Development/Plasticity/Repair

Dynamic Expression of *RGS4* in the Developing Nervous System and Regulation by the Neural Type-Specific Transcription Factor Phox2b

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Previous studies have shown that members of the family of regulators of G-protein signaling (RGS), including RGS4, have a discrete expression pattern in the adult brain (Gold et al., 1997). Here, we describe for *RGS4* a distinct, mostly transient phase of neuronal expression, during embryonic development: transcription of *RGS4* occurs in a highly dynamic manner in a small set of peripheral and central neuronal precursors. This expression pattern overlaps extensively with that of the paired-like homeodomain protein Phox2b, a determinant of neuronal identity. In embryos deficient for *Phox2b*, *RGS4* expression is downregulated in the locus coeruleus, sympathetic ganglia, and cranial motor and sensory neurons. Moreover, Phox2b cooperates with the basic helix-loop-helix protein Mash1 to transiently switch on *RGS4* after ectopic expression in the chicken spinal cord. Intriguingly, we also identify a heterotrimeric G-protein α-subunit, gustducin, as coexpressed with RGS4 in developing facial motor neurons, also under the control of Phox2b. Altogether, these data identify components of the heterotrimeric G-protein signaling pathway as part of the type-specific program of neuronal differentiation.

Key words: **RGS; neuronal differentiation; hindbrain; motor neurons; heterotrimeric G-protein; Phox2**

Introduction

Regulators of G-protein signaling (RGS) molecules are a family of GTPase-activating proteins (GAPs) for the α subunits of heterotrimeric G-proteins. They increase the kinetics of GTP hydrolysis by Gα subunits, thus fostering the reassociation of an inactive GDP-bound trimeric complex and the termination of both Gα and G$\beta\gamma$-mediated signaling. One of their main functions is therefore thought to shorten, sharpen, or otherwise attenuate signals transduced by heterotrimeric G-protein-coupled receptors (GPCRs) (for review, see De Vries et al., 2000; Ross and Wilkie, 2000). Furthermore, the complex interplay of enzyme kinetics and mutual affinities of GPCRs, G-proteins, and RGS proteins, as well as various levels of feedback regulation allow them to act as versatile modulators of G-protein signaling dynamics: they could sharpen not only the termination of signaling but also its onset, maintain the strength of signaling in the presence of an ongoing signal (Ross and Wilkie, 2000), extinguish spontaneous (i.e., receptor-independent) G-signaling (Siekhaus and Drubin, 2003), or translate a continuous stimulus into an oscillatory response (Luo et al., 2001). Many roles have been proposed for RGS proteins in the adult nervous system, based on inactivation in *Caenorhabditis elegans*, overexpression in cell cultures or correlative expression studies. In contrast, very little is known on possible functions of RGS proteins in differentiating neurons (see Discussion), and, to our knowledge, no *RGS* expression pattern has been reported so far in the embryonic nervous system of vertebrates.

The paired-like homeodomain protein Phox2b (Pattyn et al., 1997) is required for the early differentiation phase of several classes of neurons, including all noradrenergic neurons, cranial branchial motoneurons, and most relays of the autonomic reflex pathways in mouse (Pattyn et al., 1999, 2000a,b; Dauger et al., 2003) (for review, see Brunet and Pattyn, 2002), and in humans, as suggested by the clinical consequences of *PHOX2B* mutations (Amiel et al., 2003). Here, in an attempt at dissecting the genetic program of cranial branchial motoneuron (bm) differentiation, we identify *RGS4* as a gene lying downstream of *Phox2b*. More generally, we show that *RGS4* expression in the developing nervous system substantially overlaps with that of *Phox2b* and is controlled by the latter at sites of coexpression and after ectopic expression in the spinal cord. *RGS4* thus defines part of a "core" *Phox2b*-dependent program of neuronal differentiation. Finally, we identify gustducin, a heterotrimeric G-protein α-subunit, as another Phox2b direct or indirect transcriptional target, coexpressed with RGS4 in differentiating facial motoneuronal precursors.

Materials and Methods

Mouse strains. *Phox2b*$^{LacZ/+}$ (Pattyn et al., 1999) and *Phox2a*$^{tauLacZ/+}$ (Jacob et al., 2000) mice were bred, and their progeny genotyped as

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described previously. For histological analysis on adult tissue, animals were anesthetized by intraperitoneal injection of Avertine and fixed by transcardial perfusion of 50 ml of 4% paraformaldehyde (PFA).

Construction and screening of a rhombomere 4-derived cDNA library. The hindbrains of the embryonic day 10.5 (E10.5) progeny of *Phox2b*$^{LacZ/+}$ intercrosses were dissected and treated with fluorescein di-(β-D-galactopyranoside) (Sigma, St. Louis, MO) (Nolan et al., 1988). From each hindbrain, the *LacZ*$^{+}$ ventral domain of rhombomere 4 (r4) was precisely excised under a green fluorescent protein (GFP) binocular (the rest of the tissue being used for genotyping), and total RNA (~200 ng) was extracted with hot phenol (Brunet et al., 1991). Individual samples were reverse-transcribed and PCR-amplified for 19 cycles with the SMART PCR kit (Clontech, Palo Alto, CA). Two *Phox2b*$^{LacZ/+}$-derived cDNA populations were independently subtracted with two *LacZ/Phox2b*^{LacZ}-derived cDNA populations by the suppression-subtractive hybridization technique (Diatchenko et al., 1996) using the PCR-Select Subtraction kit (Clontech). They were assayed on virtual Northern blots for enrichment of differentially expressed sequences using *Phox2b*, *Phox2a*, *Math3*, *Ebf1*, *Ebf2*, *Ebf3,* and *Islet-1* as probes (Pattyn et al., 2000b) and for depletion of nondifferentially expressed sequences using a PCR-amplified fragment of *G3PDH*. The most efficiently subtracted cDNA population was used to generate a library of ~1800 clones by T/A cloning in pGEM-T (Promega, Madison, WI). Replicas were hybridized in parallel in Church buffer at 6.10^{6} dpm/ml with a forward-subtracted (heterozygous minus homozygous) probe and a reverse-subtracted (homozygous minus heterozygous) probe. Two hundred seventy-five differentially hybridizing clones were rescreened by dot-blotting their PCR-amplified inserts in triplicates and hybridizing them with the forward and reverse probes as well as with nonsubtracted *Phox2*$^{LacZ/+}$ cDNA. The 25 clones (corresponding to 20 different cDNAs) that behaved like the positive controls *Phox2b*, *Islet-1,* and *Math3* (i.e., were detected by the forward subtracted probe but neither by the reverse nor the nonsubtracted probes) were kept for further study. Two of them are analyzed in this paper: pGEM-F168 which contained a 653 bp *Rsa*I insert including the 3′ half of the open reading frame of *RGS4*, and pGEM-F217 that contained a 650 bp *Rsa*I insert corresponding to the 5′ half of the *gustducin* mRNA.

