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Generation and Characterization of *Rgs4* Mutant Mice

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RGS proteins are negative regulators of signaling through heterotrimeric G protein-coupled receptors and, as such, are in a position to regulate a plethora of biological phenomena. However, those have just begun to be explored in vivo. Here, we describe a mouse line deficient for *Rgs4*, a gene normally expressed early on in discrete populations of differentiating neurons and later on at multiple sites of the central nervous system, the cortex in particular, where it is one of the most highly transcribed *Rgs* genes. *Rgs4*$^{lacZ/lacZ}$ mice had normal neural development and were viable and fertile. Behavioral testing on mutant adults revealed subtle sensorimotor deficits but, so far, supported neither the proposed status of *Rgs4* as a schizophrenia susceptibility gene (by showing intact prepulse inhibition in the mutants) nor (unlike another member of the *Rgs* family, *Rgs9*) a role of *Rgs4* in the acute or chronic response to opioids.

RGS proteins are negative regulators of signaling through heterotrimeric guanidine nucleotide-binding proteins (G proteins). They act via their so-called RGS domain as GTPase-activating proteins for Gα subunits, most commonly of the Gi or Gq class (10, 40). Twenty family members have been described so far, falling into nine structural classes according to the presence and nature of protein motifs other than the RGS domain, endowing them with a variety of additional functions (9). RGS4, one of the most extensively studied RGS proteins at the structural, biochemical, and cell biological levels, defines the R4 or B class (see reference 32 for a review) in which the RGS domain is simply flanked by N and C terminal sequences with no recognizable motif other than an N-terminal amphipathic helix allowing for membrane attachment and some degree of G protein-coupled receptor specificity (50). Little is known about the in vivo roles of *Rgs4*. Expression and gain-of-function studies have suggested a role during gastrulation in *Xenopus laevis* (48), a role in the hypertrophic response of the heart to hypertension (39), and a capacity to modulate neurotransmitter receptors in the rat brain (5HT-1 autoreceptors in the dorsal raphe nucleus) (2). More speculatively, *Rgs4* being widely but differentially expressed in the central nervous system, most notably in the cerebral cortex (12, 16, 33), it is a candidate regulator of several types of neurotransmission acting through G protein-coupled receptors, such as dopaminergic, serotonergic, noradrenergic, glutamatergic, or opioid. Moreover, expression in certain brain areas is modulated by morphine (3, 13, 15), suggesting a role in opioid-induced adaptations. Along a different line of research, *Rgs4* has emerged, through human genetic studies, as a candidate susceptibility gene for schizophrenia (5, 7, 30, 47) and has been reported to be downregulated in postmortem cortices of schizophrenic patients (28). More recently, we described an exquisitely discrete and dynamic neural expression pattern during embryonic development, partially under the control of the neural type determinant *Phox2b*, which prompted us to hypothesize roles in neuronal differentiation (17).

To explore those various leads in vivo, we engineered an *Rgs4* knockout mouse. Here, we report that *Rgs4* null pups are viable, do not display any obvious developmental defect, and give rise to fertile adults. Behavioral studies failed to detect any decrease in prepulse inhibition (PPI) (a widely accepted schizophrenia-like trait in rodents) or altered responses to acute morphine or withdrawal from chronic morphine. No other behavioral anomaly was detected in a large set of other tests, except a subtle and complex sensorimotor deficit.

MATERIALS AND METHODS

Targeting strategy. The targeting vector, derived from a 129/OLA cosmid encompassing the *Rgs4* locus (RZPD Resource Center, Berlin, Germany), consisted of an 11-kb 5′ homology arm and a 1.12-kb 3′ homology arm separated by an Flp recognition target-flanked *neo* cassette itself preceded by a *loxP*/splice-acceptor site/promotorless *lacZ* cassette (Fig. 1A). The *neo* and *lacZ* cassettes replaced a 1.2-kb region containing low-complexity sequences with no similarity to the human locus, located 0.6 kb downstream of the *Rgs4* poly(A) signal. Upstream (4.5 kb) of these two cassettes, a second *loxP* site and a third *FRT* site were introduced in the second intron of *Rgs4*, so that recombination by the Flp or Cre recombinase would interrupt the *Rgs4* coding sequences before the RGS domain (Fig. 1A).

Production of mice and genotyping. A homologous recombinant clone of E14-1 embryonic stem (ES) cells (129/OLA) was obtained by electroporation and verified for the correct recombination event by Southern blotting using both internal and external probes (Fig. 1C and not shown). Injection of the clone into C57Bl/6 blastocysts produced nine male chimeras, four of which transmitted the recombinant locus. Genotyping of *Rgs4*$^{loxFRTlacZ/+}$ mice was performed by PCR on genomic tail DNA using primers which surround the *loxP*/*FRT* insertion site in the second intron (SensInt2, 5′-GGTGAGGGTACACAATTGTAG-3′; antiEx 4, 5′GCTTTGAAAGCTGCCAGTCC-3′) and produce a 566-bp and a 693-bp band from the wild type (wt) and the targeted allele, respectively (Fig. 1B and D). *Rgs4*$^{loxFRTlacZ/loxFRTlacZ}$ males were crossed with heterozygous *PGK-Cre* females (26) (on a B6D2 background), and the offspring was genotyped by PCR using two pairs of primers: one amplifying a 780-bp band from the Cre-recombined allele (SensInt2 [see above] and AntilacZ: 5′-GGCGCATCGTAACCGT

