adult sea lampreys (Figs. 1A-E, 2A-G, 3A-P). *SSTa* positive CSF-c cells were only detected in the spinal cord of adult lampreys (Figs. 1F, 3Q).

In the hypothalamus (secondary prosencephalon), numerous *SSTa*-expressing cells are located in the periventricular cell layer extending from the anterior (postoptic) hypothalamic recess to the tuberal region (ventral hypothalamus) (Fig. 1A-B, 2A-C, 3A-E). Most of these cells probably correspond to CSF-c neurons because of their bipolar appearance and their location close to the ependymal layer (Fig. 3C and E). This population was observed from larvae (Figs. 2A-B, 3D-E) to mature adults, although in the latter they appear more extended and narrowed (compare Figs 3A-E), as it occurs with the periventricular cell layer.

In the diencephalon, the ventral tier or caudal part of the thalamus exhibits numerous *SSTa*-expressing neurons in the thick-layered periventricular region (Figs. 1C, 2C-E, 3F-I). These cells form a rather compact elongated group in larvae and young adults located ventral (rostral) to the fasciculus retroflexus, whereas it appears as a more extended cell lamina in mature adults.

Two *SSTa*-expressing neuronal populations were observed in the rostral rhombencephalon. One of them was a large group of small *SSTa*-expressing cells located in the isthmus tegmental region just caudal to the mid-hindbrain border (Figs. 1D, 2F, 3J-L). Here, numerous *SSTa*-positive reticular cells appear in a mediolateral region at the level of the interpeduncular nucleus. This population has its caudal limit just rostral to the giant II isthmus cell. The second *SSTa*-expressing neuronal population was a smaller neuronal group in the anterior rhombencephalic reticular nucleus extending to levels ventral to the trigeminal motor nucleus (Figs. 1E, 2G, 3M-P). These cells are larger and exhibit stronger staining than those in the rostral isthmus. In mature

adults, migrated *SSTa* positive cells are also observed in the ventral midline neuropil (interpeduncular nucleus neuropil) (not shown).

In the rostral spinal cord of mature adult sea lampreys *SSTa*-positive CSF-c cells are located in the intermediate-lateral region of the periventricular cell layer around the central canal (Figs. 1F, 3Q). These cells are clearly stained in mature adults but only faintly stained in young adults, whereas positive hybridization signal was not noted in the larval spinal cord.

SSTb expression

The expression of *SSTb* transcript was restricted to postoptic-tuberal hypothalamus in adult and larval lampreys (Figs. 1G-H, 2H-K, 4), although in the former some expression was also observed in the obex-spinal cord transition (not shown).

In lamprey larvae, the hypothalamic population of *SSTb* cells extends in the ventricular wall of the hypothalamus between the anterior (postoptic) hypothalamic recess and the rostral level of the hypophysis. These numerous neurons are located in the thick periventricular cell layer (Fig. 2H-K, 4E-G). In adults, these cells are located in the rostral part of the ventral hypothalamus, at intermediate levels in the periventricular region around the anterior (postoptic) hypothalamic recess (Figs. 1G-H, 4A-D). In adults, the periventricular cell mantle is separated in this region from the ependyma by a thin neuropil layer. Most *SSTb*-positive neurons are located in the cell mantle but a few *SSTb*-positive neurons are located in the neuropil or among the cells of the ependymal layer. *SSTb*-positive cells are in the same general region where the *SSTa*-expressing hypothalamic population is observed (compare Figs. 3A-C and 4), but if they colocalize in the same cells or not has not been assessed.

SSTc expression

The *SSTc* transcript showed wider expression than the other two *SSTs* transcripts in the adult lamprey brain. Thus, in adults, *SSTc*-expressing cells were found in the telencephalon and hypothalamus (secondary prosencephalon), diencephalon, mesencephalon, rhombencephalon and spinal cord (Figs. 1I-V, 5, 6, 7). *SSTc* expression is more reduced in the larval brain, especially in the telencephalon where *SSTc*-expressing populations are lacking (Figs. 2L-T, 8). We will first describe in detail the

SSTc-positive populations in adults, indicating summarily the main differences observed in larvae.

In the adult telencephalon, the most conspicuous *SSTc*-expressing populations were observed in the lateral pallium and the striatum. Numerous cells showing weak staining with the *SSTc* probe were also observed in the thin periventricular cell band of the medial pallium (Figs. 1I-K, 5A-B). In the striatum, the *SSTc*-expressing neurons were observed in periventricular location, but a few also in the characteristic striatal cell band away from the ventricle (Fig 1I, 5C). Sparse *SSTc* positive cells were found in the subhippocampal lobe (Figs. 1J, 5D) and also in the ventro-medial region of the lateral pallium near the striatum (Fig. 1I, 5D). In the olfactory bulb, no *SSTc*-expressing cells were observed. *SSTc* expression was not observed in the telencephalon of larvae (not shown).

Three groups of *SSTc*-expressing cells were found in the preoptic region of adult lampreys, which is comprised between the striatum and the optic chiasm. One of the *SSTc*-expressing populations is located dorsal to the magnocellular preoptic nucleus (medial preoptic nucleus) adjacent to the striatal population (Figs. 1I, 5C). Most of the *SSTc*-expressing cells of this group are located laterally in the periventricular cell layers, but some cells are also located in the inner cell layers (Fig. 5C). Another *SSTc*-expressing population forms a compact group located just rostral (dorsal) to the base of the preoptic recess and ventral to the magnocellular preoptic nucleus (Figs. 1I, 5C,E). It consists of small cells showing strong *SSTc* expression. The third preoptic group is formed by a layer of cells just in the caudal (ventral) wall of the preoptic recess and rostral (dorsal) to the optic chiasm (caudal preoptic nucleus). These cells are larger and show weaker *SSTc* expression than those of the rostral group (Fig. 5C,E). Some positive cells were observed in this region of the brain of larvae (not shown).

In the hypothalamus of adult lampreys, a group of *SSTc*-expressing cells was observed in the nucleus of the tract of the postoptic commissure, both in dorsal and ventral parts of the nucleus (Figs. 1K, 5F,H,I). Numerous *SSTc*-expressing cells were found scattered in the periventricular layers of the ventral hypothalamus and another smaller population was found in inner periventricular layers of the dorsal hypothalamus, both rostral to the location of the neurohypophysis (Figs. 1L, 5G). Some *SSTc*-expressing cells were also present in the nucleus of the postoptic commissure and rostral hypothalamus in larvae (Figs. 2L-M, 8A).

In the diencephalon of adults, a conspicuous band with strongly *SSTc*-expressing cells was found extending from the prethalamus to the posterior tubercle nucleus (Figs. 1M, 6A). This population was also conspicuous in larvae (Figs. 2Q, 8B). A population of *SSTc*-expressing cells was also observed in the adult nucleus of the medial longitudinal fascicle (Figs. 1N, 6B-C). In the adult pretectum, numerous *SSTc*-expressing neurons were scattered in the lateral pretectal area near the optic tract (Figs. 1N-O, 6D-E). The cell populations of the medial longitudinal fascicle and pretectum were not observed in larvae.

