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Imprinted *Rasgrf1* expression in neonatal mice affects olfactory learning and memory

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Abstract

Rasgrf1 is genomically imprinted; only the paternally-inherited allele is expressed in the neonatal mouse brain until weaning, at which time expression becomes biallelic. Whereas *Rasgrf1* has been implicated in learning and memory via knockout studies in adult mice, the effect of its normal imprinted expression on these phenotypes has not yet been examined. Neonatal mice with experimentally manipulated patterns of imprinted *Rasgrf1* expression were assessed on an associative olfactory task. Neonates lacking the normally-expressed wildtype paternal allele exhibited significant impairment in olfactory associative memory. Adult animals in which neonatal imprinting had been manipulated were also behaviorally assessed; while neonatal imprinting significantly affects body weight even into adulthood, no learning and memory phenotype attributable to imprinting was observed in adults. Additional analyses of neonates revealed imprinted *Rasgrf1* transcript selective to olfactory bulb even in mice that were null for *Rasgrf1* in the rest of the brain, and showed that *Rasgrf1* affects Ras and Rac activation in the brain. Taken together, these results indicate that *Rasgrf1* expression from the wildtype paternal allele contributes to learning and memory in neonatal mice.

Keywords

genomic imprinting; olfactory learning; development; memory consolidation; conflict hypothesis

INTRODUCTION

Rasgrf1 is an imprinted gene that is expressed solely from the paternally-inherited allele in neonatal mouse brain until weaning (postnatal day 21; P21), at which time its expression becomes biallelic (Drake *et al.* 2009; Plass *et al.* 1996). *Rasgrf1* imprinting is controlled by a binary switch consisting of a differentially-methylated domain (DMD) and a series of repeats immediately 3' of the DMD. These repeats direct the placement of methylation on the paternal DMD, which regulates gene transcription at the *Rasgrf1* locus. In contrast, the maternal DMD is unmethylated, which permits CTCF binding and results in the inhibition of gene transcription from the maternal allele (Yoon *et al.* 2005; Yoon *et al.* 2002). Hence, only the paternal allele is normally expressed in neonates. *Rasgrf1* is most highly expressed

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in central nervous system neurons (Sturani *et al.* 1997; Zippel *et al.* 2000), with lower expression levels in other somatic tissues (Font de Mora *et al.* 2003; Plass *et al.* 1996). Its product, RasGRF1 protein, is a guanine nucleotide exchange factor for Ras and Rac (Cen *et al.* 1993; Innocenti *et al.* 1999), activating these G-proteins in response to cellular calcium influx (Farnsworth *et al.* 1995) or serine phosphorylation (Mattingly *et al.* 1999; Yang *et al.* 2003) in pathways downstream of muscarinic receptor activity (Mattingly & Macara 1996), heterotrimeric G-protein subunit dissociation (Kiyono *et al.* 2000; Shou *et al.* 1995), and neurotrophin binding to TrkA, TrkB, and TrkC receptors (MacDonald *et al.* 1999; Robinson *et al.* 2005).

Rasgrf1 is involved in multiple neuronal learning and plasticity mechanisms, including Ras-MAPK-dependent memory consolidation and long-term plasticity in the amygdala (Brambilla *et al.* 1997) and Ras-ERK pathway activation (Fasano *et al.* 2009; Krapivinsky *et al.* 2003). Correspondingly, adult *Rasgrf1* knockout mice are impaired in several learning and memory tasks (Brambilla *et al.* 1997; Fasano *et al.* 2009; Giese *et al.* 2001). These results suggested that the normal neonatal imprinting of *Rasgrf1* expression may affect learning performance in neonatal mice – a prediction consistent with the conflict hypothesis of genomic imprinting (Moore & Haig 1991; Wilkins & Haig 2003). Here, we evaluated the effects of perturbation of *Rasgrf1* imprinting on olfactory associative learning during the neonatal period when *Rasgrf1* expression is normally imprinted. Specifically, we engineered two mutant *Rasgrf1* alleles that, respectively, prevented expression of the paternally-inherited allele or forced expression of the maternally-inherited allele; with these we generated neonates expressing *Rasgrf1* from either, neither, or both parentally-inherited alleles and tested them with an olfactory associative learning assay. Additionally, as *Rasgrf1* imprinting in neonates affects postnatal growth well into adulthood (Drake *et al.* 2009), we also measured the effects of neonatal imprinting on a battery of behavioral assays in adults.