In situ *hybridization and immunohistochemistry.* Staged embryos (vaginal plug was recorded as E0.5), or brains and lumbar dorsal root ganglia (DRGs) of adult animals were processed for *in situ* hybridization or by combined *in situ* hybridization and immunohistochemistry on 12-μm-thick cryosections or whole-mount *in situ* hybridization as described (Tiveron et al., 1996). BrdU (Sigma) was injected intraperitoneally (6 mg/mouse) into pregnant mice 1 hr before removing the embryos. Antisense RNA probes were synthesized from pKS-mRGS4 (resulting from the subcloning of the coding sequence of *RGS4* from pCR2.1-mRGS4) (Nomoto et al., 1997), pKS-cRGS4 (see infra), pKS-mGust (resulting from the subcloning of the pGEM-F217 insert), and *EGFP* (Clontech), using a DIG-RNA labeling kit (Roche Products, Hertforshire, UK). Anti-Phox2a (Tiveron et al., 1996), anti-Phox2b (Pattyn et al., 1997), and anti-BrdU (Sigma) antibodies were used for immunohistochemistry.

Cloning of a chicken RGS4 cDNA. A cDNA phage library was made from 2 μg of polyA(+) RNA from stage HH23–24 chick hindbrains using the SMART cDNA library construction kit (Clontech). Of 1.6 10^{5} pfu screened with the insert of pGEM-F168, two clones were isolated and sequenced. They were identical (GenBank accession number AY297457) and showed most identity, at the amino acid level, to mouse RGS4 (69% overall and 84% within the RGS domain; the next closest mouse relative being mRGS5 with 49% overall identity). A 474 bp *Pst*I-*Apa*I cDNA fragment was subcloned into pKS to create the plasmid pKS-cRGS4.

Expression vectors and electroporation in chicken embryos. The coding regions of mouse *Mash1* (Cau et al., 1997) and *mPhox2b* (Pattyn et al., 1997) were cloned into the pCAGGS vector that drives expression by a cytomegalovirus–actin hybrid promoter (Koshiba-Takeuchi et al., 2000). *GFP* was expressed from the pCAGGS-AFP vector (Momose et al., 1999). Chick embryos 44- to 52-hr-old (HH 12–14) were electroporated *in ovo* essentially as described (Dubreuil et al., 2000). The expression vectors were used at 1 mg/ml except for pCAGGS-AFP (0.8 mg/ml). Coinjection of pCAGGS-AFP was used to visualize the transfected area. Embryos were allowed to develop at 38°C for 20 or 48 hr, then fixed in 4% paraformaldehyde, embedded in gelatin, and analyzed on transverse sections at the transfected level. Correct expression of all constructs was verified by *in situ* hybridization with the appropriate probes. In all cases, expression of the transfected gene was coextensive with that of *GFP*.

Results

Identification of *RGS4* as a candidate *Phox2b*-regulated gene in facial motoneurons

To identify genes expressed in facial motoneuron precursors under the control of *Phox2b*, we used a differential cloning strategy. We prepared total RNA from the ventral part of r4, containing the facial branchial motoneuronal (FBM) precursors, which were dissected from either heterozygous or homozygous *Phox2b* mutant E10.5 embryos (see Material and Methods). First-strand cDNA was synthesized, amplified by PCR, and subjected to suppression subtractive hybridization (Diatchenko et al., 1996; Gurskaya et al., 1996). Two independently generated *Phox2b*$^{+/-}$ cDNA populations were subtracted with *Phox2b*$^{-/-}$ cDNA and tested by "virtual Northern" analysis for the depletion and enrichment of, respectively, the housekeeping gene *G3PDH* and *Phox2b* or genes known to be activated by Phox2b such as *MATH3* and *Ebf1* (Pattyn et al., 2000b) (data not shown). The cDNA population that proved the most efficiently enriched was T/A cloned, and 1800 clones were screened with subtracted cDNA populations, both "forward" (i.e., heterozygous minus homozygous) and "reverse" (i.e., homozygous minus heterozygous). A total of 25 independent clones (corresponding to 20 distinct genes) were selected (see Material and Methods for details). Two of these are the subject of the present report: one containing a 653 bp *Rsa*I cDNA fragment of *RGS4*, the other, a 650 bp fragment of *gustducin*.

Expression of RGS4 in cranial motoneuronal precursors

We first examined the expression of *RGS4* in the *Phox2b*$^{+}$ ventral r4 region from which we cloned it. During the time window of neuroepithelial *Phox2b* expression, the pMNv domain of r4 gives rise to two populations of neuronal precursors: FBM precursors (Ericson et al., 1997; Pattyn et al., 2000b) and the small population of inner ear efferent (IEE) precursors (Simon and Lumsden, 1993; Tiveron et al., 2003). After exit from the neuroepithelium, *Phox2b*$^{+}$ postmitotic FBM precursors undergo a unique pattern of migration, caudally through r5 and then radially to the pial surface of r6 (Auclair et al., 1996; Pattyn et al., 1997), whereas the IEE precursors (also *Phox2b*$^{+}$) migrate contralaterally and dorsally in r4 (Fritzsch, 1996; Tiveron et al., 2003). At E10.5, *RGS4* expression was detectable in ventral r4 (Fig. 1*A*), in a subpopulation of Phox2b^{+} precursors localized at the border between the ventricular zone, which they had probably just exited, and the mantle layer (Fig. 1*B,C*). As confirmed by double *RGS4*/BrdU labeling those cells had already undergone their last S phase (Fig. 1*C*). However, the more superficially located, thus presumably older postmitotic precursors, had already lost *RGS4* expression (Fig. 1*B,C*). This pattern was unchanged at E11.5 (data not shown), that is after all IEEs are born (A. Pattyn, personal communication) and have started their migration (Tiveron et al., 2003), and while FBMs are still being generated (Taber Pierce, 1973; Pattyn et al., 1997). No signal could be detected in those FBMs which have, by then, started their migration through r5 (Fig. 1*D*), or in migratory IEEs (data not shown). Therefore, *RGS4* is very transiently expressed in FBM precursors (and possibly IEEs) concomitant with or immediately subsequent to cell cycle exit and is downregulated before their postmitotic migra-

