The Shark Basal Hypothalamus: Molecular Prosomeric Subdivisions and Evolutionary Trends

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The hypothalamus is a key integrative center of the vertebrate brain. To better understand its ancestral morphological organization and evolution, we previously analyzed the segmental organization of alar subdivisions in the catshark Scyliorhinus canicula, a cartilaginous fish and thus a basal representative of gnathostomes (jawed vertebrates). With the same aim, we deepen here in the segmental organization of the catshark basal hypothalamus by revisiting previous data on ScOtp, ScDlx2/5, ScNkx2.1, ScShh expression and Shh immunoreactivity jointly with new data on ScLhx5, ScEmx2, ScLmx1b, ScPitx2, ScPitx3a, ScFoxa1, ScFoxa2 and ScNeurog2 expression and proliferating cell nuclear antigen (PCNA) immunoreactivity. Our study reveals a complex genoarchitecture for chondrichthyan basal hypothalamus on which a total of 21 microdomains were identified. Six belong to the basal acroterminal region, the rostral-most point of the basal neural tube; seven are described in the tuberal region (Tu/RTu); four in the perimamillar region (PM/PRM) and four in the mamillar one (MM/RM). Interestingly, the same set of genes does not necessarily describe the same microdomains in mice, which in part contributes to explain how forebrain diversity is achieved. This study stresses the importance of analyzing data from basal vertebrates to better understand forebrain diversity and hypothalamic evolution.

INTRODUCTION

The hypothalamus is an important physiologic center of the brain. It integrates information from limbic, endocrine and autonomic sources to elaborate different kinds of homeostatic and behavioral responses such as feeding or reproduction. Its organization has been elusive for neuroanatomists as result of complex patterning processes converging at this point.

Abbreviations: ABB, alar-basal boundary; Ah, adenohypophysis; AHy, alar hypothalamus; ap3, prosomere 3, alar part; BA, basal acroterminal subdomain; HDB, hypothalamic-diencaphalic border; hp1, prosomere hp1 or peduncular; hp2, prosomere hp2 or terminal; IHB, intrahypothalamic border; MM, mammillary area; Nh, neurohypophysis; P, pallium; p3Tg, prosomere 3, tegmental part; Pa, paraventricular area; PRM, perirhinal-mammillary area; PSPa, subparaventricular area, peduncular part; PThE, prethalamic eminence (ap3); RM, retromammillary area; RTu, retrotuberal area; Sp, subpallium; SpA, subparaventricular area; Sv, saccus vasculosus; T, telencephalon; TPa, paraventricular area, terminal part; TSPa, subparaventricular area, terminal part; Tu, tuberal area; zli, zona limitans intrathalamica.
Scyliorhinus canicula

work, we sketched prosomeric organization in the catshark of the vertebrate brain (Coolen et al., 2009). In previous
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2017; Domínguez et al., 2013, 2014, 2015; González et al., 2017; la-Torre et al., 2011; Medina et al., 2011; Moreno et al., 2012, comparati
ekay for homologies establishment and is largely accepted as a
microdomains proposed by the prosomeric framework rely
segments, boundaries, histogenetic domains, subdomains and
the development of structures like the optic chiasm or the
plates meet, a region referred as acroterminal is recognized.
Furthermore, at the rostral-most hp2, where the alar and basal
subparaventricular area (TSPa/PSPa); tuberal and retrotuberal
subparaventricular area (TPa/PPa); terminal and peduncular
also indicated by the particle "retro"): terminal and peduncular
separated by the alar-basal boundary (ABB). Moreover, these
dorso-ventral domains can be further subdivided into two rostro-caudal subdomains (terminal or peduncular; the last also
indicated by the particle "retro"): terminal and peduncular
paraventricular area (TPa/PPa); terminal and peduncular
paraventricular area (TPsa/TPsa); tuberal and retrotuberal
area (Tu/RTu); perimammillary and periretromammillary area
(PM/PRM); mammillary and retromammillary area (MM/RRM;
Morales-Delgado et al., 2011, 2014; Puelles et al., 2012; Díaz
et al., 2015; Ferrán et al., 2015; Rodríguez-Moldes et al., 2017).
Noteworthy, the underlying logic of
The updated prosomeric view of the hypothalamus understands it to be organized into five longitudinal histogenetic
domains dorso-ventrally arranged into two alar and three basal
domains (Puelles et al., 2012; Puelles and Rubenstein, 2015)
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Morales-Delgado et al., 2011, 2014; Puelles et al., 2012; Puelles
et al., 2015). As a result, the prosomeric framework became
key for homologies establishment and is largely accepted as a
comparative tool (Puelles and Rubenstein, 2003; Martínez-de-
la-Torre et al., 2011; Medina et al., 2011; Moreno et al., 2012,
2017; Dominguez et al., 2013, 2014, 2015; González et al., 2017;
Pombal and Megias, 2017; Rodríguez-Moldes et al., 2017).
Cartilaginous fishes, also known as Chondrichthians, are
a key group for evo-devo studies. They are among the most
basal extant groups of gnathostomes (jawed vertebrates) being
the closest out-group to osteichthians (the other major phylum
of gnathostomes, which includes bony fishes and tetrapods).
Therefore, they are essential to address the ancestral condition
of the vertebrate brain (Coolaen et al., 2009). In previous
work, we sketched prosomeric organization in the catshark
Scyliorhinus canicula to better understand the ancestral condition
of the vertebrate hypothalamus (Santos-Durán et al., 2015).