MATERIALS AND METHODS

Animals

Mice utilized for behavioral experiments carried various combinations of wildtype and two mutated alleles designed to alter the pattern of neonatal imprinting (allele-specific expression). One mutated allele, *Rasgrf1*^{tm1Pds}, or *tm1*, prevents *Rasgrf1* expression in neonates when paternally inherited (Yoon *el al.* 2002). The second mutated allele, *Rasgrf1*^{tm2Pds}, or *tm2*, activates expression of *Rasgrf1*, including expression from the normally silent maternal allele. The *Pgk* enhancer enforces expression of the *tm2* allele (Yoon *et al.* 2005). Mutations were prepared using J1 ES cells (129S4Jae background); mice bearing the mutations were backcrossed a minimum of 10 times onto the C57BL/6J background before the additional crosses described herein were performed. For imprinting tests, we crossed C57BL/6J mice with FVB/NJ mates.

Animals used in behavioral experiments were derived from two crosses. First, +/*tm1* males were bred with *tm2*/+ females, which generated four genotypes and facilitated the use of littermate controls. Specifically, in addition to the wildtype genotype, in which neonatal *Rasgrf1* expression is monoallelic and derived from the paternal allele (MP, +/+ or *wt*), mutant genotypes were generated that exhibited biallelic expression (B, *tm2*/+; maternal allele is listed first), were null for *Rasgrf1* expression (N, +/*tm1*), or that exhibited monoallelic expression from the maternal allele (MM, *tm2/tm1*). Second, +/+ females were crossed with *tm2*/+ males, which generated a fifth genotype, +/*tm2*, as well as +/+ (MP) littermates to use as controls. The +/*tm2* genotype was constructed to replicate wildtype (MP) imprinted expression of *Rasgrf1* in behavioral experiments so as to control for any effects of the manipulations used to express the *tm2* allele. Both *tm1* and *tm2* alleles were maintained on a C57BL/6J background. Mice used for RNA quantification were derived by

crossing *tm2/tm2*, *tm1/tm1*, and +/+ homozygotes to generate the five genotypes (*tm2*/+, *tm2/tm1*, +/*tm1*, +/+, and +/*tm2*).

Mice were maintained on a regular 12:12-hour light/dark cycle and had ad libitum access to food and water except where specified in Materials and Methods. All experiments were carried out under a protocol approved by the Cornell University Institutional Animal Care and Use Committee in accordance with NIH guidelines.

Neonatal olfactory associative learning assay

Training—Mouse pups, eight days postnatal (P8, with P1 defined as the day of birth), were assessed for olfactory associative learning using established methods (Armstrong *et al.* 2006); except as noted, all procedures used were identical to those described therein. Briefly, two odor stimuli were prepared: 2-furyl methyl ketone (FMK) and *n*-hexyl acetate (HA), differentially diluted in mineral oil to concentrations theoretically emitting vapor-phase partial pressures of 5.0 Pa (Cleland *et al.* 2002); the corresponding liquid-phase (vol/vol) dilution ratios were 13.0×10^{-3} for FMK and 11.4×10^{-3} for HA. Mouse pups (individually identified on P2 using footpad tattoos) were separated from their dam by removing the dam from the home cage for 90 minutes. One of the diluted test odorants was then applied to all of the dam's nipples, after which she was returned to the home cage so that the pups could suckle and thereby associate the experimental odorant with a milk reward. This training procedure was repeated daily from P3 through P8. Whereas each litter was presented with the same odor CS across the five training trials, odorants were counterbalanced across litters, such that half of the litters associated reward with FMK and the other half with HA.