In the midbrain, *SSTc*-expressing cells were found in the torus semicircularis and in the mesencephalic tegmentum (Figs. 1O-R, 6F-I). The torus semicircularis population is comprised of a part that is periventricular and a lateral part that extends caudally (Figs. 1Q-R, 6F-G). A group of numerous small *SSTc*-expressing cells was also observed in the periculomotor region surrounding and caudal to the giant M3 cell (Figs. 1O-P, 6H-I). Periculomotor and toral cells were also observed in the larval midbrain (Figs. 2R, 8C), but the toral *SSTc*-expressing cells are much less numerous than in adults.

The rhombencephalon of adult lampreys shows some *SSTc*-expressing cells, mostly in its rostral part. In the isthmus, a population of strongly stained *SSTc*-expressing cells was found in the ventral reticular area from caudal to the mid-hindbrain boundary till levels rostral to the trigeminal motor nucleus (Figs. 1Q-R, 6J-L, 7A). At the level of the isthmus II giant cell there were numerous *SSTc* expressing cells (Fig. 6K). In the rostro-dorsal rhombencephalon just caudal to the trochlear nucleus, a conspicuous *SSTc*-positive population was found in the medial nucleus of the octavolateralis area (Figs. 1S, 7B-C). In the larval rhombencephalon, the most conspicuous *SSTc*-expressing population was that of the medial octavolateralis area (Figs. 2S, 8D), and the ventral isthmus cells were scarce (Fig. 2S).

In more caudal regions of the rhombencephalon (at the level of the glossopharyngeal and vagal nerves), scattered *SSTc*-expressing neurons were located near the lateral surface of the medulla below the octavolateralis region and near the descending root of the trigeminal nerve (Figs. 1T, 7D-E). At the transition between the obex and the spinal cord and in the rostral spinal cord, a few *SSTc*-expressing cells were found near the giant reticulospinal axons; in sections they appear over, lateral or between the two medial longitudinal fascicles (Figs. 1U-V, 7F). These cells were also observed in larval lampreys (Figs. 2T, 8E-F).

Analysis of the relationship of lamprey SSTs with SSTs from gnathostomes

To investigate relationships of the sea lamprey SSTs with gnathostome SSTs, we used a previously published database of SST precursors (Tostivint et al. 2019) and performed a cluster-based analysis of the precursor sequences using CLANS (Fig. 9a). This analysis revealed different clusters: 1) the cluster containing the SST1, SST3 and SST5 precursors, which contains very defined cluster of sequences; and 2) a cluster containing the SST2 and SST6 precursor sequences, which seem to be very closely related to each other (Fig. 9a). Teleost SST4 and lamprey SSTc sequences are so divergent to the rest of SST precursors that it was not possible to recognize their relationship to any of the other SST sequences (Fig. 9a). However, we were able to identify that the lamprey SSTa precursor shares close relationship with the SST1 cluster (Figure 9a). Furthermore, an alignment of the predicted mature peptide from lamprey SSTa and SST1 from Gnathostome species show a high degree of conservation in all species, with the exception of the SST1 precursor from the Australian elephant shark (*C. milii*) that contains a non-synonymous mutation in the 5th position of the predicted mature peptide (Fig. 9b). In the case of the lamprey SSTb, we found a connection with the sequences belonging to the SST6 precursors (Fig. 9a). However, as this cluster contains sequences belonging to the SST2 cluster we performed an alignment with the predicted mature peptide from the lamprey SSTb and the SST2 and SST6 sequences (Fig. 9c and 9d). The comparison between SSTb and SST2 sequences shows that there is more variability between the peptides, and SSTb shows 2 non-synonymous mutations when compared to the rest of the mature SST2 peptide sequences. Lamprey SSTb shares the most similarity with the SST6 mature peptide, with only 1 non-synonymous mutation in the 2nd position of the mature peptide (Fig. 9d). This evidence strongly suggests that lamprey SSTa is orthologous to the SST1 precursors and peptides and that lamprey SSTb is homologous of the cluster containing the sequences SST2 and SST6 and is likely an ortholog of the SST6 precursor.

Discussion

In lampreys, three *SST* genes have been identified and were called *SSTa*, *SSTb* and *SSTc*, but their phylogenetic status was unclear (Tostivint et al. 2016). A very deep analysis of the evolution of SST precursors in vertebrates was performed in 2019 by Tostivint and coworkers, but it was still not possible to identify the homology of the

lamprey SST precursors with those of gnathostomes (Tostivint et al. 2019). Presumably the phylogeny was unable to define the relationship of lamprey SSTs with the precursors from other species because lampreys are the most basal vertebrate on the list of sequences used in those analyses and there was no outgroup for the phylogeny (Tostivint et al. 2019). Also, due to the short length of the precursor genes is not always easy to identify homology between precursor genes. Here, we used a different approach in order to identify potential homology between the lamprey and gnathostome SST precursors. CLANS (Cluster Analysis of Sequences) is a software that performs all-vs-all pairwise analysis and represents sequences by nodes in a graph, placed in a 3D space. Clustering is performed using attractive forces proportional to the negative logarithm of the BLAST-P values, and a uniform repulsive force (Frickey and Lupas, 2004). Despite being inferior to a phylogeny for the inference of homology, it has previously been used to identify orthologues and to reconstruct orthologue families of neuropeptide precursors and receptors in which a phylogeny is challenging (Jékely, 2013; Thiel et al. 2017; Yáñez-Guerra et al. 2020). According to the data published by Tostivint and coworkers (2019), SST3 and SST6 have independently evolved by tandem duplications of SST1 and SST2 respectively. Our clustering analysis is consistent with this idea, as the SST3 and SST1 clusters are more closely related to each other, than to any other SST precursor, and the same occurs with SST2 and SST6, which cluster together.

Using this clustering approach, we were able to identify that lamprey SSTa precursors share the most similarities with the SST1 precursors from other species, the same result was obtained with the alignment of the mature peptides. This suggests that lamprey SSTa is an ortholog of SST1. In the case of the lamprey SSTb, we identified that this precursor shows connection to the SST6 precursors. As this cluster is less defined than the clusters containing the SST1, SST3 and SST5 sequences, we aligned the mature peptide from SSTb with the two precursors conforming this cluster (SST2 and SST6). The alignment shows more similarity between lamprey SSTb and SST6, thus we infer that lamprey SSTb could be an ortholog of SST6 precursors. Such a result strongly suggests that SST6 arose before SST2, instead of the other way round. In contrast, our study failed to clarify the orthologous status of SSTc. In this respect, it is noteworthy that the temporal relationship between the two WGD events and the cyclostome-gnathostome divergence is still unclear and several plus or minus contradictory scenarios have been proposed (Kuraku et al. 2009, Mehta et al. 2013,

Smith et al. 2013 and Smith and Keinath, 2015, Sacerdot et al., 2018, Simakov et al. 2020, Cardoso et al. 2020). However, since SSTc does not appear as an ortholog of SST5, one can hypothesize that SSTc emerged specifically in the cyclostome lineage, after the cyclostome-gnathostome divergence. In any case, since we still could not clearly clarify the phylogenetic relationships of the 3 lamprey SST genes, we decided to maintain the *SSTa*, *SSTb* and *SSTc* nomenclature. However, the *SSTa*/*SST1* and *SSTb*/*SST2/6* relationships identified in our study will be kept in mind during the discussion of the expression data below.