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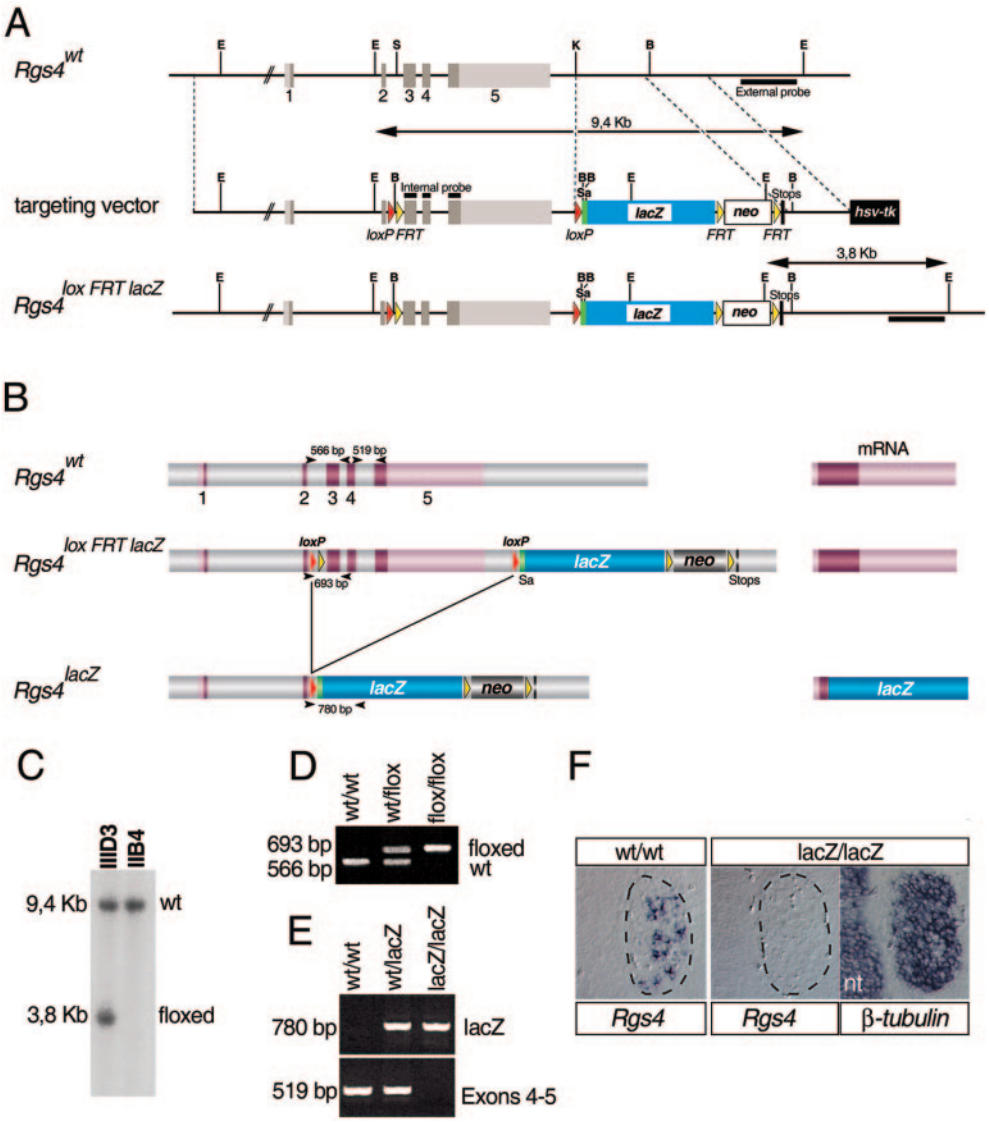

FIG. 1. Generation of *Rgs4* deficient mice. (A) Targeting strategy. (Top) Wild-type *Rgs4* allele with exons 1 to 5 (boxes, coding sequences in dark shade, non coding sequences in light shade). The RGS domain is encoded by exons 3, 4, and 5. Selected sites for EcoRI (E), BamHI (B), KpnI (K), and SwaI (S) are indicated. (Middle) Targeting vector. A loxP/BamHI/FRT cassette has been inserted in the SwaI site. A cassette (*loxP* site/splice acceptor [Sa]/promoterless *lacZ* open reading frame/FRT site/*neo* open reading frame under the control of the pGK promoter/FRT site/three out-of-phase stop codons) replaces the KpnI/BamHI fragment downstream of the fifth exon. A thymidine kinase cassette under the control of the *hsv* promoter was added at the 3′ end of the construct. (Bottom) Recombined locus. (B) PCR genotyping strategy of the wild-type, targeted, and Cre recombined loci. Primers are represented by arrowheads. (C) Southern blot of the DNA of a recombinant (IIID3) and a wild-type (IIB4) ES clone, digested by EcoRI and probed with the external probe depicted in panel A. floxed, recombined allele. (D) PCR analysis of tail DNA of wt, $Rgs4^{loxFRTlacZ/+}$ (wt/flox), and $Rgs4^{loxFRTlacZ/loxFRTlacZ}$ (flox/flox) pups with the primers depicted in panel B. (E) PCR analysis of tail DNA of wild-type, $Rgs4^{+/lacZ}$ (wt/lacZ), and $Rgs4^{lacZ/lacZ}$ (lacZ/lacZ) pups with the primers depicted in panel B (see Materials and Methods for details). (F) Transverse sections through wild-type and homozygous mutant E11.75 embryos, showing that the expression of *Rgs4* mRNA is lost in the mutants, even though the ganglionic cells are present, as assessed by β-tubulin expression. nt, neural tube.