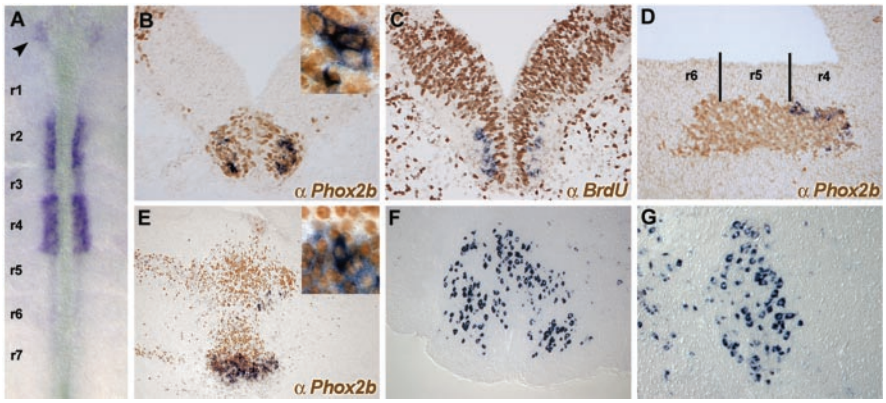

Figure 1. Expression of *RGS4* in hindbrain motoneurons examined by *in situ* hybridization (*A*, *F*, *G*) or *in situ* hybridization combined with anti-Phox2b (*B*, *D*, *E*) or anti-BrdU (*C*) immunohistochemistry. *A*, Flat-mount of the hindbrain at E10.5 showing expression in the ventral column of r2-r4 and in the anlage of the trochlear nucleus, in the isthmus (arrowhead). *B*, *C*, (and inset showing a higher magnification), Cross-section through r4 at E10.5, showing that *RGS4* is expressed in a subset of postmitotic facial motoneuronal precursors situated in between the mantle layer and the ventricular zone. *D*, A parasagittal section (anterior to the right) at E12.5 shows that RGS4 is extinguished before motoneuronal precursors have started to migrate through r5. *E* (and inset at higher magnification), Parasagittal section (anterior to the right) at E13.5 showing that *RGS4* is switched on in facial motoneuronal precursors as they settle in the facial nucleus anlage. *F*, *G*, Coronal sections through an adult brain showing high level of expression of *RGS4* in the facial (*F*) and trigeminal (*G*) motor nuclei.

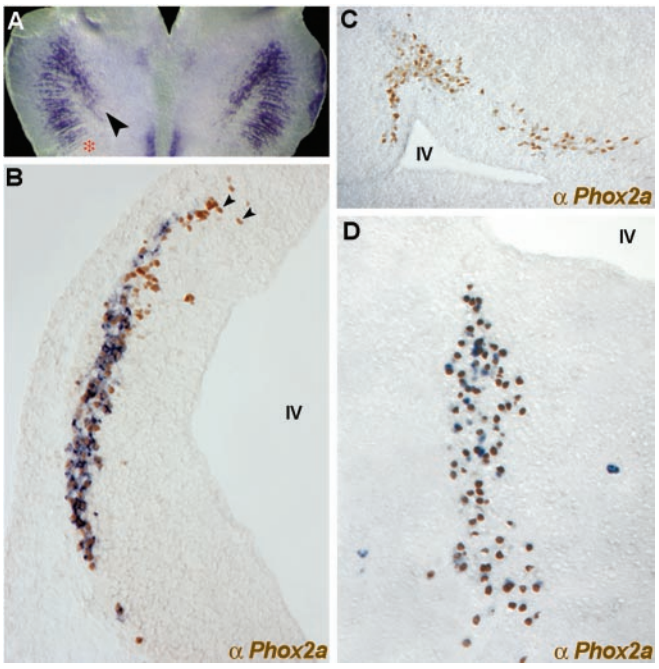

Figure 2. Biphasic expression of *RGS4* in the locus coeruleus examined by *in situ* hybridization (*A*) or *in situ* hybridization combined with anti-Phox2a immunohistochemistry (*B–D*). *A*, Flat-mount of the metencephalon at E10.5 showing expression in the locus coeruleus (arrowhead) and in a neighboring population of interneurons (asterisk). *B*, Transverse section through the first rhombomere at E10.75 showing that LC precursors, which are born dorsally and start expressing Phox2a as soon as they exit the neuroepithelium (arrowheads), switch on *RGS4* soon after, as they migrate ventrally. *C*, Parasagittal section through the LC at E13.5 showing that *RGS4* is extinguished at this stage. *D*, Coronal section (medial to the right) through an adult brain showing that mature LC neurons express *RGS4*. IV, IVth ventricle.

tion. Remarkably, 2 d later, when a sizable contingent of FBM precursors had reached the pial surface after their radial migration through r6, they switched on *RGS4* again (Fig. 1*E*). *RGS4* expression was virtually extinguished again by E15.5 (data not shown) but expressed at strong levels in facial motoneurons throughout adult stages (Fig. 1*F*).

In the medulla at E10.5, *RGS4* expression was not confined to FBM precursors but was also detected in the motoneuronal column of r2 and r3, that is, in the precursors of the trigeminal motor nucleus (Fig. 1*A*). Just like in FBMs, expression was very transient, but the trigeminal nucleus expressed strong levels of RGS4 in postnatal life (Fig. 1*G*). Caudally to r4, only an occasional RGS4$^+$ cells was detected in the pMNv domain (data not shown).

In the isthmic region, RGS4 was detected in the trochlear nucleus, located in r1 (Fig. 1*A*) but not the nearby oculomotor nucleus, located in the caudal mesencephalon (data not shown).

No expression was detected in embryonic spinal motoneuronal precursors, whether somatic or visceral, at least until E15.5 (data not shown).

Other sites of *RGS4* expression in the embryonic CNS

Apart from cranial motor neurons, *RGS4* expression was detected in several classes of differentiating central neurons. In the rostral hindbrain, the precursors of the locus coeruleus (LC) (the main noradrenergic center of the brain) switched on *RGS4* at E10.5 (Fig. 2*A*,*B*) simultaneously with Phox2b (data not shown), that is soon after Phox2a (Fig. 2*B*), itself the earliest postmitotic marker known for the LC (Pattyn et al., 1997). At E13.5 the locus coeruleus had lost *RGS4* expression (Fig. 2*C*) but had recovered it by postnatal stages (Fig. 2*D*) (Gold et al., 1997; Ni et al., 1999), although we did not determine the exact timing of re-expression. Just dorsal to the LC, *RGS4* was expressed in another population of neurons that we did not identify (Fig. 2*A*).