In this study, the expression of ScNkx2.1, ScDlx2/5, ScShh
and ScOtp led to the identification of alar and basal domains,
apparently homologous to the murine ones. However, this
work also suggested that alar organization seems to be more
conserved than basal one, what correlates with the development
of conserved and divergent adult structures, respectively
(Santos-Durán et al., 2015; Rodríguez-Moldes et al., 2017). In subsequent
work we deeply tested prosomeric assumptions in the alar
hypothalamus on the light of additional makers. Our findings
suggested conserved traits that can be traced back to the
agnathan-gnathostome transition (Santos-Durán et al., 2016).
Now, we revisit the organization of the basal hypothalamus
with similar aims: (i) to look for further prosomeric molecular subdivisions; (ii) to test if new data on gene expression patterns
support previous prosomeric interpretations; and (iii) to obtain
some insights on the evolution of this region by comparative
analysis. Noteworthy, conserved adult structures (i.e., tracts
of the hypothalamic-hypophyseal system, a median eminence
or the neurohypophysis) and divergent ones (i.e., inferior
hypothalamic lobes and the saccus vasculosus) emerge from
this territory offering an attractive scenario for evolutionary
insights. To address these questions, previous data on ScNkx2.1,
ScDlx2/5, ScOtp, ScShh expression and Shh immunoreactivity
were revised jointly with new data on ScLhx5, ScEmx2, ScLmx1b,
ScPitx2, ScPitx3a, ScFoxa1, ScFoxa2 and ScNeurog2 expression
and proliferating cell nuclear antigen (PCNA) immunoreactivity
patterns. Here we were able to identify a plethora of subdomains
(microzones) in the catshark hypothalamus. A comparative
analysis of microzone identity in catshark is made with
mammals but not with other vertebrates due to the lack of
detailed data. However gross comparisons among vertebrates
prompt the idea that the caudal border of the hypothalamus,
as it is currently defined, could be a derived character
rather than a conserved one, a feature that deserves further
investigation.

MATERIALS AND METHODS
Experimental Animals
Some embryos of the catshark (lesser spotted dogfish; S. canicula)
were supplied by the Marine Biological Model Supply Service of the
CNRS UPMC Roscoff Biological Station (France). Additional
embryos were kindly provided by the Aquaria of Gijón (Asturias,
Spain), O Grove (Pontevedra, Spain) and Finisterrae (A Coruña,
Spain). Embryos were staged by their external features according
to Ballard et al. (1993). For more information about the
relationship of embryonic stages with body size, gestation and birth,
see Table 1 in Ferreiro-Galve et al. (2010). Sixty-nine
embryos from stages 28 to 32 were used in this study. Eggs from
different broods were raised in seawater tanks in standard
conditions of temperature (15–16°C), pH (7.5–8.5) and salinity
(35 g/L). Adequate measures were taken to minimize animal
pain or discomfort. All procedures conformed to the guidelines
established by the European Communities Council Directive of 22 September 2010 (2010/63/UE) and by the Spanish Royal
Decree 53/2013 for animal experimentation and were approved
by the Ethics Committee of the University of Santiago de Compostela.

**Tissue Processing**

Embryos were deeply anesthetized with 0.5% tricaine methane sulfonate (MS-222; Sigma, St. Louis, MO, USA) in seawater and separated from the yolk before fixation in 4% paraformaldehyde (PFA) in elasmobranch's phosphate buffer [EPB: 0.1 M phosphate buffer (PB) containing 1.75% urea, pH 7.4] for 48–72 h depending on the stage of development. Subsequently, they were rinsed in phosphate buffer saline (PBS), cryoprotected with 30% sucrose in PB, embedded in OCT compound (Tissue Tek, Torrance, CA, USA), and frozen with liquid nitrogen-cooled isopentane. Parallel series of sections (12–20 µm thick) were obtained in transverse planes on a cryostat and mounted on Superfrost Plus (Menzel-Glasser, Madison, WI, USA) slides.

**Single and Double Immunohistochemistry on Sections and Whole Mounts**

For heat-induced epitope retrieval, sections were pre-treated with 0.01 M citrate buffer (pH 6.0) for 30 min at 95°C and allowed to cool for 20–30 min at room temperature (RT). Sections were then rinsed twice in 0.05 M Tris-buffered saline (TBS; pH 7.4) for 5 min each and incubated overnight with the primary antibody (polyclonal rabbit anti-Sonic Hedgehog [anti-Shh], Santa Cruz Biotechnology, Santa Cruz, CA, USA, diluted 1:300; monoclonal mouse anti-proliferating cell nuclear antigen [anti-PCNA] Sigma, St. Louis, MO, USA, diluted 1:500). Appropriate secondary antibodies (horseradish peroxidase [HRP]-conjugated goat anti-rabbit and anti-mouse, BIORAD, diluted 1:200) were incubated for 2 h at RT. The immunoreaction was developed with secondary antibodies (horseradish peroxidase [HRP]-conjugated goat anti-rabbit, BIORAD, diluted 1:200 in TSTM) was incubated overnight. After a final washing in TST, the embryos were pre-incubated with 0.25 mg/mL dianobenzidinetetrachloride (DAB, Sigma) in TST with 2.5 mg/mL nickel ammonium sulfate for 1 h, and then allowed to react with DAB in TST containing 0.00075% H2O2 for 20–40 min at RT. The reaction was stopped using Tris-HCl buffered saline and specimens were post-fixed with 4% PFA overnight at 4°C. Epidermis and mesodermic derivatives were carefully removed and specimens were rinsed in graded series of glycerol (25%, 50%, 75% and 100%) and observed under the stereomicroscope.