Expression of SSTs in lampreys

Present results show for the first time the distribution of neurons expressing the *SSTa*, *SSTb* and *SSTc* genes in the brain of the sea lamprey *Petromyzon marinus*. These three genes showed clear differential expression in the larval and adult sea lamprey brain. *SSTc* showed a broader expression than *SSTa* and *SSTb*, the latter being exclusively expressed in hypothalamic neurons. The patterns of expression are largely conserved between larval and adult stages in most brain regions but there are also some significant changes from larvae to adults. Large differences in the neuroanatomical expression of the different *SST* genes of non-mammalian species were also reported in the chicken (Trabucchi et al. 2003), frog (Tostivint et al. 1996), the sturgeon *Acipenser transmontanus* (Trabucchi et al. 2002), the goldfish (Canosa et al. 2004) and the catshark *Scyliorhinus canicula* (Sobrido-Cameán et al. 2020a). Results in the sea lamprey and other non-mammalian species appear to indicate that different *SST* genes become specialized in different neural circuits.

Comparison with results of immunohistochemical studies in lampreys

The distribution of SST-like-ir neurons and fibers in the lamprey brain was previously studied with antibodies raised against mammalian SST-14 (Wright 1986; Cheung et al. 1990; Yáñez et al. 1992, Cheung et al. 2011), which is identical to the peptide sequence predicted by the *SSTa* (*SST1*) gene. SST-like-ir positive perikarya were observed in the nucleus of the tract of the postoptic commissure, ventral hypothalamus, thalamus (dorsal thalamus), isthmus tegmentum (rostral and caudal groups) and spinal cord central canal, a pattern consistent with the distribution of *SSTa* transcript expression reported here. Thus, previous immunohistochemical studies probably revealed the distribution of the *SSTa*-expressing neurons. Since the

hypothalamic expression of *SSTb* is rather similar to that of *SSTa*, there exists the possibility that the SST-14 antibody also recognized neurons expressing this gene. On the other hand, the expression of *SSTc* is clearly different from that reported by immunohistochemical studies with the SST-14 antibody and represents a new somatostatinergic system. Moreover, this system differs greatly between larvae and adults in some brain regions, suggesting that it has evolved in response to their different ways of life. Cheung et al. (1990) employed an antibody raised against SST-34, which revealed additional populations to those observed with anti-SST-14, notably in the telencephalon. These SST-34-like-ir cells appear widely distributed in the olfactory bulb, which is clearly different from the distribution of the *SST* transcripts reported here in the telencephalon of the same lamprey species. This suggests that in the study by Cheung and coworkers (1990) the SST-34 antibody cross-reacted in the telencephalon with a different protein, or that the expression of *SST* transcripts in these cells was under the detection level of the present ISH procedure.

Comparative aspects

Telencephalon

Of the three *SST* genes of the sea lamprey, *SSTc* is the only one expressed in the telencephalon. In the adult pallium, abundant cells express *SSTc* in the medial pallium. A few cells were also observed in ventral regions of the telencephalic hemispheres (lateral pallium). In ventral lateral pallial regions, they are distributed in the inner zone, which also shows GABAergic cells (Robertson et al. 2007; Villar-Cerviño et al. 2011; Suryanarayana et al. 2017), but if GABA and *SSTc* are co-located in the same cells was not investigated. With regards to the lamprey medial pallium, its equivalence with any cortical regions of jawed vertebrates is unclear and even recent work has proposed that it represents the homolog of the prethalamic eminence of jawed vertebrates (Pombal et al. 2009). In any case, the important topographical differences with the distribution of GABAergic cells reported in the medial pallium (Robertson et al. 2007) indicates that *SSTc*-expressing cells are very probably non-GABAergic cells. This is unlike the cortex of mammals, where *SST* and cortistatin expression have been reported in interneurons of the telencephalon (Fitzpatrick-McElligott et al. 1988, 1991; Naus et al. 1988; de Lecea et al. 1997), which are GABAergic and take part in inhibitory cortical circuits (Urban-Ciecko and Barth, 2016; Adler et al. 2019). *SST*-ir or *SST*-positive cells have also been reported in the pallium of birds (Trabucchi et al. 2003), reptiles (Reiner and

Oliver, 1987), amphibians (Tostivint et al. 1996), lungfish (Vallarino et al. 1997), teleosts (Sas and Maler, 1991; Becerra et al. 1995; Canosa et al. 2004) and elasmobranchs (Sobrido-Cameán et al. 2020a), but if they are GABAergic was not investigated.

The subpallium of adult sea lampreys shows *SSTc*-expressing cells mainly in the striatum. The lamprey striatum shares important features with the mammalian striatum such as its premotor nature, dopaminergic innervation and excitability properties of its neurons (Ericsson et al. 2013). The finding of *SSTc*-expressing cells in lamprey adds new layers of neurochemical complexity to this conserved center. In mammals, a population of GABAergic interneurons that express SST controls the feed-forward activity of glutamatergic projection neurons in the dorsomedial striatum (Fino et al. 2018). It is possible that the *SSTc*-expressing striatal neurons of lampreys may have similar roles in control of the striatal output. On the other hand, the expression of *SSTc* in some cells of the subhippocampal lobe deserves special attention because it is considered a pallidal homolog (Stephenson-Jones et al. 2012) and is the origin of a major afferent projection to the habenula (Yáñez and Anadón, 1996; Stephenson-Jones et al. 2012). Similar to the subhippocampal lobe of lampreys, the entopeduncular nucleus is the main telencephalic afferent nucleus to the habenula in teleosts (Yáñez and Anadón, 1994; Turner et al. 2016) and the Arabian bamboo shark (*Chiloscyllium arabicum*) (Giuliani et al. 2002); this nucleus shows cells expressing *SST3* and *SST1* in goldfish (Canosa et al. 2004) and *SST1* in catshark (Sobrido-Cameán et al. 2020a). Our results indicate that jawless and jawed vertebrates opted for the use of different *SST* genes in this region.