It is notable that numerous sites of adult expression, such as the cortex (Gold et al., 1997) were undetectable embryonically, at least until E15.5 (data not shown).

RGS4 expression in the developing peripheral nervous system

In contrast to its very restricted central distribution, *RGS4* was broadly expressed in the peripheral nervous system in complex spatiotemporal patterns.

Autonomic ganglia

RGS4 was first switched on in most sympathoblasts as early as their arrival at the dorsal aorta, at approximately E10.5 (Fig. 3*A*). At E13.5, a majority of cells were found positive in the superior cervical (SCG) and stellate ganglia (SG) (Fig. 3*B*) (data not shown) and fewer in the trunk sympathetic chain (data not shown). Positive cells, rather than being dispersed in a salt and pepper pattern, tended to be clustered (Fig. 3*B*) (data not shown). This pattern also prevailed in most of the prevertebral chain with the exception of two ganglionic masses ventrolateral and caudal to the celiac ganglion, which were almost entirely *RGS4*$^+$ (Fig. 3*C*,*D*).

At the same stage (E13.5) most parasympathetic ganglia, which have just formed, expressed *RGS4* at high level: sublingual (Fig. 3*E*), submandibular, paracardiac, and pulmonary (data not shown). However, the most rostral ones (ciliary, sphenopalatine, and otic) contained very few positive cells (data not shown).

In the enteric nervous system, *RGS4* was very transiently de-

tected in a sparse subset of Phox2b$^+$ migrating precursors at E10–E10.5 (data not shown). Later on, at E11.5 no expression was detected, and at E13.5 only rare Phox2b$^+$ cells were found to express *RGS4* (Fig. 3*F*).

Cranial and spinal sensory neurons

All Phox2a$^+$/Phox2b$^+$ neuronal precursors destined to form the distal ganglia of the VIIth, IXth, and Xth nerves (respectively the geniculate, petrosal, and nodose ganglia), switched on *RGS4* soon after their delamination from the epibranchial placodes (Fig. 4*A–C*). Expression had virtually faded out by E13.5 (Fig. 3*B*).

At E10, the otic placode-derived sensory precursors also expressed *RGS4* (Fig. 4*A,B*) as did the trigeminal ganglion (Fig. 4*A*), both of which never express *Phox2* genes. Finally, all DRGs expressed high levels of *RGS4* in a salt and pepper pattern from E10.5 (Fig. 4*A*) until E13.5 (Fig. 4*D*). Expression had faded out in the vast majority of cells at E15.5 (data not shown). Postnatally, expression occurred at high level in a subset of small diameter neurons (Fig. 4*E*).

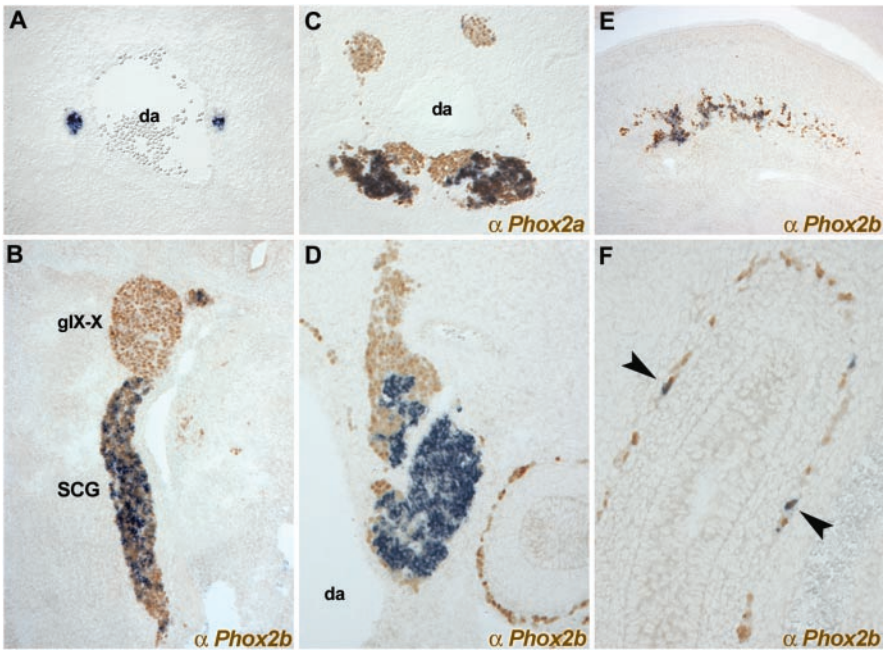

Figure 3. Expression of *RGS4* in visceral ganglia, examined by *in situ* hybridization (*A*) or *in situ* hybridization combined with anti-Phox2b (*B, D–F*) or anti-Phox2a (*C*) immunohistochemistry. *A*, RGS4 is switched on as soon as E10.5 in the aggregating sympathoblasts on each side of the dorsal aorta (da). *B*, Parasagittal section at E13.5 showing abundant *RGS4*-positive cells in the superior cervical ganglion (SCG). At this stage expression is lost in the nodose-petrosal complex (gIX-X). *C*, *D*, Transverse (*C*) and parasagittal (*D*) sections at E13.5 showing expression of *RGS4* in a prevertebral Phox2a$^+$/Phox2b$^+$ cell mass below and lateral to the celiac ganglion. *E*, RGS4 expression in sublingual parasympathetic ganglia at E13.5. *F*, RGS4 expression in a small subset of enteric neurons at E13.5.

Phox2b controls the expression of *RGS4*

We then examined the extent to which *RGS4* expression depends on Phox2b in the territories where they are coexpressed. In ventral r4 of *Phox2b* mutants, at E10.5 a reduced population of postmitotic neurons is produced which display none of the phenotypic markers of motoneurons (Pattyn et al., 2000b) but, rather, differentiates into serotonergic neurons (which normally do not arise in r4) (Pattyn et al., 2003; Tiveron et al., 2003). Accordingly, *RGS4* was completely undetectable in the postmitotic progeny of mutant ventral r4 (Fig. 5*A–D*), as it was in fact throughout r2 and r3 (Fig. 5*C,D*). Therefore *RGS4* is directly or indirectly under the control of Phox2b in trigeminal and facial motoneuronal precursors. Similarly, in *LacZ/Phox2b^{LacZ}* embryos, *RGS4* was undetectable in the precursors of the LC (Fig. 5*C,D*, arrowhead), which are still present at this stage and express Phox2a (Fig. 5*E,F*) (Pattyn et al., 2000a). More surprisingly, *RGS4* expression was also abolished in the anlage of the trochlear nucleus (Fig. 5*C,D*, asterisk), although no other aspect of the development of this *Phox2a*-dependent nucleus has been found so far to require *Phox2b*.