**Controls and Specificity of the Antibodies**

No immunostaining was detected when primary or secondary antibodies were omitted during incubations. The monoclonal anti-PCNA antibody specifically labels proliferating cells in the brain, retina and olfactory epithelium of this species (Rodriguez-Moldes et al., 2008; Ferrando et al., 2010; Ferreiro-Galve et al., 2010; Quintana-Urzainqui et al., 2014). The polyclonal anti-Shh antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was raised in rabbit against the amino acids 41–200 of the human Shh protein. We previously reported that the in situ hybridization (ISH) results were similar to those obtained by IHC, and therefore validate the specificity of the anti-Shh antibody used here (Santos-Durán et al., 2015).

**In Situ Hybridization on Sections and Whole Mounts**

We applied in situ hybridization for ScOtp (Quintana-Urzainqui, 2013; Santos-Durán et al., 2015, 2016), ScDlx2 (Quintana-Urzainqui et al., 2012, 2015; Compagnucci et al., 2013, 2016; Debias-Thibaud et al., 2013; Quintana-Urzainqui, 2013; Santos-Durán et al., 2015, 2016), ScDlx5 (Compagnucci et al., 2013; Debias-Thibaud et al., 2013; Santos-Durán et al., 2015, 2016), ScNkx2.1 (Quintana-Urzainqui et al., 2012; Quintana-Urzainqui, 2013; Santos-Durán et al., 2015, 2016), ScLhx5 (Santos-Durán et al., 2016), ScEmx2 (Derobert et al., 2002), ScLmx1b (Pose-Méndez et al., 2016), ScPitx2 (Lagadec et al., 2015), ScPitx3a, ScFoxa1, ScFoxa2 and ScNeurog2 (Santos-Durán et al., 2016) genes. These probes were selected from a collection of S. canicula embryonic cDNA library (mixed stages S9–S22), constructed in pSPORT1, and submitted to high throughput EST sequencing. Selected cDNA fragments were cloned in pSPORT vectors. Sense and antisense digoxigenin-UTP-labeled and fluorescein-UTP-labeled probes were synthesized directly by in vitro transcription using as templates linearized recombinant plasmid DNA or cDNA fragments prepared by PCR amplification of the recombinant plasmids. In situ hybridization in whole mount and on cryostat sections was carried out following standard protocols (Cooen et al., 2009). Briefly, sections were permeabilized with proteinase K, hybridized with sense or antisense probes overnight at 65°C and incubated with the alkaline phosphatase-coupled anti-digoxigenin and anti-fluorescein antibody (1:2000, Roche Applied Science, Manheim, Germany) overnight at 4°C. The color reaction was performed in the presence of BM-Purple...
**RESULTS**

**ScNkx2.1 and ScDlx2/ScDlx5 Expression. Comparison With ScShh-Expression/Shh Immunoreactivity**

An overview of the expression of ScShh, ScNkx2.1, ScOtp and ScDlx2/ScDlx5 in the basal hypothalamus of *S. canicula*, mainly in early stages of development, has been previously described.
in Santos-Durán et al. (2015). The location of the ABB was re-examined in Santos-Durán et al. (2016).

Here we revisited these data to deepen in the genoarchitectonic profile of the basal hypothalamus and further characterize possible dorso-ventral and rostro-caudal subdomains of this territory. A detailed comparative analysis of the expression of such genes in sagittal and transverse sections is presented from stages 29 to 32, when the basic mature cytoarchitecture and organization of the adult hypothalamus are clearly recognized.

**ScShh-Expression/Shh Immunoreactivity**

Since ScShh detection by means of ISH at early developmental stages yields similar results to those obtained by IHC against anti-Shh (Santos-Durán et al., 2015), here we have used the antibody to analyze additional developmental stages and to ease pattern comparisons by means of double ISH-IHC staining. As described in Santos-Durán et al. (2015), from stage 29 onwards, Shh immunoreactivity is observed in part of the rostral and dorsal Tu domain and broadly detected within the RM domain, extending from here along the diencephalic basal plate (see Figures 1A–F; see also Santos-Durán et al., 2015). Shh immunoreactivity is not observed in the SPA domain (Figures 1B,E; see also Santos-Durán et al., 2016) or in the midline (acroterminal territory) just dorsal to the developing adenohipophysis (black arrowhead in Figure 1E). Caudally, Shh immunoreactivity is only observed in a portion of the RM domain but not at its dorsal-most and ventral-most portions (Figure 1B and arrowhead in Figure 1F). At stage 30, Shh immunoreactivity is still detected in the hypothalamus and primary prosencephalon (arrowheads in Figure 1G; see also Santos-Durán et al., 2015) but it becomes reduced in the RM and basal plate of p3 (p3Tg) compared to previous stages.