GCAT-3′), the other amplifying a 519-bp band from the wild-type allele (SensEx4, 5′-CAACGAGTTCATCTCAGTGCCA-3′; AntiEx5, 5′-TGGGCTG TAACATGTTCCGG-3′) (Fig. 1B and E).

All strains of mice used in this work were bought at Janvier.

Histological methods. Embryos (the day of the vaginal plug was considered as embryonic day 0.5 [E0.5]) were stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as described by Knittel et al. (25) and for in situ hybridization combined or not combined with immunohistochemistry as described by Tiveron et al. (44). Adult tissues were treated as described by Grillet et al. (17). Golgi stains were done with the rapid GolgiStain kit (FD Neurotechnologies) according to the manufacturer's instructions.

In situ hybridization probes were *pKS-mRgs4* (17), *dopamine* β-*hydroxylase* (*Dbh*) (44), class III β-tubulin (kind gift of C. W. Ragsdale), and *lacZ*, and antibodies were specific for Phox2a (44), Phox2b (36), or Islet1/2 (kind gift of T. Jessell).

General health assessment and gross neurological examination were performed, with slight modifications, as previously described (19, 20) on 11 wt and 12 $Rgs4^{lacZ/lacZ}$ male littermates, 13 to 15 weeks old, born from matings of heterozygotes, themselves descendants of three backcrosses on C57BL/6 of the original (mixed 129/B6D2) Cre-deleted mouse. All experiments were carried out in accordance with the European Community Council Directive (24 November 1986) at the Institut Clinique de la Souris (Illkirch, France) except for the

morphine withdrawal test, which was carried out in the authors' lab. A more comprehensive description of each protocol can be found at http://www-mci.u-strasbg.fr/service_4.html.

Behavioral tests. (i) Rotating rod test. After three 60-s habituation trials on a rotating rod (Bioseb, Chaville, France) (one trial at 0 rpm and 2 trials at 4 rpm), mice were subjected to three testing trials (separated by 5- to 10-min intervals) during which the rotation accelerated from 4 to 40 rpm in 5 min and the time from latency to falling off was measured.

(ii) String test. In three consecutive trials (separated by 10-min intervals), the animals were suspended by their forepaws on a horizontally stretched wire, and the time they took to catch the wire with their hind paws was recorded.

(iii) Grip test. In three consecutive trials, the animals were allowed to grasp a grid connected to an isometric dynamometer (Bioseb) and slowly moved backwards until they released it. The maximal strength developed was expressed in grams per gram.

(iv) Tail flick test. The tail of the animal was placed under a heat source (a shutter-controlled lamp [Bioseb]) and heated three times at different sites at intervals of 1 min. The time taken by the animal to flick its tail was recorded (cutoff point at 25 s).

(v) Hot-plate test. In two trials, the mice were placed on a hot plate adjusted to 52°C (Bioseb), and the latency to the first reaction (licking, moving the paws, or leaping) was recorded. The test was ended if the mouse did not react within 30 s. In morphine experiments, the second trial duration was increased to 180 s maximum.

(vi) Shock threshold test. Mice were allowed to habituate for 30 s to a fear conditioning chamber. Behavioral responses to a 1-s foot shock (beginning at 0.05 mA and increased in 0.05-mA steps at 30-s intervals) were recorded. When both flinch (any detectable response) and vocalization were induced, shocks were increased in 0.1-mA steps (cutoff point at 1 mA) until a jump (defined as the two hind paws leaving the ground) was induced.

(vii) Open field test. Mice were tested in an automated open arena (Panlab, Barcelona, Spain) divided into central and peripheral regions homogeneously illuminated at 150 lx. Mice placed at the periphery of the open field were allowed to freely explore the apparatus for 30 min. The distance traveled, the number of rearings, and the time spent in the central and peripheral regions were recorded.

(viii) Tail suspension test. Mice were fitted in a tail suspension device (MED Associates Inc., St. Albans, Vermont). The latency to the first immobilization was determined, and the immobility time was monitored during a 6-min period in blocks of 2 min.

(ix) Auditory startle reflex reactivity and prepulse inhibition. Acoustic startle reactivity and prepulse inhibition of startle were assessed using a standard startle chamber (SR-Lab startle response system; San Diego Instruments) and 10 different trials: acoustic startle pulse alone (120 dB), prepulse trials with 70-, 80-, 85- or 90-dB stimuli presented either alone or preceding the pulse, and one trial in which only the background noise (65 dB) was presented to measure the baseline movement. The amount of PPI in the prepulse plus pulse trials was expressed as a percentage of the startle response in the pulse alone trial.

(x) Y-maze spontaneous alternation. Y-maze spontaneous alternation was assessed in a Y maze with three arms (40 by 9 by 16 cm) placed at 120° from each other and distinguishable by specific motifs on their walls. Mice were placed at the end of one arm and allowed to freely explore the apparatus for 5 min. Alternations were defined as successive entries into each of the three arms. Performance was defined as the ratio of actual alternations to possible alternations × 100.