The developmental origin and expression patterns of cortical interneurons in mammals have received much attention as deficiencies in these populations have been implicated in important neurological diseases by imbalance of excitatory and inhibitory activities in the cerebral cortex (Xu et al. 2005; Takada et al. 2014; Hu et al. 2017). Cortical SST/GABA interneurons are early-born in mice and mainly originate from the medial ganglionic eminence, which is specified by the early expression of *shh* and *nkx2.1* genes in the basal telencephalon (Puelles et al. 2016; Hu et al. 2017). Unlike jawed vertebrates, the basal telencephalon of lamprey embryos lacks expression of these genes (Osorio et al. 2005; Rétaux and Kano, 2010) and thus may lack a gene regulatory network comparable with that of mice. This may be responsible of the absence of SST expression in the larval lamprey telencephalon. The expression of *SSTc* in the adult

lamprey telencephalon is mainly found in the medial pallium and subpallial region. Cells in the ventral part of the lateral pallium are scarce and might be originated by migration of cells from the medial ganglionic eminence (Puelles et al. 2016). On the other hand, the location of *SSTc*-expressing cells in the periventricular medial pallium of adults, a region that grows only very late in larval development (Villar-Cheda et al. 2006), suggests that these somatostatinergic cells may be a pallial-derived population. Little is known about neurochemistry and functions of the medial pallium, but the abundant *SSTc* expression suggests that this substance is implicated in its control. Periventricular *SSTc* cells of the medial pallium, which are GABA (Robertson et al. 2007) and glutamate immunonegative (Villar-Cerviño et al. 2011), represent a major difference with the somatostatinergic populations of the mammalian pallium, which suggests a divergent contribution of SSTs to inhibitory mechanisms in the lamprey telencephalon.

Preoptic region and rostral hypothalamus

The preoptic region and the hypothalamus contain different somatostatinergic populations. Around the preoptic recess there are three populations expressing *SSTc* that occupy regions of the preoptic nucleus (telencephalic) and the suprachiasmatic nucleus homologue (ventral nucleus of the postoptic commissure; Weigle et al. 1996) (alar hypothalamus), although these cells were only observed in adults. One of the *SSTc* preoptic populations has a similar location to GnRH-III neurons described in the sea lamprey (Nozaki et al. 2000), but whether they are related to sexual maturation is not known. In the catshark, positive cells of these areas express *SST1* and *SST6*, respectively, indicating higher complexity in the somatostatinergic systems of this region of cartilaginous fishes. With regard the basal hypothalamus, the three *SST* genes are expressed in similar regions of the nucleus of the tract of the postoptic commissure and rostral hypothalamus. Although this may suggest functional redundancy of SSTs in the lamprey hypothalamus, studies in mammals reveal that SST and cortistatin have different affinity for SST receptors and ghrelin receptor 1a, allowing them to elicit differential responses on target neurons (Deghenghi et al. 2001; Ibáñez-Costa et al. 2017). Whereas immunohistochemical studies of sharks and bony fishes have revealed somatostatinergic fibers projecting to the neurohypophysis (Batten et al. 1990; Vallarino et al. 1997; Adrio et al. 2008), in the sea lamprey similar projections appear to be absent (Wright, 1986) or to be very scarce (Yáñez et al. 1992). The presence in the preoptic

region/hypothalamus of cells expressing other SSTs (*SSTb* and *SSTc*) that are probably not detected by the SST-14 antibody used in previous lamprey studies (see above) opens the possibility that a somatostatinergic preopto/hypothalamo-neurohypophysial system was already present in lampreys. This possibility needs to be addressed in future studies by using specific antibodies raised against other mature SST peptides of lampreys.

Diencephalon

Our results revealed populations expressing two different *SSTs* in regions of the three diencephalic prosomeres of the sea lamprey. Thus, a prominent *SSTa*-expressing population that corresponds with that previously reported with SST-14 immunohistochemistry (Wright, 1986; Cheung et al. 1990; Yáñez et al. 1992) was observed in the thalamus (prosomere 2), whereas two new populations expressing *SSTc* were observed in the prethalamus/posterior tubercle (prosomere 3) and in the lateral pretectum and the nucleus of the medial longitudinal fascicle (prosomere 1). This alternate distribution of *SSTa* and *SSTc* expression in diencephalic prosomeres reminds that observed in the catshark diencephalon with the *SST1* and *SST6* genes (Sobrido-Cameán et al., 2020a); although, as indicated above *SSTc* is probably not an ortholog to *SST6* of sharks, which again indicates that different vertebrates are opting for the use of different *SST* genes in similar regions. On the other hand, the sea lamprey habenula does not show *SST*-expressing cells, which is in contrast with the catshark and goldfish habenulae (Sobrido-Cameán et al., 2020a; Canosa et al. 2004).

Reticular formation

Two *SST* genes (*SSTa* and *SSTc*) are expressed in the reticular formation of the sea lamprey. Previous studies with SST-14 immunohistochemistry only observed somatostatinergic reticular cells in the isthmo-trigeminal region, forming two separate populations (Yáñez et al. 1992). This distribution corresponds well with that of *SSTa*-expressing isthmic populations reported here. The presence of additional reticular populations expressing *SSTc* in the midbrain tegmentum (perioculomotor and MRA populations), the isthmus, and the caudal rhombencephalon indicates that *SSTs* are more widely expressed by reticular populations than previously known in lampreys. The most numerous midbrain *SSTc*-expressing population is located in a tegmental region recently characterized as the lamprey homologue of the mammalian periaqueductal gray

mainly on the base of its projections on the pretectum and the afferents from the interpeduncular nucleus (Olson et al. 2017). Present *in situ* hybridization results reveal a complex pattern of *SST*-expressing reticular populations in lampreys, which reminds, although is simpler, the reticular populations expressing the *SST1* and *SST6* genes in the catshark (Sobrido-Cameán et al. 2020a). Whereas in sea lamprey these reticular populations do not appear topographically related with dopaminergic cells, in the catshark some midbrain reticular populations are closely related with the dopaminergic cells of the ventral tegmental area and substantia nigra (Sobrido-Cameán et al. 2020a).

Expression of the SST genes in sensory centers

SSTc expression was observed in cells of some sensory areas of the sea lamprey. One of the *SSTc* expressing populations is located in the torus semicircularis, which in sea lamprey receives secondary projections from the mechanosensory octavolateralis area (González et al. 1999). This mesencephalic structure, which is found in most non-mammalian vertebrates, is homologous to the inferior colliculus of mammals and probably to the lateral mesencephalic nucleus of elasmobranchs, which also exhibit somatostatinergic cells (Wynne et al. 1995; Wynne and Robertson, 1997; Sobrido-Cameán et al. 2020a). *SST* expression was also reported in cells of the torus semicircularis of frog (Tostivint et al. 1996) and goldfish (Canosa et al. 2004). Moreover, an *SSTc*-expressing population was observed in the rostral part of the medial nucleus of the octavolateralis area of the rhombencephalon. The medial nucleus of the octavolateralis area is specialized in processing lateral line mechanosensory inputs (Northcutt, 1981) and the torus semicircularis probably represents an integration/relay center for ascending mechanosensory inputs, since it also projects to the thalamus (González et al. 1999), a region that also shows *SSTc*-expressing neurons. The distribution of *SSTc* expressing cells in the medial octavolateralis nucleus, torus semicircularis, and thalamus suggests a role of its product in mechanosensory processing centers in lampreys.