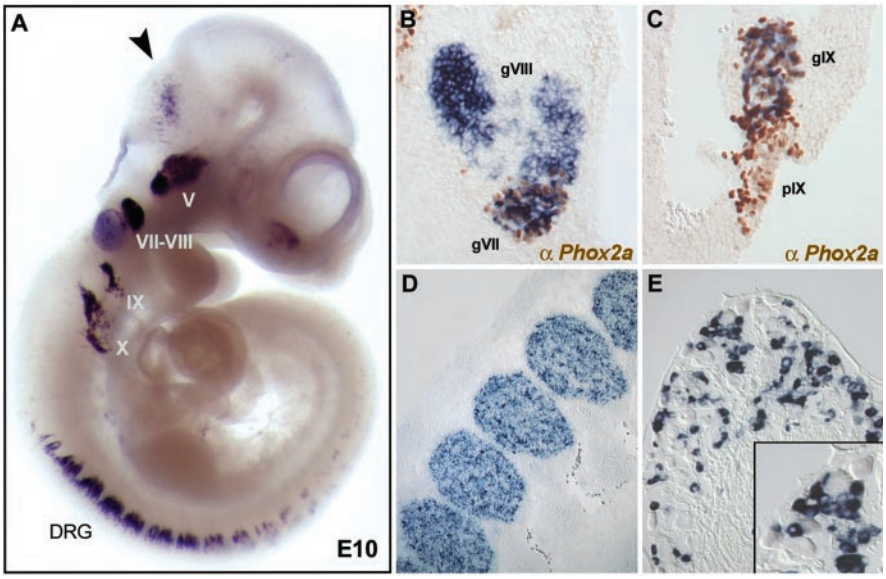

Figure 4. Expression of *RGS4* in sensory ganglia examined by *in situ* hybridization (*A, D, E*) or *in situ* hybridization combined with anti-Phox2a immunohistochemistry (*B, C*). *A*, Whole-mount E10 embryo showing expression in trigeminal (*V*), geniculate and acoustic (VII-VIII), glosopharyngeal (IX), and vagal (*X*) cranial ganglia and in the nascent dorsal root ganglia (DRG). Expression in the LC has just started (arrowhead). *B*, Transverse section close to the otic vesicle showing expression of *RGS4* in the acoustic (gVIII) ganglion and the geniculate ganglion (gVII), which also expresses Phox2a. *C*, Transverse section at E10.5 showing Phox2a$^+$ cells delaminating from the glossopharyngeal placode (pIX) and switching on *RGS4* as they aggregate to form the ganglion anlage (gIX). *D*, Parasagittal section at E13.5 showing a widespread expression, albeit at variable levels, of *RGS4* in the DRGs. *E*, Section through an adult DRG showing strong expression in a subset of small-diameter (see inset) DRG neurons.

In the peripheral nervous system of E10.5 *Phox2b$^{LacZ/LacZ}$* mutants, *RGS4* expression was extinguished in the aggregating sympathoblasts (Fig. 5*G,H*) that are still present at this stage (Fig. 5*H*, inset) (Pattyn et al., 1999). In the delaminating geniculate, petrosal and vagal ganglionic precursors *RGS4* were downregulated but not extinguished (Fig. 6*A,B*), possibly reflecting the fact that the early development of these ganglia only partially depends on *Phox2b* (Pattyn et al., 1999) and also requires its paralog *Phox2a* (Morin et al., 1997). In

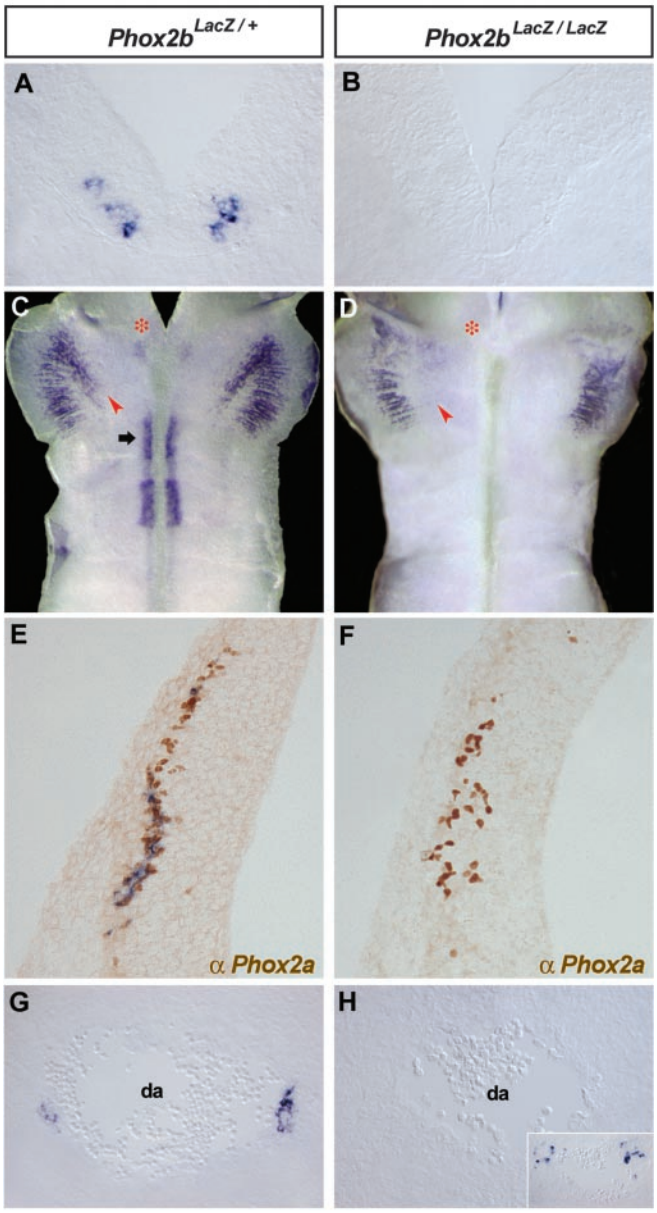

Figure 5. Dependence of *RGS4* expression on Phox2b studied by *in situ* hybridization on transverse sections through r4 (*A, B*), r1 (*E, F*) or the thoracic region (*G, H*) and a flat-mount of the hindbrain (*C, D*) at E10.5. Expression of *RGS4* in facial motoneuronal precursors in ventral r4 at E10.5 (*A, C*), in precursors of the trochlear (*C,* asterisk) and trigeminal (*C,* black arrow) motor nuclei and the locus coeruleus (*C,* arrowhead, *E*) and in forming sympathetic ganglia (*G*) is abrogated in *Phox2b*$^{LacZ/LacZ}$ mutants (*B, D, F, H*). Inset, *In situ* hybridization with *LacZ* on an adjacent section shows the presence of sympathetic precursors at E10.5 in *Phox2b* homozygous mutants. da, Dorsal aorta.