(xi) Pavlovian fear conditioning. Pavlovian fear conditioning was assessed in 18.5- by 18- by 21.5-cm operant chambers (Coulbourn Instruments, Allentown, PA) with aluminium side walls and Plexiglas rear and front walls. A loudspeaker and a bright light, inserted on opposite walls, provided the cues during conditioning and cue testing. The activity of animals was monitored by infrared cells in the ceiling and recorded using the Graphic State software (Coulbourn). After a 4 min acclimatation, a light/tone (10 kHz) (conditional stimulus) was presented for 20 s and coterminated by a mild (1 s, 0.4 mA) foot shock (unconditional stimulus). Another conditional stimulus-unconditional stimulus pairing was presented 2 min later, and mice were returned to their home cages 2 min later. Twenty-four hours after the conditioning session, mice were tested for context by placing them back into the operant chamber and allowing them to explore it for 6 min. Testing for the cue was done 5 h after testing for context: mice were placed in a new chamber (with different wall color, odor, and floor texture), allowed to habituate for 2 min, and presented with light/auditory cues for 2 min. This sequence was repeated once.

(xii) Morphine withdrawal. Mice were injected intraperitoneally twice a day, 10 h apart, for 5 days with escalating doses of morphine (two injections of 20, 40, 60, 80, and 100 mg/kg of body weight on days 1, 2, 3, 4, and 5, respectively). On the sixth day, withdrawal was precipitated 2 h after one administration of morphine at 100 mg/kg, by subcutaneous injection of naloxone at 1 mg/kg. Withdrawal behaviors were monitored and scored over a period of 30 min according to Celerier et al. (4).

Statistical analysis. For quantitative parameters showing normal distribution, data were analyzed using the unpaired Student *t* test or repeated measures of analysis of variance (ANOVA) with one between factor (genotype) and one within factor (time). Qualitative parameters (e.g., some clinical observations) were analyzed using the χ^2 test. The level of significance was set at a P value of <0.05. Morphine analgesia and withdrawal scores were analyzed using two-way ANOVA with genotype and treatment as factors of variation. When appropriate, one-way ANOVA and Student-Newman-Keuls post hoc analysis were performed.

RESULTS AND DISCUSSION

Generation of *Rgs4* mutant mice. Since *Rgs4* is expressed at gastrulation in *Xenopus laevis* (48), potentially reflecting an embryonic early vital requirement in vertebrates, we modified the *Rgs4* locus so as to allow for tissue-specific knockouts. We sought to circumvent the potential mosaicism or incompleteness of the tissue-specific Cre-mediated recombination by the detection of effectively recombined cells. We thus designed the targeting vector so that (i) the action of a Cre recombinase on the conditional allele ($Rgs4^{loxFRTlacZ}$) would generate an in-frame fusion with the *lacZ* reporter ($Rgs4^{lacZ}$) (Fig. 1A and B) and (ii) the Flp recombinase could be used to generate a simple null allele ($Rgs4^{KO}$) (Fig. 1A) which, in combination with the conditional one, insures that all $lacZ^+$ cells are indeed $Rgs4^{-/-}$. In the present study, we analyzed the *Rgs4* null phenotype in $Rgs4^{lacZ/lacZ}$ mice. A homologous recombinant ES clone (Fig. 1C) was used to generate a chimera which gave rise to $Rgs4^{loxFRTlacZ/+}$ pups (Fig. 1D). These in turn were crossed with *PGK-Cre* mice (26) to produce $Rgs4^{lacZ/+}$ offspring (Fig. 1E) which were viable and fertile. Intercrosses of $Rgs4^{lacZ/+}$ animals produced homozygous mutants in which the disruption of the *Rgs4* locus was further verified by the fact that *Rgs4* was undetectable by in situ hybridization (Fig. 1F).

$Rgs4^{lacZ/+}$ embryos and adults examined by X-Gal staining or *lacZ* in situ hybridization displayed a pattern of *lacZ* expression matching and refining the previously described pattern of *Rgs4* expression during development (17) and in the adult nervous system (16, 33) (Fig. 2A to E and data not shown). A previously undescribed neuronal site was a subset of ganglion cells in the retina (Fig. 2F). The main discrepancy between our observations and published expression data concerns reports of *Rgs4* mRNA in heart tissue, cardiomyocyte primary cultures (6, 24, 51), and even individual atrial myocytes (11). Based on this expression and its modulation during congestive heart failure as well as on gain-of-function experiments, *Rgs4* has been proposed to mediate a counterregulatory mechanism during cardiac hypertrophy (29, 33, 35, 39, 41). However, we could not detect any *lacZ* expression in the heart muscle of $RGS4^{lacZ}$ adult mice (Fig. 2G), nor could we detect *Rgs4* mRNA expression by in situ hybridization (not shown). This, barring some species difference between mouse and rat or human (which were studied in previous reports), suggests that *Rgs4* expression, if real, is extremely low and represents an improbable pharmacological target in cardiomyocytes. In contrast, we detected high levels of expression in the walls (presumably smooth muscle cells) of the large vessels of the heart and coronaries (Fig. 2G and H and not shown), which could