Unlike mechanosensory centers, in the visual system of the sea lamprey brain *SSTs* expression is scarce. Thus, none of the three *SSTs* was expressed in cells of the optic tectum, either larval or adult, and only the pretectum showed *SSTc*-expressing cells in its lateral area. Some *SST*-positive cells were also present in the MRA, which extends lateroventrally to the optic tectum (De Miguel et al. 1990; Rodicio et al. 1995). The MRA and Schober's M5 nucleus contain retinopetal cells and thus are related to

visual circuits (De Miguel et al. 1990; Rodicio et al. 1995). The lack of *SST* expression in the lamprey optic tectum is in marked contrast with the optic tectum of chicken, frog, sturgeon, goldfish and catshark, in which *SST* genes are expressed in tectal cells (Tostivint et al. 1996; Trabucchi et al. 2002, 2003; Canosa et al. 2004; Sobrido-Cameán et al. 2020a). Somatostatin mRNA expression has also been reported in the superior colliculus (the mammalian homolog of the optic tectum) in the rat (Harvey, et al. 2001), camel (Mensah-Brown and Garey, 2006) and the tree shrew (Ranc et al. 2012). The major differences in tectal expression of *SST* genes between lampreys and jawed vertebrates suggests an ancient divergence in the involvement of *SSTs* in visual circuits.

Spinal cord

The lamprey spinal cord contains a well-characterized system of somatostatinergic CSF-c cells (Buchanan et al. 1987, Christenson et al. 1991; Jalalvand et al. 2014, 2016a, 2016b) that is involved in control of movements through its lateral projections on the edge cell system. Our results show that these cells express *SSTa* but not the other two *SSTs*, which agrees with immunostaining of these cells using *SST-14* antibodies. Similar somatostatinergic CSF-c cells are found in the catshark spinal cord expressing only *SST1* (Sobrido-Cameán et al. 2020a). These catshark spinal cells also give rise to the somatostatinergic fibers innervating the marginal glomerular fields of the spinal cord contacted by marginal neurons (Anadón et al. 1995). *SST-ir* CSF-c neurons were also observed in the spinal cord of sturgeon (Adrio et al. 2008), but the presence of mechanosensory marginal neurons or neuropil similar to that described in lampreys and sharks has not been reported in ray-finned fishes.

Our results also show some *SSTc*-expressing neurons in the lamprey spinal cord that were not of CSF-c type, mostly located near the giant reticulospinal axons, indicating that the spinal somatostatinergic systems is more diverse than previously known in lampreys. In the catshark spinal cord there are also non-CSF-c *SST6*-expressing neurons, which may indicate a conserved pattern (Sobrido-Cameán et al. 2020a). At rhombencephalic levels, *PSS1*- and *PSS2*-expressing cells were observed in the medial longitudinal fasciculus of the sturgeon (Trabucchi et al. 2003) and *PSS-I* expressing cells surround the medial longitudinal fasciculus of goldfish (Canosa et al. 2004). The location of somatostatinergic cells next to the reticulospinal axons suggest that they can play a role in modulating descending brain-spinal inputs in different vertebrates.

Conclusions

The present study of expression of the three somatostatin genes in the central nervous system of the sea lamprey (*Petromyzon marinus* L) by *in situ* hybridization has revealed a clear differential pattern, since only in the rostral hypothalamus the three genes were expressed in similar neuronal populations. The *SSTc* gene was the only *SST* gene expressed in the telencephalon, with positive populations located in the pallium and subpallium that may be implicated in control of higher brain functions. Whereas *SSTa* hypothalamic cells are probably not related with the hypophysis on the base of results with SST-14 antibodies, the presence of *SSTb* and *SSTc* in cells of the preoptic region and/or hypothalamus suggest that other somatostatins may be used to control the secretion of pituitary hormones (Sower, 2018). In the diencephalon, the expression of *SSTa* and *SSTc* genes is differentially related with its segmental organization. Thus, *SSTa* positive cells were only observed in the thalamus (prosomere 2), whereas *SSTc* positive cells were distributed in the prethalamus/posterior tubercle (prosomere 3), and in the pretectal area and nucleus of the medial longitudinal fascicle (prosomere 1). In the midbrain, only *SSTc* positive cells were observed (in the torus semicircularis, lateral reticular area and perioloculomotor midbrain tegmentum), whereas unlike in jawed vertebrates the optic tectum lacks expression of *SSTs*. In the isthmus, different *SSTa* and *SSTc* positive neuronal populations were observed in the tegmentum. *SSTc* neurons were also observed in the rostral region of the octavolateralis area and in caudal rhombencephalic reticular regions associated to the medial longitudinal fascicle. In the spinal cord, *SSTa* was expressed in some cerebrospinal fluid-contacting (CSF-c) neurons whereas *SSTc* expression was observed in non-CSF-c interneurons in medial regions. The three genes were expressed in both larval and adult lampreys with similar overall pattern except in a few regions. Comparison with previously published neurochemical results on sea lamprey with anti SST-14 shows that SST-14 immunoreactive populations only correspond with the *SSTa* positive populations revealed here. On the other hand, the *SSTc* positive populations described here do not correspond with any SST-immunoreactive population reported previously in lampreys and thus represent a new somatostatinergetic system. Moreover, comparison of *SSTs* sequences of lampreys and other vertebrates suggest that *SSTa* corresponds to *SST1* and that *SSTb* is related to *SST2/6*, whereas *SSTc* is distantly related with the *SSTs* of gnathostomes. The lamprey gene regulatory networks responsible of the expression patterns of somatostatins revealed here need to be investigated. Our results also open

the opportunity to investigate the differential contribution of lamprey SSTs to CNS circuits in lampreys.

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Figure Legends

Figure 1. Schematic drawings of transverse sections of the adult sea lamprey brain and spinal cord showing the distribution of cells expressing *SSTa* (A-F), *SSTb* (G-H) and *SSTc* (I-V) transcripts. Cells are represented as dots on the right side of sections. Anatomical regions are presented on the left side. Asterisk, ventricle. For abbreviations, see the list. The planes of sections are represented in the

squared figurine representing a lateral view of the brain. Scale bars: A-V, 200 μm ; figurine, 1 μm .

Figure 2. Schematic drawings of transverse sections of the larval sea lamprey brain showing the distribution of cells expressing *SSTa* (A-G), *SSTb* (H-K) and *SSTc* (L-T). Cells are represented as dots on the right side of sections. Anatomical regions are represented on the left side. Asterisk, ventricle. For abbreviations, see the list. The planes of sections are represented in the 3 figurines representing a lateral view of the brain. Scale bars: a-t, 200 μm .