**ScNkx2.1 Expression**

From stage 29 onwards, ScNkx2.1 is expressed ventral to the optic stalk through the whole basal hypothalamus except in the RM compartment (see Figures 1A–C,H–K). While ScNkx2.1 expression and Shh immunoreactivity co-distribute in part of the Tu domain (Figures 1C,I), Shh immunoreactivity does not match the dorsal border of ScNkx2.1 expression (black arrows in Figure 1I). In contrast to Shh, ScNkx2.1 is additionally expressed in the acroterminal territory dorsal to the adenohipophysis (arrowhead in Figure 1I). ScNkx2.1 expression in the MM abuts the RM, but it does not meet Shh immunoreactivity since Shh is absent from the ventral-most portion of RM, which creates a gap between both (Figures 1C,J; see also Santos-Durán et al., 2015). Of note, ScNkx2.1 expression in the ventricular zone forms a clear-cut border between the positive MM and
the negative RM domain that is more evident on sagittal sections (Figure 1K), though ScNkx2.1-expressing cells can be detected in the mantle of the RM domain (black arrowhead in Figures 1J,K).

**ScDlx2/ScDlx5 Expression**
In the basal plate, ScDlx2/5 is intensely expressed in a restricted subdomain of the Tu/RTu and the p3Tg domains (Figure 1L). In the hypothalamus, it is expressed in a subdomain spreading from the RTu to the neurohypophysis (Figures 1L–N). Rostrally, ScDlx2/5 expression in the basal hypothalamus co-distributes with Shh immunoreactivity in a subdomain of the Tu (Figures 1C,M). Note that neither ScDlx2/5 expression nor Shh immunoreactivity can be observed in the acrotterminal territory co-extensive with the adenohypophysis (arrowhead in Figure 1M) but it is expressed in the neurohypophysis (Figure 1L). ScDlx2/5 expression in the caudal-most RTu almost abuts Shh immunoreactivity in the RM although a gap exists (Figure 1C; arrow in Figure 1N). In the most caudo-ventral part of Tu, individual and dispersed ScDlx2/5-expressing cells can be recognized almost reaching the rostral and ventral-most part of the PM (arrowheads in Figures 1L,N) including the primordium of the saccus vasculosus. These cells are less intensely labeled but still observable at stage 31 (arrowhead in Figure 1O). At stage 32 the basic pattern described for ScDlx2/5 is maintained although its expression becomes reduced in intensity (see below).

**ScLhx5 and ScOptp Expression**

**ScLhx5 Expression**
From stage 29 onwards, in the basal plate, ScLhx5 is observed in a subdomain of the dorsal-most and rostral-most Tu domain (Figures 2A,B,I,J). Dispersed ScLhx5-expressing cells are also observed in the most caudo-ventral part of Tu where individual and dispersed ScDlx2/5-expressing cells were observed (compare Figure 2D with Figure 1O). ScLhx5 expression can also be observed in the PM/PRM and MM domains (Figures 2A–D,I,J). Note that ScLhx5 expression in the MM domain describes a clear-cut border with the RM domain (Figure 2D), though ScLhx5-expressing cells can be recognized in the mantle of the RM domain (arrowheads in Figures 2C,D).
ScOptp Expression
In the basal plate, ScOptp has been identified in Tu and PM/PRM domains (Santos-Durán et al., 2015). From stage 29 onwards ScOptp is expressed in the rostral-most part of the Tu domain, from the optic stalk to the primordial neurohypophysis (Figure 2E). Specifically, it is restricted to the acroterminal territory of the Tu domain just dorsal to the adenohypophysis (Figures 2E, I; arrowhead in Figure 2F) codistributing with ScNkx2.8 (arrowhead in Figure 2F). In the PRM domain, ScOptp expression abuts the RM but not the Shh immunoreactivity of this domain (Figures 2G, J). In the PM, ScOptp is also expressed in the acroterminal territory (Figure 2E). Note that the expression of ScOptp in the PM faces the MM (Figure 2H). Marginal ScOptp-expressing cells can be recognized in the RM (black arrowheads in Figures 2G, H) and p3Tg (not shown). This pattern is maintained until stage 32.

ScEmx2 Expression
The expression of ScEmx2 has been analyzed by Derobert et al. (2002) in the brain and related tissues from early stages of development (stage 19) until mid-gestation stages (stages 28–30). Here we analyze in detail the expression of ScEmx2 in the basal hypothalamus from stage 29 until stage 31. From stage 29 onwards, ScEmx2 is expressed in the basal hypothalamus in a well-defined domain spreading into part of rostral and ventral-most Tu domain, the PM/PRM and the MM domains (Figures 3A–D). Of note, its expression lacks in midline domains of the Tu (acroterminal territory) such as the neurohypophysis.
ScLhx5 and ScOtp in the PM/PRM (compare ScLhx5 in the PM/PRM and MM (compare expressing domain includes the caudal domain expressing the RM domain (compare expressing domain in the PRM is fairly complementary to Shh in Figures 3E–G (arrowheads in domain (arrowheads in immediately caudal to the last (arrowheads in Figures 3A,E)). The caudal border of ScEmx2 expression in the MM domain (arrowheads in Figures 3A,E) correlates with a domain of reduced PCNA immunoreactivity in the ventricular zone (arrowheads in Figures 3E–G). The caudal border of ScEmx2 expression in the PRM domain also correspond with a domain of restricted PCNA immunoreactivity (compare arrowheads in Figures 3D,H). Thus, a band of reduced or negative proliferation seems to spread from the rostral and dorsal border of the RM (Figures 3F–H), p3Tg and zona limitans intrathalamic (zli; not shown).