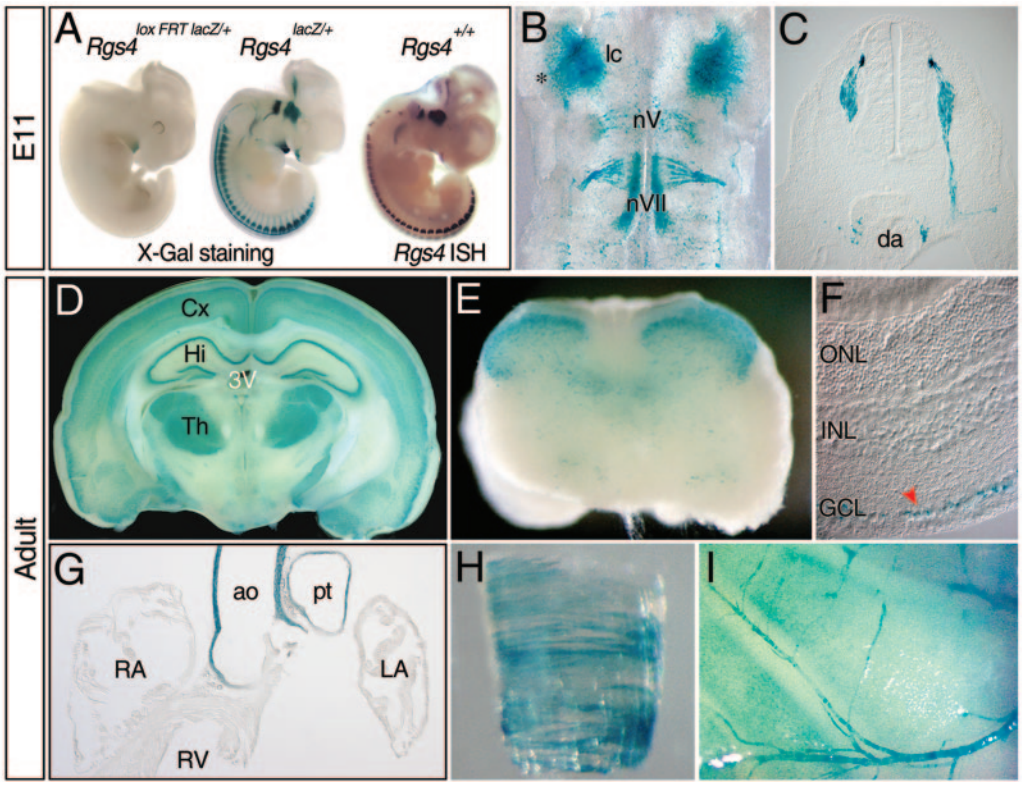

FIG. 2. Expression pattern of *Rsg4* monitored by X-Gal staining in $Rgs4^{lacZ/+}$ embryos and mice. (A) Whole mounts of $Rgs4^{loxFRTlacZ/+}$ E11 embryos without (left) or with (middle) the action of the Cre recombinase (provided by a *PGK-Cre* female). (Right) In situ hybridization (ISH) with an *Rgs4* probe on a wild-type embryo. A slight localized leakage of *lacZ* expression from the $Rgs4^{loxFRTlacZ}$ locus is visible in the mandibles of $Rgs4^{loxFRTlacZ/+}$ embryos. The action of the Cre recombinase triggers *lacZ* expression in a pattern indistinguishable from that of *Rgs4*. (B) Flat mount of a hindbrain from an $Rgs4^{lacZ/+}$ E11 embryo showing expression of the *Rgs4* locus in the forming trigeminal (nV) and facial (nVII) motor nuclei, as well as in the locus coeruleus (lc) and unidentified interneurons in the metencephalon (star). (C) Section of the thorax of an E11 embryo showing expression of $Rgs4^{lacZ}$ in the dorsal root ganglia and in the anlagen of sympathetic ganglia surrounding the dorsal aorta (da). (D) Coronal section of the forebrain of an adult $Rgs4^{lacZ/+}$ mouse. Cx, cortex; Hi, hippocampus; Th, thalamus; 3V, third ventricle. The expression in hippocampus is comparable to that in cortex and stronger than reported by Gold et al. (16). (E) Section of the spinal cord of an adult $Rgs4^{lacZ/+}$ mouse showing expression of *lacZ* in the dorsal horns. (F) In the retina, a subset of ganglion cells (arrowhead) express *Rgs4*. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. (G) Sagittal section of the heart of an adult $Rgs4^{lacZ/+}$ mouse treated with X-Gal, showing intense expression in the aorta (ao) and pulmonary trunk (pt) but none in the heart muscle. LA, left atrium; RA, right atrium; RV, right ventricle. (H) Close-up of $Rgs4^{lacZ}$ expression in a whole mount of aorta, suggestive of smooth muscular expression. (I) *Rgs4* expression in blood vessels on the surface of the cerebellum.

underlie at least part of the findings cited above. This strong vascular expression, also found at other sites (Fig. 2I) (and also reported for *Rgs5* [1] and *Rgs2* [6]) suggests an involvement in normal vascular function and the regulation of blood pressure, as was shown for *Rgs2* (42).

$Rgs4^{lacZ/+}$ mice were intercrossed to produce homozygous mutants which were recovered at a lower than Mendelian frequency (16%; $n = 136$). Roughly the same proportion of homozygotes were recovered at E8.5 (17%; $n = 36$) as at later stages. These figures, contrasted with the full viability of homozygotes resulting from intercrosses of $Rgs4^{loxFRTlacZ/+}$ mice (26%; $n = 83$) suggests an early, incompletely penetrant lethality of *Rgs4* null embryos. However, such a lethality would be dependent on the DBA/2 background (brought by the deleter mouse) and/or the E129 background (brought by the recombinant ES cell) since, after three backcrosses on a C57BL/6 background, a Mendelian frequency was obtained.