Testing—After the final training session on P8, pups were allowed to suckle for 45 minutes and then were again separated from the dam for 120 minutes before being assessed for an associatively learned odor preference (Alleva & Calamandrei 1986; Armstrong *et al.* 2006). Briefly, pups were tested for place preference in a 32 × 19 cm plexiglass arena (13 cm wall height) with a wire mesh placed above two adjacent 12 × 19 × 7 cm deep compartments. One compartment contained a Kimwipe (Kimberly-Clark, Neenah, WI, USA) saturated with 500 μL of diluted FMK, the other the same but with diluted HA; note that for any given pup one of these odorants had been used for training whereas the other had never before been presented. The two compartments were separated by 0.7 cm (wall thickness) where they met under the center of the arena; the pup was placed on the mesh atop this wall, facing away from the experimenter, such that their right limbs were placed over one compartment and their left limbs were placed over the other compartment.

The exploratory behavior of each pup was recorded for 180 seconds (s). Each pup was scored as investigating a compartment whenever the pup moved its muzzle or limbs completely off the center wall and directly over a compartment. Pups were replaced upon the midline when the following criteria were met so that their limited mobility would not dominate the assessment of preference: if a pup fell over so as to be unable to regulate its movements, reached the external wall of the arena, froze for 3 s without head movements, or began grooming, it was replaced on the midline in the opposite orientation. The total accumulated time spent sniffing or moving over each of the two compartments was recorded by stopwatch. The orientation of the two compartments was varied with respect to both odor identity and odor contingency between test trials; furthermore, the spatial orientation of the scented compartments was also varied with respect to the room to control for differences in external cues. The dam and littermates of the pup being tested were removed to a distant location to avoid possible distraction via ultrasonic calls. The experimenter was blind to genotype during testing.

Immediately following testing, tail biopsies were taken for genotyping. The group sizes for neonatal behavioral testing were as follows: 68 wildtype/MP, 59 biallelic (B), 31 monoallelic maternal (MM), 46 null (N), and 29 *+/tm2* mutants.

Rasgrf1 Quantification

For quantification of *Rasgrf1* transcript levels, brains were collected from P8 neonates and the olfactory bulbs and hippocampi were dissected out. *Rasgrf1* transcript levels were separately measured by quantitative PCR in olfactory bulb, hippocampus, and in the remainder of the brain (ROB, comprising the whole brain excepting olfactory bulbs, hippocampus, hypothalamus and pituitary gland) for each of the five genotypes. Specifically, RNA was extracted, reverse-transcribed, quantified in triplicate using an ABI Taqman© probe specific for *Rasgrf1*, and normalized to 18S rRNA levels. Alpha levels for statistical significance were Bonferroni-corrected for ten multiple comparisons such that $p < 0.005$ indicated significance.

Rasgrf1 Imprinted Expression

RNA was separately isolated from the olfactory bulbs and the rest of the brain (in this case including all structures other than the olfactory bulbs) of P8 neonates bred from reciprocal crosses between C57BL/6J and FVB/NJ males and females. RNA was reverse-transcribed into cDNA, and PCR amplified with the following primers and cycling conditions: (F) 5'-ggctcatgatgaatgccttt-3' (R) 5'-tacagaagcttggcgttgtg-3'; 95 °C × 3 minutes, followed by 40 cycles of 95 °C × 20 seconds, 58 °C × 30 seconds, 72 °C × 50 seconds, followed by 72 °C × 5 minutes. PCR products were then digested with 10U *Aci*I, which recognizes a restriction site (C'CGC) in exon 14 that distinguishes expression derived from either of the two parental strains (SNP ID rs29947965). C57BL/6J-derived expression was indicated by bands of 210/146 bp, and FVB/N-derived expression was indicated by bands at 226/130 bp.