Finally, we compared ScEmx2 expression with that of other genes usually expressed in the basal hypothalamus to better understand its organization. A comparison with Shh immunoreactivity (Figure 3I) revealed that the ScEmx2-expressing domain in the PRM is fairly complementary to Shh in the RM domain (compare Figures 3A,I). Besides, this ScEmx2-expressing domain includes the caudal domain expressing ScLhx5 in the PM/PRM and MM (compare Figures 3A,J) and ScOtp in the PM/PRM (compare Figures 3B,L). Of note, ScEmx2, ScLhx5 and ScOtp define consecutively more restricted domains (compare Figures 3B,K,L). Moreover, the expression of ScEmx2 abuts that of ScDlx2/5 in dorsal and caudal positions (RTu domain) while they co-distribute in more rostral and ventral positions (not shown).

**ScLmx1b, ScPitx2, ScPitx3a, ScFoxa1, ScFoxa2 and ScNeurog2 as Markers of the RM**

ScFoxa1 and ScFoxa2 are expressed in fairly the same spatial and temporal patterns in the regions and stages considered in this study and thus are conjointly referred as ScFoxa1/2.

At stage 29 ScLmx1b, ScPitx2, ScPitx3a, ScFoxa1/2 and ScNeurog2 are expressed in a similar pattern spreading caudally from RM into the diencephalon including the zli in the case of ScPitx2, ScPitx3a, ScFoxa1/2 and ScNeurog2 (Figures 4A–D,B′,C′,O). On transverse sections the expression of these genes dorsally abuts the PRM (Figures 4E–H,F′). Rostrally these genes also abut the MM (arrowheads in Figures 4E–H). From stage 29 onwards this general pattern persists although some differences emerge. At stage 31, ScLmx1b becomes downregulated being restricted to the floor plate (Figure 4I) while Shh immunoreactivity is still found in the basal plate (arrowheads in Figure 4I). At stage 31 ScPitx2 (Figure 4J) is still expressed in the pattern observed at stage 29, while ScPitx3a is restrictedly expressed in the caudal-most diencephalon (not shown). In the case of ScFoxa1/2, there is slight dorsal and ventral downregulation but the main pattern persists through RM and diencephalon (Figure 4K). From stage 30 onwards, ScNeurog2 becomes downregulated in the mentioned territories although it can be recognized in the zli and habenulae (data not shown). Finally, ScLmx1b, ScPitx2 and ScFoxa1/2 still present a sharp border of expression that abuts the MM domain at this developmental stage (arrowheads in Figures 4L–N).

**DISCUSSION**

In a previous work, we identified the shark basal hypothalamus harboring three domains (Tu/RTu, PM/PRM and MM/RM) based on the basal expression of ScNkx2.1, ScShh, ScOtp and ScDlx2/5 (Santos-Durán et al., 2015). In the present work we revisit such analysis on the light of ScLhx5, ScEmx2, ScLmx1b, ScPitx2, ScPitx3a, ScFoxa1, ScFoxa2 and ScNeurog2 expression, besides Shh and PCNA immunoreactivity. Different subdomains were identified within the aforementioned domains and within the basal acroterminal region, the basal rostral-most neural tube (Figures 5, 6; Table 1). These genes present a robust and conserved expression across vertebrates. However, their roles and functions are still poorly understood and their expressions do not necessarily define domains per se. Therefore, caution has to be borne in mind during the following analysis.

**Basal Acroterminal Domains**

The acroterminal domain involves the alar and basal plate spreading from the rostral-most roof plate to the rostral-most floor plate. It has been suggested that specialized structures like...
the **lamina terminalis**, the optic chiasm and the neurohypophysis emerge here under particular signaling events (Puelles et al., 2012; Puelles and Rubenstein, 2015). We have identified at least 6 subdomains inside the basal acroterminal region named 1–6 from dorsal to ventral (BAt1–6; Figure 6B). Noteworthy, the acroterminal territory (medial) is easily distinguishable from the remaining hypothalamus (lateral) due to genes differentially expressed at these locations (compare Figures 6A,B).

The two dorsal-most domains (BAt1–2) are positive for **ScNkx2.1** (Figure 1I) and **ScOtp** (Figure 2F) but only BAt1 shows expression of **ScLhx5** (Figure 3J). Furthermore, ScOtp co-distributes with ScNkx2.8 (Figures 2F,F'). BAt1–2 subdomains are negative for other genes broadly expressed in the Tu such as **ScDlx2/5** and Shh (compare Figure 1M with Figure 2F). Of note, at later stages ScLhx5 is absent from the midline (arrowhead in Figure 3K). Noteworthy, BAt1–2 are almost co-extensive with the developing adenohypophysis (Figures 11, 6B), which in part is co-extensive with negative subdomains for ScDlx2/5-expression and Shh immunoreactivity (Figure 1M). Of note, these gaps are as wide as the adenohypophysis, which has been noted in other vertebrates even for different adenohypophysis sizes (see Figure 2N in Manning et al., 2006), suggesting a role for the adenohypophysis in the local patterning of the hypothalamus. In shark, both the gaps of ScDlx2/5-expression and Shh immunoreactivity and the expression of ScNkx2.8 are wider than the medio-lateral extension of ScOtp-expression (Figures 2F,F'), which suggests the existence of additional medio-lateral subdomains.
BAt3 (the acroterminal region at the level of the neurohypophysis) is also Shh immunonegative and also expresses ScNkx2.1 (Figures 1H,1), but differently from BAt1–2, it expresses ScDlx2/5 (Figures 1L, 6B).