Figure 3. Photomicrographs of *in situ* hybridized transverse sections showing the expression of *SSTa* mRNA in the brain and spinal cord of mature (A, B, F, G, J, M, Q), juvenile (C, H, K, N, O) and larval (D, E, I, L, P) sea lampreys. **A-E**, Sections of the rostral hypothalamus ventral to the postoptic commissure. B is a magnification of A. **F-I**, Sections throughout the diencephalon showing abundant positive cells in the ventral tier of the thalamus. Section in G is caudal to F. **J-L**, Sections throughout the rostral isthmic tegmentum. **M-P**, Sections throughout caudal regions of the isthmic tegmentum/rostral trigeminal tegmentum. **Q**, Section of the spinal cord showing positive CSF-c neurons in the lateral walls of the central canal. **Inset**, Detail of positive cells. Asterisk, ventricle. For abbreviations, see the list. Scale bars: 100 μm (A, I, L, P, Q), 50 μm (B, C, E, F, G, H, J, K, M, N, O, and inset in Q), 250 μm (D).

Figure 4. Photomicrographs of *in situ* hybridized transverse sections showing the expression of *SSTb* mRNA in cells of the hypothalamus of mature (A, B), juvenile (C, D) and larval (E, F, G) sea lampreys. Asterisk, ventricle. For abbreviations, see the list. Scale bars: 100 μm (A, E), 50 μm (B, C, D, F, and G).

Figure 5. Photomicrographs of *in situ* hybridized transverse sections of juvenile sea lampreys (j) showing the expression of *SSTc* mRNA in cells of the telencephalon and hypothalamus (secondary prosencephalon). **A-B**, Sections through the medial pallium showing small positive neurons in the periventricular layer. **C**, Section through the basal telencephalon and preoptic region showing abundant positive neurons in the striatum and dorsal and ventral parts of the preoptic

recess nucleus. **Inset**, detail of positive cells. **D**, Section through the caudal telencephalon showing some positive neurons in the subhippocampal lobe and lateral pallium. **E**, Detail of the *SSTc* positive neuronal populations located around the preoptic recess. **F**, Positive neurons in the ventral part of the nucleus of the tract of the postoptic commissure. **G**, Positive neurons in the anterior hypothalamus. **H**, Neurons in the dorsal nucleus of the tract of the postoptic commissure. **I, J**, Sections showing differences between positive cells of the dorsal and ventral regions of the nucleus of the posterior commissure. Asterisk, ventricle. For abbreviations, see the list. Scale bars: 100 μm (A, C, D, H, I), 50 μm (B, E, F, G, J, inset in C).

Figure 6. Photomicrographs of *in situ* hybridized transverse sections of juvenile sea lampreys (j) showing the expression of *SSTc* mRNA in cells of the diencephalon (A-E), mesencephalon (E-I) and isthmus (J-L). **A**, Compact group of positive cells in the posterior tubercle nucleus. **B, C**, Positive cells in rostral and caudal regions of the Nmlf. **D, E**, Scattered positive cells in the lateral pretecal area. **F, G**, *SSTc* positive neurons in rostral and caudal levels of the TS, respectively. **H-I**, Abundant *SSTc* neurons in the periculomotor region, close to the M3 cell. **J-L**, *SSTc* positive populations in rostral and caudal levels of the isthmus tegmentum. Asterisk, ventricle. For abbreviations, see the list. Scale bars: 100 μm (A, B, C, D, E, G, H, I, J, K, L), 50 μm (F).

Figure 7. Photomicrographs of *in situ* hybridized transverse sections of juvenile (j) and mature (m) adult sea lampreys showing the expression of *SSTc* mRNA in cells of the hindbrain (A-E) and spinal cord (F). **A**, Section showing ventral cells in the ARRn. **B-C**, Sections showing abundant positive neurons in the rostral region of the OLA. **D-E**, Sections showing reticular cells (arrows) near the lateral hindbrain surface at levels of the glossopharyngeal and vagal motor nuclei. **F**, Section showing positive neurons located over the giant axons of the mlf. Asterisk, ventricle. For abbreviations, see the list. Scale bars: 50 μm (A, B, C, F), 100 μm (D, E).

Figure 8. Photomicrographs of selected *in situ* hybridized transverse brain sections of larval sea lampreys (l) showing *SSTc* expression in the rostral hypothalamus (A),

basal diencephalon (B), mesencephalon (C), rostral and caudal rhombencephalon (D-E) and spinal cord (F). Asterisk, ventricle. For abbreviations, see the list. Scale bars: 50 μm (A, D), 100 μm (B, C, E, F).

Figure 9. Analysis of the relationships of lamprey SST precursors and peptides with those from other species. **A**, BLOSUM62 cluster map of SST precursors. Nodes correspond to the precursors and are colored according to the key. Edges correspond to BLAST connections of P value $>1\text{e-}20$. **B**, Alignment of the lamprey SSTa predicted mature peptide with the predicted mature peptides from different gnathostome SST1 precursors. Basic cleavage sites are also included with the mature peptide sequences. **C**, Alignment of the lamprey SSTb predicted mature peptide with the predicted mature peptides from gnathostome SST2 precursors. **D**, Alignment of the lamprey SSTb predicted mature peptide with the predicted mature peptides from gnathostome SST6 precursors. Sequences were highlighted in taxon-specific colors: blue (agnathans), purple (mammals), orange (sauropsids), yellow (lobe-finned fishes), green (ray-finned fishes), pink (cartilaginous fishes). Species names in the alignments are as follows: Pmar (*Petromyzon marinus*), Ljap (*Lethenteron japonicum*), Lflu (*Lampetra fluviatilis*), Hsap (*Homo sapiens*), Btau (*Bos taurus*), Ggal (*Gallus gallus*), Acar (*Anolis carolinensis*), Lcha (*Latimeria chalumnae*), Locu (*Lepisosteus oculatus*), Drer (*Danio rerio*), Gpet (*Gnathonemus petersii*), Scan (*Scylorhinus canicula*), Rtyp (*Rhincodon typus*), Cmil (*Callorhinchus milii*).

Supplementary figure 1. Raw figure of the cluster analysis presented in Figure 9A. BLOSUM62 cluster map of somatostatin precursors. Nodes correspond to the precursors and are colored according to the key. Edges correspond to BLAST connections of P value $>1\text{e-}20$. The names of the sequences are included in the figure.