Ras and Rac signaling assays

To determine whether Ras or Rac activation was influenced by mutations at *Rasgrf1*, we quantified the levels of the active forms of both G proteins in the olfactory bulb, hippocampus, and isocortex of P8 wildtype (MP) and null mutant (N) neonates. Similarly-sized tissue samples of olfactory bulb, hippocampus, and isocortex were removed from extracted brains and placed in Krebs-Ringer solution (11.1 mM glucose, 1.1 mM $MgCl_2$, 1 mM Na_2HPO_4, 1.3 mM $CaCl_2$, 25 mM $NaHCO_3$, 120 mM NaCl, 4.7 mM KCl). To extract protein, tissues were homogenized in 0.5 ml of extraction buffer on ice using a Dounce 1-ml homogenizer with a tightly fitting pestle. The extraction buffer included 1 mM sodium orthovanadate (Na_2VO_4), 25 mM sodium fluoride (NaF), and EDTA-free protease inhibitor tablets (Roche) in magnesium-containing buffer supplied by Millipore (#20–168; 125 mM HEPES, pH 7.5, 750 mM NaCl, 5% IGEPAL CA-630, 50 mM $MgCl_2$, 5 mM EDTA, and 10% glycerol). Extracts then were affinity purified using Ras and Rac activation assay kits (Millipore 17–218 and 17–283 respectively). Purified protein extracts then were SDS-PAGE electrophoresed, blotted, and probed using α-Ras (05–516) and α-Rac (05–389) antibodies supplied with the kits in conjunction with goat α-mouse HRP-conjugated IgG secondary antibody (Millipore 12–349). Blots were visualized using SuperSignal West Dura Substrate (Pierce 34075), captured using a LAS-4000 imager's CCD camera and chemiluminescent detection function, and quantified using MultiGauge software. The amounts of precipitated (active) protein were normalized to the amounts of input (total) protein for each structure, and then normalized to wildtype levels; N=4 mice for all comparisons.

Adult behavioral assays

Adult mice (37–52 days old) of four genotypes (B, MM, MP, N) were tested on a battery of standard behavioral phenotyping tasks in order to assess whether a learning deficit phenotype such as that observed in neonates with abnormal *Rasgrf1* imprinting persisted into adulthood (i.e., after the onset of biallelic *Rasgrf1* expression in all genotypes). Such persistent effects of neonatal *Rasgrf1* imprinting have been observed for body mass (Drake *et al*. 2009). Specifically, measurements of working memory, olfactory nonassociative memory, and short- and long-term fear memory tasks dependent on amygdala and hippocampus were performed; additionally, several control studies assessing motor function and basal activity and anxiety levels were performed to aid interpretation of the learning task results. The same mice were used for all studies except fear conditioning (inhibitory avoidance, cued and contextual conditioning), for which tests the mice were divided into groups such that each animal was subjected to only one shock. Moreover, in fear conditioning studies, mice were either assessed at 0.5 hours after training (short-term fear memory) or 24 hours (long-term fear memory), but not both.

Mice were assessed on all behavioral tasks in the following order across four consecutive days. Day 1: neurological screening, open field, balance beams, wire forelimb suspension, vertical pole, hanging wire grip test. Day 2: olfactory habituation, visual cliff, prehabituation to fear-conditioning test cage, spontaneous alternation, social recognition. Day 3: fear conditioning (either light/dark inhibitory avoidance or auditory tone-cued conditioning) and 0.5 hour assessment. Day 4: 24-hour assessment of fear conditioning. Detailed methods for behavioral tests other than learning and memory tests are provided in Supplemental Materials.

Learning and memory tests—A spontaneous alternation test was performed to assess working memory (King & Arendash 2002). Mice were placed in a radially symmetric plexiglass Y-maze, with arms 4 cm wide × 21 cm long and with 40 cm high walls, and allowed to explore for 300 seconds. The total number of arm entries was recorded as an additional measure of baseline activity. Spontaneous alternation was measured as the proportion of arm choices differing from the previous two choices; i.e., the number of such choices divided by the total number of opportunities to alternate (the total number of arm entries minus two). The score for spontaneous alteration reflects each animal's memory of their exploration, since mice tend to avoid re-entering the arm that they explored most recently.