Rgs4 mutants had lower body weight than their wt littermates ($t_{21} = 4.46$, $P < 0.001$) (Table 1) but overall were in good general health (normal body temperature, well-groomed coat, and normal body posture and righting reflex) (Table 1 and not shown). Gross neurological examination of animals revealed no sign of modified sensory functions as assessed in basic tests of vision, audition, olfaction, and touch sensitivity. However, it should be noted that most wt mice (8/11) but only

TABLE 1. Body weight, temperature, and neurological tests

Parameter	Avg value for indicated mouse group[a]	
	Wild type	$Rgs4^{lacZ/lacZ}$
Body weight (g)	33.7 ± 0.8	30.2 ± 0.3***
Body temperature (°C)	37.5 ± 0.2	37.3 ± 0.2
Time to fall off rotating rod	111.3 ± 4.9	82.2 ± 6.3**
Time to complete string test (s)	16.7 ± 5.8	18.5 ± 4.2
Grip strength (g/g)	6.7 ± 0.3	6.7 ± 0.2

[a] Asterisks indicate significant difference from corresponding wild-type values (unpaired Student *t* test). **, $P < 0.01$; ***, $P < 0.001$.

2/12 mutants vocalized during handling ($\chi^2 = 7.3$; $P < 0.01$). *Rgs4* mutants had poorer performance in the rotating rod test than wt mice ($t_{21} = 3.62$; $P < 0.01$) (Table 1), while they performed correctly in the grip and string tests (Table 1), suggesting an alteration in balance/motor coordination. It is of note that inactivation of either Gαq (34) or Gαo (22), both potential targets of RGS4, also leads to coordination deficits, which are, however, much more pronounced.

A host of biological roles for *Rgs4* have been proposed based on the expression of the gene and on the in vitro cell biological properties of the protein. In this study we have explored several of those, mostly with negative results.

Normal neuronal differentiation in *Rgs4*$^{lacZ/lacZ}$ mice. We previously showed an exquisitely specific pattern of *Rgs4* expression in the developing nervous system, partially under the control of a well characterized determinant of neuronal identity, the homeobox gene *Phox2b* (17). In spite of the lack of a gross developmental phenotype in homozygous mutant mice, we looked for subtle differentiation anomalies or delays in *Rgs4*$^{+}$ neuronal precursors. Using the neuronal transcription factors Phox2a (44), Phox2b (36), and Islet1/2 (43) or *lacZ* as markers, we could not detect any defect in shape, size, or timing of formation of the facial and trigeminal nuclei, the locus coeruleus (LC), sympathetic and parasympathetic ganglia, or cranial ganglia VII, IX, and X (Fig. 3A to C' and data not shown). The noradrenergic differentiation of sympathetic ganglion cells or the locus coeruleus were unaffected (Fig. 3D and D' and not shown) as well as sympathetic axonal projections examined on *Rgs4*$^{lacZ/lacZ}$; *Phox2a*$^{tau lacZ/+}$ embryos (21) (not shown).

At later stages of development and in the adult, *Rgs4* is switched on in many telencephalic and diencephalic structures. The gross histological structure of the brain, the pattern of *lacZ* expression, and Nissl and Golgi stains on the cortex of heterozygous and homozygous mutants failed to reveal any difference between them (Fig. 3E and E' and data not shown).

No obvious interference of the *Rgs4* mutation with opioid signaling. Endogenous or exogenous RGS proteins have been shown in vitro to dampen signaling through the μ-opioid receptor (9, 37, 38). It follows that *Rgs4* could regulate antinociception by endogenous or exogenous opioids, both presynaptically in nociceptor neurons of the dorsal root ganglia and postsynaptically in second-order sensory neurons on which they project, two sites of strong *Rgs4* expression (13, 17) (Fig. 2E). We therefore tested the sensitivity of wt mice and mutants first to pain and then to the analgesic effects of morphine treatment.

Mutant mice had normal pain sensitivity in the tail flick test and showed first-reaction latency comparable to that of wt mice in the hot-plate test (Table 2). In the shock threshold test, subtle differences between wt mice and mutants were detected: the shock levels at which mice first detected the shock (flinch) were comparable in wt and *Rgs4*$^{lacZ/lacZ}$ mice (Table 2), but the shock intensity required to induce jumping was significantly increased in *Rgs4*$^{lacZ/lacZ}$ mice compared to wt ($t_{21} = 2.76$; $P < 0.05$, Student *t* test) (Table 2). Thus, the proportion of mutants displaying a jumping reaction before the cutoff point (41.6%) was significantly reduced compared to wt mice (90.9%) ($\chi^2 = 6.1$; $P < 0.05$), suggesting an effect of the mutation on central processing of painful stimuli.