Ventrally to BAt3, we identified BAt4 as a subdomain that corresponds to the primordium of the saccus vasculosus (Figure 6B; see also Van de Kamer and Shuurmans, 1953; Sueiro et al., 2007). The initial tiny domain expands becoming morphologically distinguishable (stage 29, Figures 1H, 2E; stage 30, Figure 1I). This domain is characterized by the expression of ScNkx2.1 and dispersed ScDlx2/5-expressing cells (Figure 1L). Since ScDlx2/5 is involved in the development of a GABAergic phenotype (Anderson et al., 1999), its expression in the saccus vasculosus could explain the existence of GABAergic cells at this point (Sueiro et al., 2007). Besides, GFAP-immunoreactivity has been described to be restricted to BAt4 (the developing saccus vasculosus), and it is not observable in more caudal subdomains (Sueiro et al., 2007). Finally, BAt4 is also characterized by lack of ScEmx2 (Figure 3B) which, however, is present in more caudal acroterminal subdomains (Figures 3C,D; see also BAt5 in Figure 6B) and in lateral (non-acroterminal) domains (Figures 6A,D). Noteworthy, in S. canicula, the tip of the notochord has been described to reach the primordium of the saccus vasculosus (BAAt; Figure 1 in Van de Kamer and Shuurmans, 1953) suggesting a causal relationship to saccus vasculosus development.

BAt4 shares ScNkx2.1 expression and dispersed ScDlx2/5-expressing cells with the domain ventral to it (BAAt5; Figure 6B). However, as commented above, BAAt5 differentially presents a lack GFAP-immunoreactivity and the absence of ScEmx2 expression (Figures 3C,D; see also Figure 6B) at late stages of development.

The ventral-most acroterminal domain is BAt6 which express ScNkx2.1, ScEmx2, ScOtp and ScLhx5, but not ScDlx2/5 (see Figure 6B). However, we cannot discard that this territory could be interpreted as the floor plate of the MM (Figure 6D) that in mouse (but not in shark; compare Figures 7E,F) differentially expresses Shh (see Figure 8.9B in Puelles et al., 2012).

### Tuberal (Tu/RTu) Subdomains

The dorsal and rostral-most domain is Tu1 and expresses genes like ScNkx2.1 and ScLhx5 (Figures 1H, 2A, 6C). Caudally to it, we have distinguished a similar domain lacking ScLhx5 but expressing ScDlx2/5, named as Tu2 (compare Figure 1L and Figure 2A; see Figure 6C). These two subdomains belong to the subliminal part of the basal hypothalamus and so, they lack ScShh (compare Figures 2L, J; see Figure 6C) and they express ScNkx2.8 and ScLhx9 (see Figure 5B in Santos-Durán et al., 2016). More ventrally, two subdomains, Tu3 and Tu4, appear as the ventral extension of Tu1 and Tu2 respectively, since they share with them either ScLhx5 or ScDlx2/5 expression. However, they additionally express ScShh (Figure 6C) but lack ScNkx2.8 and ScLhx9 expression (see Figure 5B in Santos-Durán et al., 2016). The expression of ScLhx5 in the Tu1/Tu3 appears complementary to that of ScDlx2/5 in Tu2/Tu4 (compare Figure 1L with Figure 2A; see Figure 6C). Complementary patterns between Dlx and Lhx5 have been previously described in the mouse forebrain (Sheng et al., 1997), which suggests a conserved inhibitory relationship between both genes. Moreover, a small domain ventral (and caudal) to Tu4, which expressed ScNkx2.1 and ScDlx2/5 but was negative to Shh immunoreactivity, was referred as Tu5 (Figure 6C).

A more ventral subdomain, referred as Tu6 is characterized by the expression of ScEmx2 and a dispersed distribution of ScDlx2/5- and ScLhx5-expressing cells (compare Figure 1L with Figures 2D and 3A; see also Figure 6C). The dorsal and caudal-most subdomain identified is RTu1 (Figure 6C), which

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TABLE 1 | Microzone histogenetic codes of the shark basal hypothalamus corresponding to schemes in Figure 6.  

| ScNkx2.1 | ScEmx2 | ScOtp | Shh/ScShh | ScDlx2/5 | ScLhx5 | ScLmx1b | ScPitx2 | ScPitx3a | ScFoxa1 | ScFoxa2 | ScNeurog2 |
|-------|-------|-------|-----------|---------|--------|--------|---------|---------|---------|---------|---------|
| BA1   | +     | −     | +         | −       | +      | −      | −       | −       | −       | −       | −       |
| BA2   | +     | −     | +         | −       | +      | −      | −       | −       | −       | −       | −       |
| BA3   | +     | −     | +         | −       | +      | −      | −       | −       | −       | −       | −       |
| BA4   | +     | −     | +         | −       | +      | −      | −       | −       | −       | −       | −       |
| BA5   | +     | −     | +         | −       | +      | −      | −       | −       | −       | −       | −       |
| BA6   | +     | −     | +         | −       | +      | −      | −       | −       | −       | −       | −       |
| Tu1   | +     | −     | −         | +       | −      | +      | −       | −       | −       | −       | −       |
| Tu2   | +     | −     | −         | +       | −      | +      | −       | −       | −       | −       | −       |
| Tu3   | +     | −     | −         | +       | −      | +      | −       | −       | −       | −       | −       |
| Tu4   | +     | −     | −         | +       | −      | +      | −       | −       | −       | −       | −       |
| Tu5   | +     | −     | −         | +       | −      | +      | −       | −       | −       | −       | −       |
| Tu6   | +     | −     | −         | +       | −      | +      | −       | −       | −       | −       | −       |
| RM1   | +     | −     | −         | +       | −      | +      | −       | −       | −       | −       | −       |
| RM2   | +     | −     | −         | +       | −      | +      | −       | −       | −       | −       | −       |
| RM3   | +     | −     | −         | +       | −      | +      | −       | −       | −       | −       | −       |