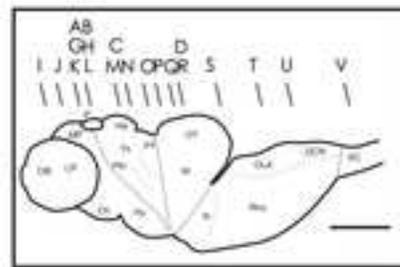
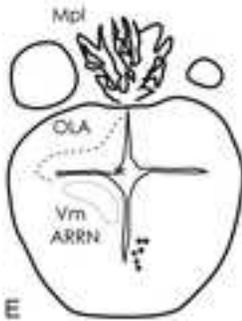
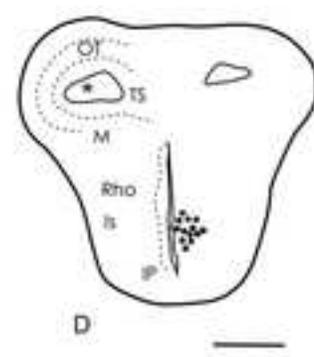
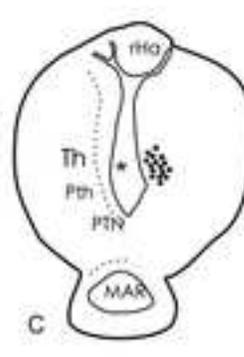
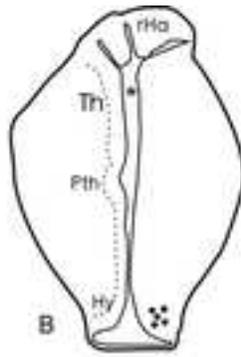
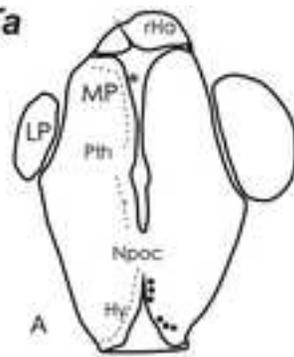
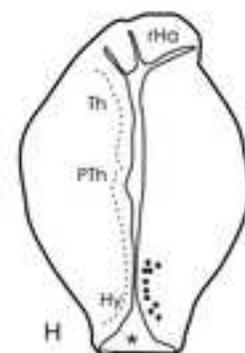
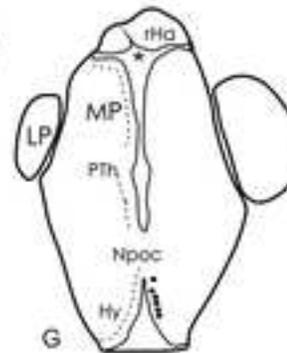
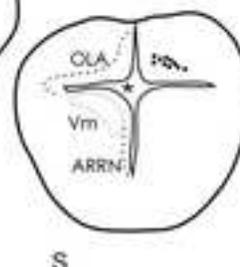
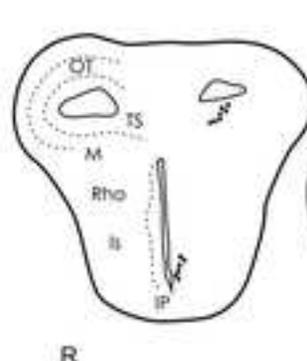
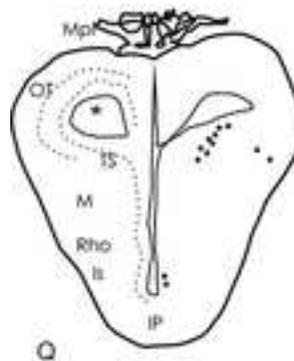
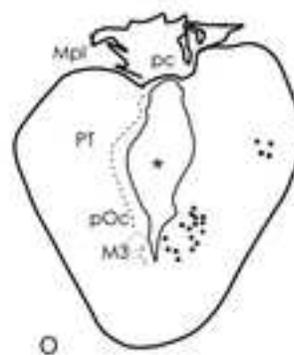
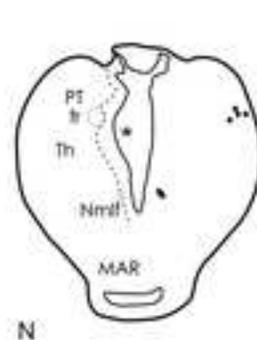
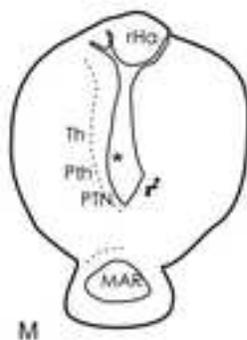
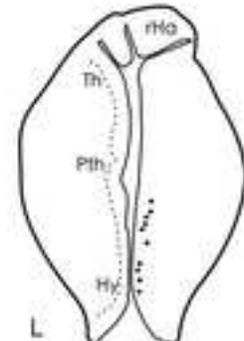
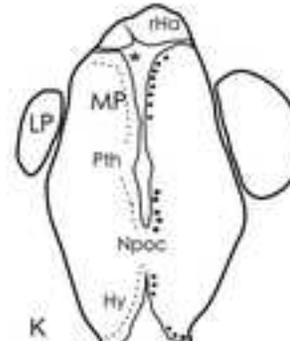
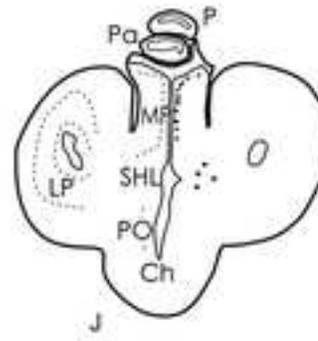
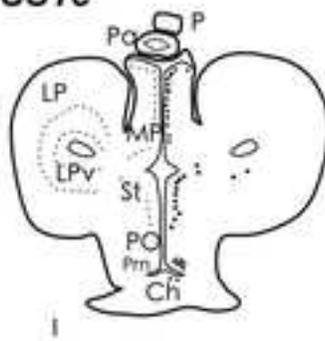
Supplementary file 1. Sequences used for the cluster analysis and alignment performed and shown in figure 1.

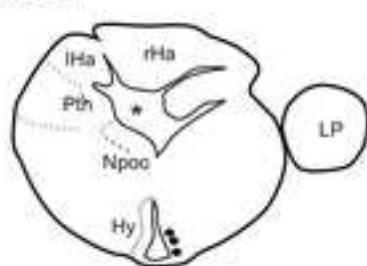
Abbreviations used in the figures

ARRN anterior rhombencephalic reticular nucleus

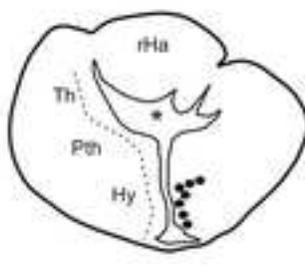
Cc	central canal of spinal cord
Ch	optic chiasm
DC	dorsal column
DCN	dorsal column nucleus
DIG	dorsal isthmic grey
dNpoc	dorsal part of the nucleus of the tract of the postoptic commissure
fr	fasciculus retroflexus
Ha	habenula
Hy	hypothalamus
Hyp	hypophysis
I1	I1 isthmic giant reticulospinal neuron
III	oculomotor nucleus
IP	interpeduncular nucleus
Is	Isthmus
IXm	glossopharyngeal motor nucleus
j	juvenile (postmetamorphic adult)
l	larva
lHa	left habenula
LP	lateral pallium
LPv	ventral region of the lateral pallium
m	mature adult (upstream migrating)
M	mesencephalon
MAR	mamillary recess
M2	M2 giant Müller reticulospinal cell
M3	M3 giant Müller reticulospinal cell
M5	Schober's M5 nucleus of the mesencephalon
mlf	medial longitudinal fascicle
MP	medial pallium (prethalamine eminence of Pombal et al. 2009)
Mpl	mesencephalic choroid plexus
MRA	mesencephalic reticular area
Nmlf	nucleus of the medial longitudinal fascicle
Npoc	nucleus of the tract of the postoptic commissure
OB	olfactory bulbs
OLA	octavolateralis area