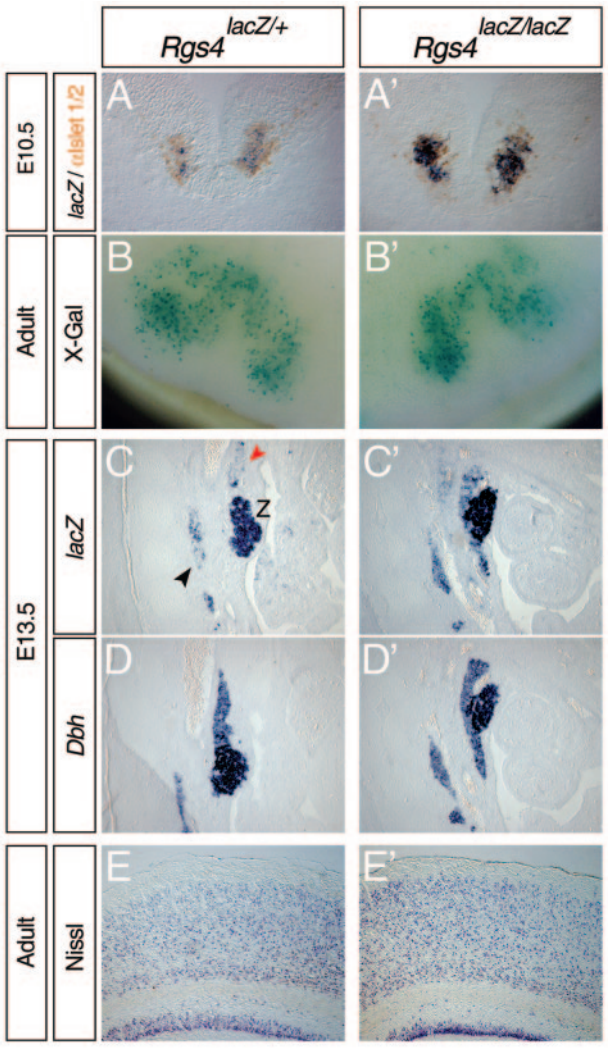

FIG. 3. Absence of a neuronal developmental defect in *Rgs4*$^{lacZ/lacZ}$ embryos. (A and A') Combined in situ hybridization for *lacZ* and immunohistochemistry for the motoneuronal marker Islet1/2 on transverse sections at the level of the fourth rhombomere, showing an intact differentiation of facial motoneurons. (B and B') Transverse sections through the pons of adult mice stained with X-Gal and showing morphologically intact facial nuclei in the homozygote mutants. (C to D') Sagittal sections of E13.5 embryos hybridized with a *lacZ* or *Dbh* probe, showing intact morphology and *Dbh* expression of the paravertebral (black arrowhead) and prevertebral (red arrowhead) sympathetic chains and organ of Zuckerkandl (z) in homozygous (C' to D') compared to heterozygous (C to D) mutants. (E and E') Nissl stain of sections of the cortex, showing normal cytoarchitecture in *Rgs4* null mutants.

Systemic injection of morphine at the dose of 8 mg/kg induced comparable antinociceptive effects in wt mice and mutants in the tail flick assay [treatment effect, $F(1,19) = 12.9$ (numbers in parentheses represent degrees of freedom corresponding to each experiment) and $P < 0.01$; genotype effect, $F(1,19) = 0.2$ and $P > 0.05$; genotype × treatment interaction, $F(1,19) = 0.6$ and $P > 0.05$] as well as in the first reaction to the hot-plate test [treatment effect, $F(1,19) = 17.5$ and $P < 0.001$; genotype effect, $F(1,19) = 0.4$ and $P > 0.05$; genotype × treatment interaction, $F(1,19) = 0.4$ and $P > 0.05$].

TABLE 2. Pain sensitivity

Parameter	Avg value for indicated mouse group	
	Wild type	*Rgs4*$^{lacZ/lacZ}$
Time to complete tail flick test (s)	6.0 ± 0.7	5.0 ± 0.3
Time to complete hot plate test (s)	9.3 ± 0.6	8.8 ± 0.5
Shock threshold (mA)		
Flinching	0.05 ± 0.00	0.05 ± 0.00
Jumping	0.57 ± 0.06	0.82 ± 0.07*

[a] An asterisk indicates significant difference ($P < 0.05$) from the corresponding wild-type value (unpaired Student *t* test).

Another potential role of *Rgs4* connected to its biochemical capacity to regulate opioid signaling is suggested by its expression in the LC (16, 17). The LC, the main noradrenergic center of the brain, has a high level of opioid receptors and is hypothesized on numerous grounds to be a mediator of the opioid withdrawal syndrome (reviewed in reference 31), although the evidence has been extensively questioned (reviewed in references 8 and 46). We therefore tested a role for *Rgs4* in somatic signs of abstinence to opioids according to the procedure described in reference 4 (see Materials and Methods). No difference between mutants and wt mice was found, except for an almost complete suppression of the sniffing reflex (Fig. 4). It is of note that sniffing, a minor sign of abstinence, also stood out in the D2 dopamine receptor knockout mice as being the only withdrawal symptom affected (exacerbated, in that case) (27).