An olfactory habituation task was performed in a standard mouse housing box to assess nonassociative memory performance (Cleland *et al*. 2002). Presentation of an odorant elicits active investigation of the odor source; the extent of this investigation declines gradually over repeated presentations of the same odorant. Subsequent presentation of a different test odorant will elicit an increased investigative response depending upon the degree of similarity of the habituation and test odorants. In this study, acetic acid was used for three sequential habituation trials and the moderately similar odorant pentanoic acid for one subsequent test trial. Both odorants were diluted in mineral oil to theoretically emit vapor-phase partial pressures of 0.01 Pa, and were presented for 60 s per trial with 120 s intertrial intervals.

For light/dark inhibitory avoidance conditioning, mice were placed in the brightly-lit side of an automated, two-chamber light/dark shuttle cage (Coulbourn Instruments, Whitehall, PA, USA) to which they had been prehabituated on the previous day. After 10 seconds, the door between the two chambers was opened, and the latency for mice to enter the dark side was recorded. Once the mouse entered the dark side, the door was closed and a 2 s, 0.5 mA footshock was delivered. After an additional ten seconds, the mouse was returned to its

home cage. Half of the mice were then re-tested 30 minutes later (short-term fear memory), while the other half were tested 24 hours later (long-term fear memory); the latency to enter the dark side of the shuttle cage was again recorded (up to a maximum of 180 s).

Finally, a joint cued/contextual learning paradigm was administered to mice that had not undergone inhibitory avoidance conditioning. Freezing behavior was assessed during each of four training/testing epochs, noted parenthetically below by name. To score freezing, mice were assessed every 5 seconds and scored as freezing or non-freezing; scoring was based on the average of these assessments. Mice were first placed into a square enclosure to which they had been previously habituated and given 120 s to explore (*Pre*). An 80 dB white noise auditory cue was then delivered for 30 s; during the last 2 s of cue presentation a 0.5 mA footshock was delivered. After an additional 60 s in the training enclosure, the mouse was removed to its home cage. Half of the mice began testing 30 minutes later (short-term memory) while the other half began testing 24 hours later (long-term memory). For testing at either latency, mice were again placed in the training enclosure for 120 s during which freezing was measured (*Context*), and then returned to the home cage. One hour later, mice were placed in an octagonal enclosure that was dissimilar from the training enclosure in floor texture, shape, wall design, lighting, and odor. After measuring freezing for 120 s (*Switch*), another 80 dB white noise cue was delivered for 30 s (with no shock), after which freezing was scored for an additional 120 s (*Cue*).

Statistical analysis

Neonatal behavior and most adult strength/motor and exploratory/sensory tests were analyzed with nonparametric tests (Mann-Whitney *U* for neonates, Kruskal-Wallis *H* for adults) because of the imposed maximum times or arbitrary scoring methods used. The normal approximation was used for the Mann-Whitney U-test ($n > 20$ in all cases), hence z-scores rather than the U statistic are reported. In nearly all cases (except where noted in Results), initial testing determined that sex was not a significant factor and the sexes were grouped together for analysis. The social recognition, spontaneous alternation, and inhibitory avoidance tests were analyzed by parametric ANOVA. Olfactory habituation and cued/contextual conditioning tests were analyzed using repeated-measures ANOVA (Wilks' lambda criterion). Sex was included as a factor in parametric ANOVA designs but was never significant. Post hoc testing for ANOVA was performed using Tukey's honestly significant difference (HSD) criterion. Body weight trajectories were analyzed using repeated-measures ANOVA. Expression assays were analyzed using Student's *t*-test; the alpha criterion for the QPCR analyses was Bonferroni-corrected to account for multiple comparisons.