Asterisks indicate that cells expressing this gene are not located in the ventricular zone. For abbreviations, see list.
FIGURE 7 | Comparative representations of microzones defined in mammals at E13.5 (A,C,E) and chondrichthyans at stage 29 (B,D,F) by sets of ortholog genes. Comparisons consider genes expressed in prosomeric histogenetic domains (A,B, Tu/RTu; C,D, PM/PRM; E,F, MM/RM). For simplicity schemes represent markers (Continued)
expresses the same genes as Tu2 and Tu5 (ScNkx2.1, ScDlx2/5; see Figure 6C).

Perimamillar (PM/PRM) Subdomains

Ventral to Tu6, we identified PM1 a domain where ScNkx2.1/ScEmx2/ScLhx5 are co-expressed (compare Figures 1K, 3C,K; see Figure 6D). We term PM2 (PM-like in Santos-Durán et al., 2015) the subdomain expressing ScNkx2.1/ScEmx2/ScLhx5/ScOtp (compare Figures 1K, 3C,K,1; see Figure 6D). The caudal continuation of PM1 and PM2 are referred as PRM1 and PRM2 (PM-like in Santos-Durán et al., 2015) and express the same genes (Figure 6D).

Mamillar (MM/RM) Subdomains

MM1 (MM-like in Santos-Durán et al., 2015) expresses ScNkx2.1/ScEmx2/ScLhx5 (Figures 1K, 2D, 3A) but not ScOtp (Figures 2H, 6D). Of note, the genes expressed in the MM1 show a clear-cut border with those expressed in the RM domain (RM-like in Santos-Durán et al., 2015; see Figure 6D,E).

We have identified three dorso-ventral subdomains in the ventral and caudal-most part of the basal hypothalamus here referred as RM1, RM2 and RM3 (Figure 6E), which together fairly correspond to the previously defined RM-like territory (Santos-Durán et al., 2015; see also Figure 5). The dorsal-most domain, RM1, may be defined based on lack of Shh immunoreactivity at stage 29 (as does not reaches the ABB; see Santos-Durán et al., 2016) and the expression of ScLmx1b/ScPitx2/ScPitx3a/ScFoxa1/ScFoxa2/ScNeurog2 (compare Figure 1A with Figures 3A–D; see Figure 6E). In the RM2, these genes co-distribute with Shh immunoreactivity while the ventral-most domain, RM3, is again characterized by the lack of Shh immunoreactivity (compare Figure 1F with Figures 1E–H; see Figure 6E). Of note, from stage 30 onwards, ScPitx2 is expressed in the whole RM (Figures 4J,M) but the downregulation of other genes suggest the existence of even more dorso-ventral subdomains. Finally, RM3 also presents ScLhx5- and ScOtp-expressing cells likely born in neighbor domains (Figures 2D,H, 6E; Table 1).

Evo-Devo Considerations Concerning the Basal Hypothalamus

The prosomeric model offers a key tool to study homologies and brain evolution (Puelles and Rubenstein, 2003, 2015). Counterparts of the genes here considered have been also studied in other vertebrates. Nevertheless, the lack of detailed data in prosomeric terms makes difficult to perform comparisons with most groups, except for mammals, at the level of microdomains. Below, detailed comparisons are made with mammals and we assume they are mostly transferable to other amniotes (Figure 7) due to the similarity of several patterns observed between mice and birds (Manning et al., 2006; Bardet et al., 2008, 2010; García-Calero et al., 2008; Abellán et al., 2010; also reviewed in Domínguez et al., 2014). Gross comparisons are also made with anamniotes that are, however, still informative (Figure 8).

Comparisons With Amniotes

In mouse, different works have addressed the expression of the orthologs here considered [Shh, Nkx2.1, Dlx5, Otp (Morales-Delgado et al., 2011, 2014; Puelles et al., 2012), Lhx5 (Szabó et al., 2009; Abellán et al., 2010; Puelles et al., 2012), Emx2 (Shimamura et al., 1995; Suda et al., 2001; Szabó et al., 2009), Lmx1b (Asbreuk et al., 2002; Martínez-Ferre and Martínez, 2012; Puelles et al., 2012), Pitx2 (Martin et al., 2004; Puelles et al., 2012), Foxa1 (Diez-Roux et al., 2011; Martínez-Ferre and Martínez, 2012; Puelles et al., 2012), Foxa2 and Neurog2 (Osório et al., 2010; Puelles et al., 2012). For abbreviations, see list.}

1 http://developingmouse.brain-map.org/
The PM/PRM is characterized in both models by *Otp* and *Lhx5* expression (Figures 7C,D). Of note, in shark, this compartment can be subdivided in a rostral PM1/PRM1 that lacks ScOtp and a caudal PM2/PRM2 that does express this gene. In shark, both compartments also express ScEmx2 (Figures 7C,D). Noteworthy, the PRM of shark lacks *Shh* expression in contrast to mouse (Figures 7C,D).