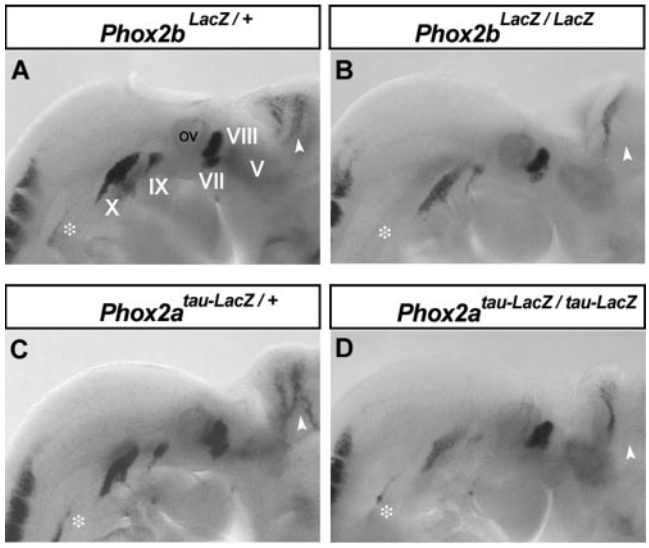

Figure 6. Dependence of *RGS4* expression on *Phox2a* and *Phox2b* in cranial ganglia. Side view of whole-mount E10.5 embryos hybridized with an *RGS4* probe. The *RGS4* signal in the nodose (*X*), petrosal (IX) and geniculate (VII) ganglia is diminished in null mutants for both, *Phox2b* (*B*) and *Phox2a* (*D*) compared with heterozygotes (*A, C,* respectively). Note that in *Phox2a* mutants, as expected, the signal is intact in the sympathetic chain (which does not depend on *Phox2a* for differentiation) (asterisk) but disappears at the level of the LC (which does not form in *Phox2a* mutants) (Morin et al., 1997) (arrowhead). ov, Otic vesicle.

line with this, a similar downregulation of *RGS4* was observed in the epibranchial-derived ganglia of *Phox2a*$^{tauLacZ/tauLacZ}$ mutants (Jacob et al., 2000) (Fig. 6*C,D*). We could not monitor *RGS4* in parasympathetic ganglia because they never form in *Phox2b* mutants (Pattyn et al., 1999). Finally, *RGS4* expression was predictably intact in DRGs (Fig. 6) (data not shown) and in the trigeminal and octaval ganglia (Fig. 6*A,B*) that never express *Phox2b*.

Together, these data show that the *Phox2b*-dependent neuronal precursors that express *RGS4* do so under the control of *Phox2b*.

Conversely, we asked whether *Phox2b* could ectopically trigger *RGS4* expression. Electroporation of *Phox2b* in the spinal cord of stage 12–15 chicken embryos did not induce *RGS4* 20 hr after electroporation (hae) (Fig. 7*A,A′*). We reasoned that Phox2b might require a cofactor to induce *RGS4*. Because *Phox2b* genetically interacts with the basic helix-loop-helix gene *Mash1* during the differentiation of several *RGS4*-expressing neuronal types, i.e., the LC, cranial motor neurons, and sympathetic and parasympathetic ganglionic cells (Pattyn et al., 2000b; Goridis and Rohrer, 2002; Tiveron et al., 2003), we tested whether coelectroporation of *Mash1* with *Phox2b* could induce *RGS4*. This was indeed the case at 20 hae (Fig. 7*B,B′*), but this expression had faded out by 48 hae (Fig. 7*D,D′*), despite the ongoing expression of *Phox2b* in electroporated cells (data not shown) (Dubreuil et al., 2002). *Mash1* electroporated on its own did not induce *RGS4* (Fig. 7*C,C′*). Therefore, Phox2b, in cooperation with Mash1, is an instructive factor for *RGS4* expression.

Gustducin is transiently expressed in differentiating facial motor neurons

Strikingly, among the genes isolated in our differential screen, we found another molecule involved in the heterotrimeric G-protein signaling pathway: the G-protein α-subunit *gustducin* which, so far, has been reported to be expressed specifically in taste receptor cells (McLaughlin et al., 1992) and a few other presumably chemoreceptive cell types (Hofer et al., 1996) and required for bitter and sweet taste transduction (Wong et al., 1996). On flat-mounts of the hindbrain at E10.5 we verified that *gustducin* was indeed expressed in FBM precursors emerging from ventral r4 and lasted until they reached the r5/r6 border (Fig. 8*A,B′*). Atypically, expression was detectable not only in the cell bodies but also in the axons converging toward the r4 facial exit point (Fig. 8*A,B,* arrowhead). Cross-sections through r4 and combined immunohistochemistry for Phox2a (which marks postmitotic bm/vm neurons) showed that *gustducin* expression starts, like *RGS4*, in facial postmitotic precursors in the mantle layer (Fig. 8*B′*). *Gustducin* expression in FBM precursors was

completely abolished in a homozygous *Phox2b* null background (Fig. 8*C*).

Discussion

Regulators of heterotrimeric G-protein signaling have been the focus of intense biochemical and cell biological studies that have steadily increased the scope of their proposed physiological functions. As GAPs for G_q and G_i classes of Gα subunits, they can impact on the vast array of Gα and G$\beta\gamma$-linked effectors. In addition, many RGS proteins have non-RGS domains, which makes them effectors in their own right (and not mere regulators) of GPCR signaling (for review, see Hepler, 1999; Burchett, 2000). Moreover, the RGS domain itself could have non-GAP roles, such as the RGS domain of axin, which binds APC within the β-catenin degradation complex (for review, see De Vries et al., 2000). Therefore, biochemical evidence opens the possibility of a dizzying variety of roles that *in vivo* experiments will be required to sort out. To this date, developmental roles for very few RGS proteins have been documented. Exceptions include *loco*, a *Drosophila* homolog of *RGS12*, required for early embryonic development (Pathirana et al., 2001) and terminal differentiation of glial cells (Granderath et al., 1999) and *Xrgs4a*, a *Xenopus* homolog of *RGS4*, suggested, on the basis of gain-of-function experiments, to act on early embryonic patterning by modulating Xwnt-8 signaling (Wu et al., 2000).