OT	optic tectum
ot	optic tract
P	pineal organ
Pa	parapineal organ
pc	posterior commissure
PO	preoptic nucleus
pOc	periculomotor region (griseum centrale of Olson et al. 2017)
poc	postoptic commissure
pOMN	posterior octavomotor nucleus
Pr	preoptic recess
Prn	preoptic recess nucleus
PRRN	posterior rhombencephalic reticular nucleus
PT	pretectum
Pth	prethalamus (ventral thalamus)
PTN	posterior tubercle nucleus
PTu	paratubercular region
rHa	right habenula
Rho	rhombencephalon
Rpl	rhombencephalic choroid plexus
SC	spinal cord
sco	subcomissural organ
SHL	subhippocampal lobe
SO	spinooccipital motor nucleus
St	striatum
T	telencephalon
Th	thalamus (dorsal thalamus)
TS	torus semicircularis
Vd	descending trigeminal tract
Vm	trigeminal motor nucleus
vNpoc	ventral part of the nucleus of the tract of the postoptic commissure
Xm	vagal motor nucleus

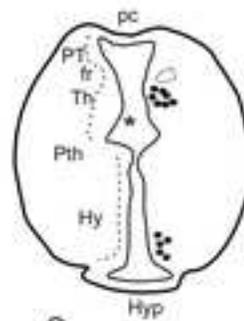
SSTa**SSTb****SSTc**

SSTa

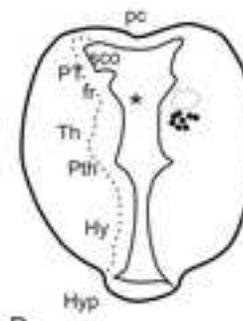
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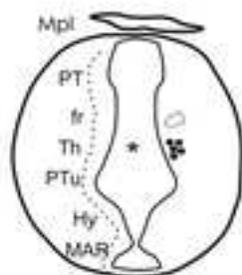
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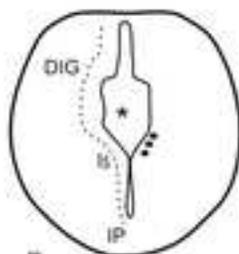
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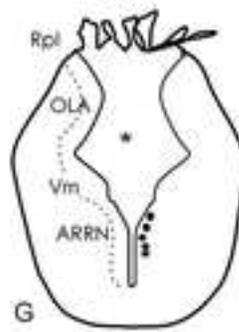
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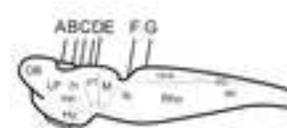
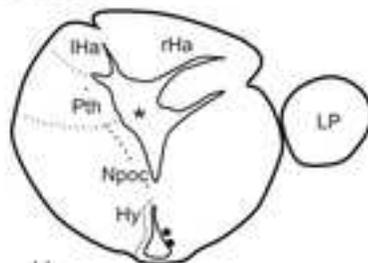
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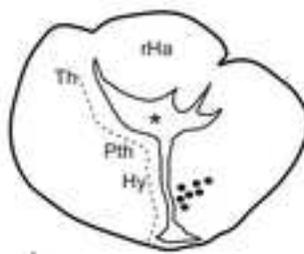
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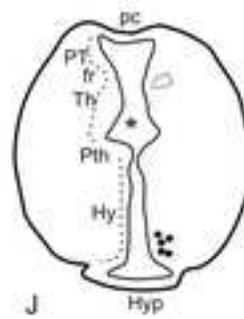
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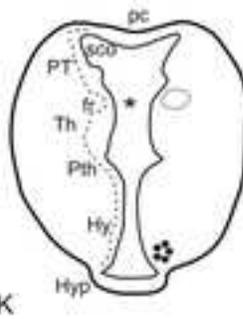
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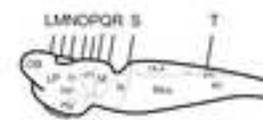
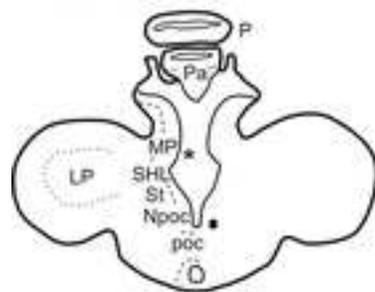
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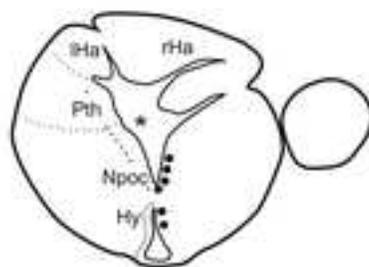
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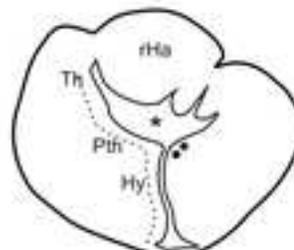
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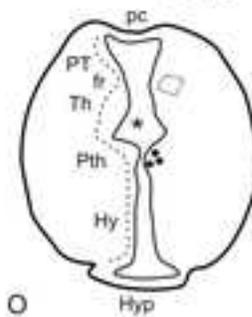
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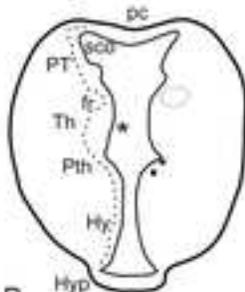
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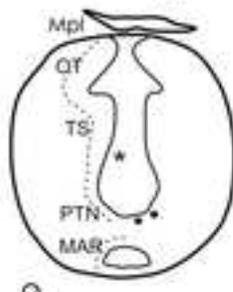
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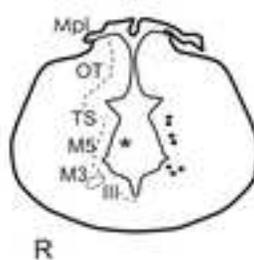
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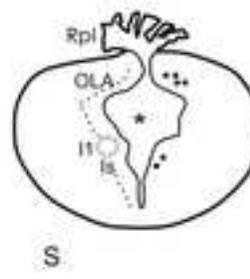
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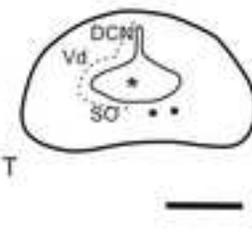
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R



S



T