In the MM/RM, in both species genes expressed in the RM, like *Lmx1b* or *Foxa1*, appear to form a clear-cut
border of expression with respect to those expressed in the MM (and even in the PRM) as \textit{Nkx2.1, Emx2} and Lhx5 (see Figures 7E,F). This seems to be a conserved feature across vertebrates (see below). However, in mouse, genes like Pitx2 and Neurog2 are expressed in both domains (Figures 7E,F) though Neurog2 is restricted to RM at earlier stages (termed as pT3g in Figures 2B,B’ in Osório et al., 2010).

Together, this analysis reveals at least two things. First, one to one comparisons are useful to understand how interspecific variability emerges. Interestingly, the same set of homologous genes defines new or different microdomains among different species. Second, the number of microdomains identified increase with the number of genes analyzed, though their significance in terms of homology (common ancestry) becomes elusive. This raises non-trivial questions concerning homology establishment (Abouheif, 1997; Puelles and Medina, 2002) and compel us to consider other possible interpretations in the context of the prosomeric model.

Comparisons With Anamniotes

Many of the genes here considered have been already studied in agnathans and teleosts (Figure 8). Though it is difficult to establish one to one comparisons at the level of subdomains, common traits do exist between these groups (including chondrichthyans). Therefore, such characters are assumed to be transferable to other anamniotes.

In anamniotes, genes expressed in the Nkx2.1-expressing hypothalamus (Emx2, Otp, Lhx5) abut those expressed more ventro-caudally (Shh, ScPitx2, ScPitx3, ScNeurog2 and ScLmx1b). Furthermore, the last group of genes describes a continuous line from the floor plate of the terminal hypothalamus and extends into the zli (Figures 6A,C and Figure 8). Such abutted expression is typical of segmental boundaries (for definition of segmental boundaries see Dahmann et al., 2011; Cavodeassi and Houart, 2012; Kiecker and Lumsden, 2012; see also Larsen et al., 2001; Puelles et al., 2012) and has not been observed at other points of the caudal secondary prosencephalon. Having this in mind we decided to look for other evidences for segmental boundaries at this point as reduced cell proliferation and the presence of signaling centers. Noteworthy, at least in sharks, reduced cell proliferation (PCNA-negative cells at the ventricular zone in Figures 3E–H) can be also detected bordering the domain where Shh and other markers are expressed in the caudal hypothalamus. Moreover, transverse \textit{Wnt} signals seem to describe such border from the zli to the MM/RM boundary in different vertebrates (Figure 8A'; see also Guérin et al., 2009; Quinlan et al., 2009) while the situation in mice remain unclear. Finally, such border also seems to be the same as that delineated by Figdor and Stern (1993) between segment D1 and D2. Noteworthy, in the lamprey (Figure 8A), the expression of \textit{Wnt} signals resemble that of other \textit{Wnt} genes between rhombomeres (Riley et al., 2004) an idea already suggested in Santos-Durán (2015). Though our results suggest that such border could get deformed on the course of evolution as shown in Figure 8A', these evidences are not necessarily supported in mice where the expression of genes like Neurog2 and Pitx2 is continuous through the MM/RM rather than restricted to the RM (Figure 7). Of note, a recent review on vertebrate forebrain development also suggests the existence of a novel secondary organizer at this point (Puelles, 2017) as previously suggested in Santos-Durán (2015).

However, the idea that the currently identified as MM/RM border could indeed represent the hypothalamic-diencephalic border implies a new model of the hypothalamus that would require re-examination of other postulated limits and thus deserves further investigation.

CONCLUSION

This work belongs to a series of articles (Santos-Durán et al., 2015, 2016) addressing the development and evolution of the chondrichthyan hypothalamus and also the new proposals of the prosomeric model (Puelles et al., 2012; Puelles and Rubenstein, 2015) on the mentioned region.

Here, our combinatorial analysis revealed the existence of many different microdomains within the main subdomains of the prosomeric basal hypothalamus of the shark (Tu/RTu; PM/PRM; MM/RM). The genes considered in this study (ScOtp, ScDlx2/5, ScNkx2.1, ScShh, ScLhx5, ScEmx2, ScLmx1b, ScPitx2, ScPitx3a, ScFoxa1, ScFoxa2 and ScNeurog2) are well conserved in vertebrates. However, detailed comparisons at the level of microdomains under the prosomeric framework can be only performed with mammals, on which abundant data are available. Such analysis reveals a number of microzones that do not exactly fit those described in mice (Ferrán et al., 2015). Understanding the homology and evolution of such microdomains results daunting and can be misleading. However, these results illustrate, at least in part, how organisms became different in spite of expressing similar set of homologous genes.

AUTHOR CONTRIBUTIONS

GNS-D, IR-M and EC designed the study and analyzed the data. SM contributed to data acquisition. GNS-D, AM and SF-G performed the experiments. GNS-D wrote the manuscript with inputs from all authors.

FUNDING

This work was supported by grants from the Spanish Dirección General de Investigación–FEDER (BFU2010-15816, BFU2014-58631–P), the Xunta de Galicia (10PXIB200051PR, IN 845B-2010/159, CN 2012/237), and the Région Centre, Région Bretagne (EVOVERT grant number 049755).
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer NM and handling Editor declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

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