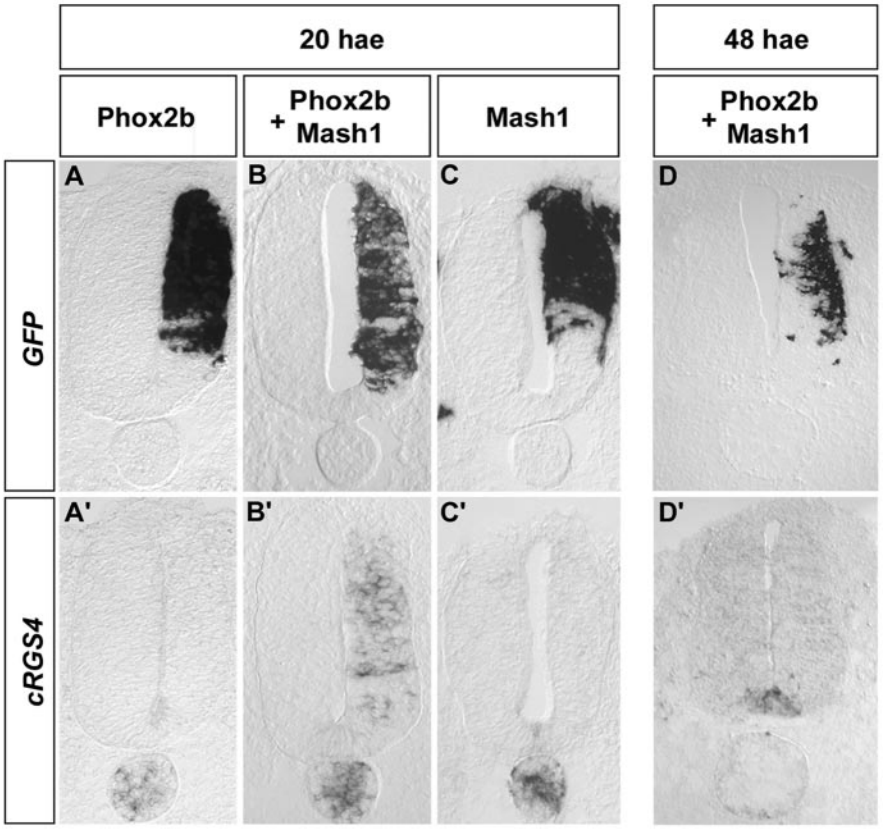

Figure 7. Phox2b can ectopically and transiently induce *RGS4* expression. Transverse sections through the spinal cord of a chick embryo 20 hr after electroporation (20 hae) of *Phox2b* (*A*, *A′*) or *Mash1* (*C*, *C′*) or 20 hae and 48 hae of *Phox2b* and *Mash1* (*B*, *B′*, *D*, *D′*, respectively) hybridized with *GFP* (*A–D*) or *cRGS4* (*A′-D′*). Note that *cRGS4* is expressed first in the notochord (*A′-C′*), then at approximately E4 switches to the floorplate (*D′*), in a manner suggestive of homeogenetic induction. This expression is not observed in mouse.

Here, we provide the first account of the expression of a *RGS* gene during the ontogeny of the nervous system (summarized in Table 1), several aspects of which are highly evocative of developmental roles.

Dynamic expression of *RGS4* in differentiating neurons

Striking features of embryonic *RGS4* expression are the very discrete set of neuronal types involved and the extremely dynamic pattern of expression, both attesting to a tight transcriptional regulation during development. In several cell types, *RGS4* is switched on and off, time and again during neuronal differentiation. One of the most extreme examples is provided by facial motoneuronal precursors that switch on *RGS4* as they exit the ventricular zone, switch it off soon after, as they start migrating caudally, switch it back on as they settle in the anlage of the facial nucleus, then largely downregulate it, and finally re-express it at high levels throughout postnatal life. A biphasic expression pattern is observed in the LC, with an early transient expression followed by a late stable re-expression. In DRGs, widespread expression primarily fades out by E15.5, and postnatal expression in small-diameter neurons is likely to correspond to a re-expression. We cannot formally exclude, however, that it represents the continuation of expression in the small subset of cells detected at E15.5 and their progeny.

These data are compatible with *RGS4* having distinct roles in differentiating and in mature neurons. It is noteworthy that the postnatal expression of *RGS4* (Gold et al., 1997; present study) although far from ubiquitous, is much wider than the developmental one.

RGS4 is regulated by Phox2b

The very discrete embryonic expression of *RGS4* considerably overlaps with that of the homeodomain protein Phox2b, specifically in sympathoadrenal and parasympathetic ganglionic cells, a fraction of enteric neuronal precursors, the locus coeruleus, epibranchial placode-derived ganglia, and trigeminal, facial and trochlear motor neurons. Strikingly, in the locus coeruleus, in which *Phox2b* is switched on after *Phox2a* and under its strict control, the embryonic phase of *RGS4* expression coincides with the transient expression of *Phox2b* (Fig. 2) (Pattyn et al., 2000a). Moreover, at all these sites, *RGS4* is under the control of *Phox2b*, as evidenced by its loss in $Phox2b^{LacZ/LacZ}$ embryos. Finally, *Phox2b* can ectopically induce *RGS4* in the spinal cord. This induction was fully detectable after 20 hr, arguing that Phox2b does not transactivate *RGS4* through a complex cascade of transcriptional events. Remarkably, induced expression of *RGS4* was transient, thus mimicking the dynamic of the endogenous *RGS4* expression and arguing that Phox2b itself participates in the extinction of *RGS4*, either directly or by promoting a postmitotic stage of motoneuronal differentiation incompatible with the maintenance of *RGS4* expression. Altogether, these data suggest that *RGS4* is a relatively direct or proximal transcriptional target of Phox2b, a determinant of neuronal identity. Clearly, other regulators must be invoked to explain the downregulation of *RGS4* in many structures even as *Phox2b* expression is main-