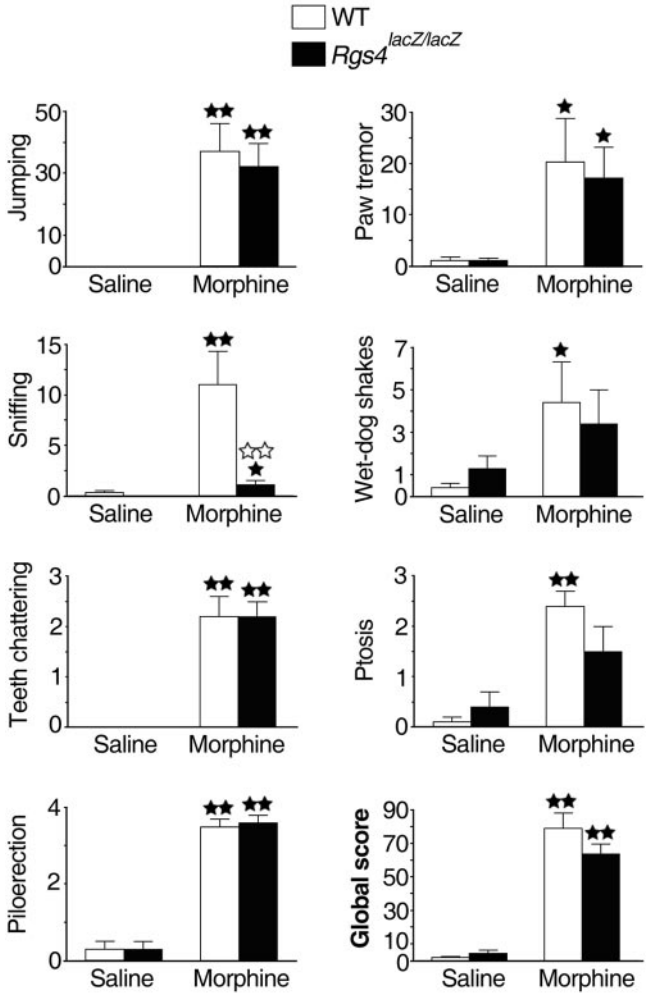

FIG. 4. Naloxone-precipitated morphine withdrawal syndrome in wild-type and *Rgs4* null mice. Data are expressed as means ± standard errors of the means. Black stars, morphine- versus saline-treated animals of the same genotype; white stars, wild-type versus mutant groups receiving the same treatment (two-tailed Student *t* test). One star, $P < 0.05$; two stars, $P < 0.01$.

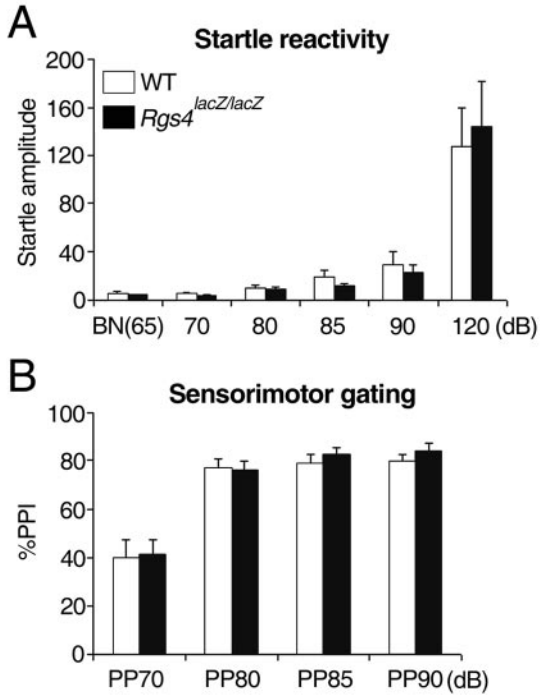

FIG. 5. Startle reactivity and prepulse inhibition in wild-type and *Rgs4* null mice. (A) The startle amplitude is measured at the background noise (BN) level or at indicated intensities. (B) The PPI is measured as percent startle response to the 120-dB pulse for prepulses of indicated intensities. Values are expressed as means ± standard errors of the means.

These data stand in stark contrast with the marked increase in morphine-induced analgesia and physical symptoms of withdrawal in *Rgs9-2* knockout mice (49). The difference could be due to class-specific effects (RGS9, belonging to the C class of RGS proteins, possesses, unlike RGS4, DEP and GGL domains) or to tissue distribution differences (the site[s] of *Rgs9-2* action on analgesia or withdrawal being presently unknown). Future studies should aim at exploring morphine tolerance (delayed in *Rgs9* knockouts [49]) and unraveling either more subtle or more integrative aspects of responses to pain in *Rgs4* mutants.

No defect in prepulse inhibition in *Rgs4* mutant mice. Along a different line of research, significant association between the *Rgs4* locus and schizophrenia has been reported in several studies (5, 7, 30, 47), and *Rgs4* transcripts have been found to be reduced in cortices of schizophrenic patients (28; see reference 18 for a review). The consequent status of *Rgs4* as a susceptibility gene for schizophrenia is strengthened by its

abundant expression in the cortex, where it could regulate dopaminergic and glutamatergic transmission, both invoked in models of schizophrenia (see references 18 and 23 for a review). We therefore tested mutant mice for PPI, a measure of sensorimotor gating abilities, whose decrease is one of the most widely accepted schizophrenia-related behaviors in rodents (see reference 14 for a review). Mutant mice displayed a normal startle response compared to wt mice at all tested acoustic stimulus intensities (Fig. 5A). They also showed a normal PPI across all prepulse intensities used (Fig. 5B). Another test of schizophrenia-like cognitive dysfunction in rodents (latent inhibition) should be performed in the future, as well as potential interactions of the *Rgs4* null mutation with schizophrenogenic environmental paradigms (45).

We also tested for depression- and anxiety-related symptoms (by the tail suspension test and the open-field test, respectively) and for associative learning and working memory defects (by Pavlovian fear conditioning and Y-maze spontaneous alternation, respectively). $Rgs4^{lacZ/lacZ}$ mice did not show any difference in their performance compared to wt mice in any of these tests (not shown).

In conclusion, subtle positive symptoms in *Rgs4* mutants (namely, lower weight, partially penetrant, genetic background-dependent early lethality, poorer sensorimotor coordination, and central integration of painful stimuli) point to developmental and physiological roles for *Rgs4*. Further purification of the genetic background may unmask other, presumably subtler, behavioral phenotypes by reducing the variability between individuals. On the other hand, combined inactivation of other *Rgs* genes, facilitated by the fertility of *Rgs4* null mice, may reveal roles hitherto masked by redundancy. Finally, postnatal inactivation allowed by our conditional knockout scheme could unveil roles developmentally compensated for in *Rgs4* null animals.

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