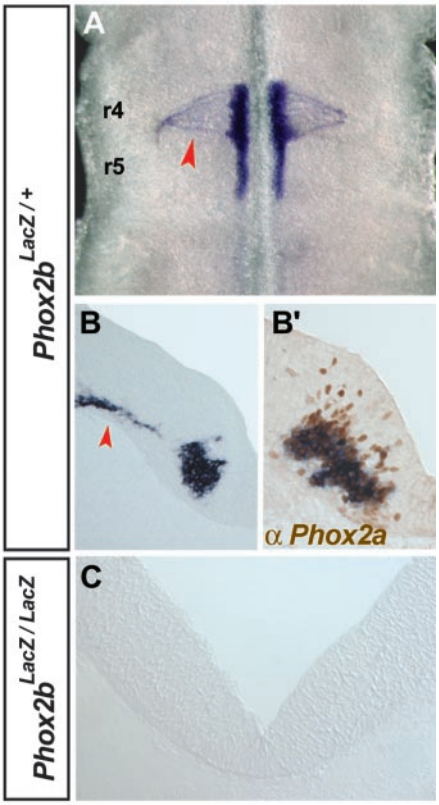

Figure 8. *Gustducin* expression in FBM precursors and its dependence on *Phox2b* studied by *in situ* hybridization on a flat-mount of the hindbrain (*A*) or on transverse sections through r4 (*B, C*) at E11. (*A, B′*) Expression of *Gustducin* in the FBM precursors of heterozygote mutants and their axons (*A*, *B*, arrowhead) starts in r4 and continues during their migration through r5. Immunohistochemistry for Phox2a confirms that *Gustducin* expression is restricted to postmitotic FBM precursors (*B′*). *C*, *Gustducin* expression is abolished in $Phox2b^{LacZ/LacZ}$ mutants.

tained, or why among cranial bm/vm motoneuron precursors, which all depend on *Phox2b*, only some express *RGS4*.

Possible roles of RGS4 in the developing nervous system

The present survey does not reveal any straightforward and general correlate between ontogenetic events and *RGS4* upregulation or downregulation. Moreover, no published data are available on the roles of RGS proteins in developing neurons to suggest functional hypotheses. In fact, very little *in vivo* data are available on the embryonic functions of the heterotrimeric G_q and G_i proteins themselves, through which RGS proteins are thought to exert their main actions. Of relevance to neural development is *Drosophila* $G\alpha_q$ whose gain-of-function leads to ectopic midline crossing of commissural axons (Ratnaparkhi et al., 2002). No study of neural development has been reported so far in knockouts of G_i and G_q family genes in mouse, except for an abnormal maintenance of Purkinje cell polyinnervation and consequent ataxia in $G\alpha_q^{-/-}$ mutants (Offermanns et al., 1997). *In vitro* assays for growth cone guidance have recently implicated signaling through heterotrimeric G-proteins (Xiang et al., 2002; Chalasani et al., 2003; Guirland et al., 2003) and *in vivo*, the chemokine SDF-1, signaling through the GPCR CXCR4 is required for neuronal precursor migration and axonal guidance (Zou et al., 1998; Bagri et al., 2002; David et al., 2002; Zhu et al., 2002). It is therefore plausible that such related phenomena as axonal navigation and neuronal migration would be affected by RGS proteins, similar to what has been shown, *in vitro*, for lymphocyte migration (Bowman et al., 1998; Reif and Cyster, 2000). In this context, we note that in at least one instance, changes in the expression of *RGS4* did correlate with cell movements; FBM precursors first expressed *RGS4* after arrival in the mantle layer of r4 where they change direction from a lateral to a caudal migration; they downregulated it during their caudal migration, then went again through a burst of expression when they settled near the pial surface of r6. Conceivably, these transient upregulations of *RGS4* could modulate the responsiveness of neuronal precursors to chemotactic cues at the transition between migratory states. Our differential screening strategy also isolated a heterotrimeric G-protein α-subunit, *gustducin*, which turns out to be a specific (if transient) marker, among cranial motoneurons for FBMs and, like *RGS4* is under the control of *Phox2b*. This finding further strengthens the notion that the heterotrimeric G-protein signaling pathway is developmentally regulated during the early phases of neuronal differentiation, in a class-specific manner.

Other roles of RGS proteins could be to foster specific differentiation stages of developing neurons, as proposed for RGS6 (Liu et al., 2002) on the basis of its effect on nerve growth factor-induced differentiation of the PC12 cell line [although this action is dependent on a GGL domain (absent from RGS4), and inde-

Table 1. Summary of the expression of *RGS4* in the developing nervous system

	E10.5	E11.5	E12.5	E13.5	E14.5	E15.5	Adult
Rhombencephalon							
nIV	+	−	−	−	−	−	ND
nV	++	−	−	−	−	−	++
nVII	++	++&−[a]	++&−	++&−	+	+/−[b]	++
LC	++	++	+	−	−	−	+
PNS							
Autonomic ganglia							
Sympathetic neurons	++	++	++&−[c]	++&−	++&−	++&−	ND
Adrenal medulla	NA	NA	NA	+	+	+	ND
Parasympathetic neurons	NA	NA	NA	++	++	++	ND
Enteric neurons	Rare	ND	ND	Rare	Rare	ND	ND
Sensory neurons							
Cranial (V, VII, VIII, IX, X)	++	++	++	+	−[d]	−[d]	ND
Spinal (DRG)	++	++	++	++	+	−	++

[a]Premigratory and postmigratory FBM precursors express *RGS4*, migratory ones do not (see Results for details).

[b]Expression tends to fade out in FBMs.

[c]From E12.5 on, sympathetic ganglia are a mosaic of *RGS4*+ and *RGS4*− *cells.*

[d]The VIIIth (vestibulo-acoustic) ganglion keeps expression of RGS4. NA, Not applicable; ND, not determined.

pendent of any interaction with G-proteins]. In this respect, it is notable that, among bm/vm neurons, *RGS4* expression was primarily restricted to the facial and trigeminal nuclei, two purely branchial motor nuclei. Transient expression of RGS4 is therefore the first marker which differentiates branchial motor neurons (with the possible exception of precursors of the nucleus ambiguus) and visceral motor neurons. We also note that *RGS4* revealed an unexpected heterogeneity among sympathoadrenal precursors, particularly evident in the abdominal region in which expression was mostly confined to one large ganglionic or paraganglionic prevertebral mass. This mass is in a position compatible with that reported for the organ of Zuckerkandl, the main location of extra-adrenal chromaffin tissue (Ober, 1983).

In conclusion, out study shows that *RGS4* is transcribed in a highly regulated manner in subclasses of developing neurons, partially under the control of a determinant of neuronal identity, Phox2b. This is suggestive of developmental roles that inactivation of the gene by homologous recombination should help decipher.

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