RESULTS

Rasgrf1 imprinting affects olfactory associative learning in neonates

In wildtype mouse neonates, *Rasgrf1* expression is monoallelic, expressed only from the paternally-inherited allele. In addition to wildtypes (MP), four mutant imprinting genotypes were generated that, as neonates, exhibited biallelic expression (B), were nominally null for *Rasgrf1* (N), that expressed *Rasgrf1* solely from the maternal allele (MM), or that monoallelically expressed the paternal allele under the control of the *Pgk* enhancer used to drive maternal allele expression in B and MM mutants (*+/tm2*) (Fig. S1). We asked whether these alterations in *Rasgrf1* imprinting influenced learning by employing an olfactory associative learning paradigm suitable for use in neonatal mice (Alleva & Calamandrei 1986;Armstrong *et al.* 2006). Among the five *Rasgrf1* imprinting genotypes studied, both genotypes with paternally-inherited wildtype alleles, the biallelics (B, $z = 2.44$, $n = 59$, $p = 0.015$) and the wildtypes (MP, $z = 4.02$, $n = 68$, $p < 0.0001$; Fig. 1), demonstrated a

significant preference for the positively conditioned odor over the neutral odor. The other three genotypes (MM, +/*tm2*, N) did not demonstrate a significant preference for the rewarded odor (MM, $z = 0.34$, $n = 31$, $p = 0.735$; +/*tm2*, $z = 1.35$, $n = 29$, $p = 0.176$; N, $z = 1.62$, $n = 46$, $p = 0.104$), suggesting an impairment in olfactory associative learning associated with reduced or abnormal *Rasgrf1* expression. Simple anosmia was ruled out as an alternative hypothesis because anosmic and hyposmic neonatal mice are suckling-impaired and often starve to death unaided (Turgeon & Meloche 2009); our neonates exhibited no such difficulties. However, hyposmia could be a contributing factor to the observed impairments, particularly given that reducing the perceived intensity of odorants reduces associative learning in adult mice (Cleland *et al.* 2009). The fact that the *+/tm2* genotype did not fully recapitulate wildtype (MP) performance further suggested that paternally-inherited expression of the *tm2* allele did not fully restore wildtype expression.

Quantification of *Rasgrf1* transcript

The *tm2* allele essentially functioned as a null allele in the neonatal associative learning task, as neither the +/*tm2* nor MM (*tm2*/*tm1*) genotypes differed from null mutants (+/*tm1*) in their performance and biallelic animals (*tm2*/+) did not differ from wildtypes. We therefore asked whether the level of *tm2*-derived *Rasgrf1* expression differed from wildtype allele expression. Using quantitative PCR (Q-PCR), *Rasgrf1* transcript levels were quantified for each of the five genotypes in three regions of the P8 neonatal brain: olfactory bulb, hippocampus, and the remainder of the brain.

The neural plasticity underlying odor preference learning in P8 neonatal rats is largely limited to the olfactory bulb (Moriceau & Sullivan 2004; Sullivan 2001; Sullivan & Leon 1987). Biallelic (B) and wildtype (MP) mice expressed significantly elevated *Rasgrf1* transcript levels in olfactory bulb compared to the other three genotypes (Fig. 2a; $p < 0.005$ for all pairwise comparisons; see Table S1 for complete statistics), correlating with the significance of olfactory preference learning in the B and MP genotypes (Fig. 1). Transcript levels in B and MP mice were similar (Table S1; $p > 0.05$). Interestingly, *Rasgrf1* expression levels in MM and +/*tm2* animals – both of which have one *tm2* allele and one inactive allele – were similar to each other (Table S1; $p > 0.05$) and intermediate between those of the B/MP animals and the nulls (Table S1; $p < 0.005$ in comparison to each), indicating that the *tm2* allele successfully produces *Rasgrf1* transcript in the olfactory bulb, but not to an extent comparable to wildtype expression. Moreover, uniquely among the brain structures tested, *Rasgrf1* transcript was detected in the olfactory bulbs of null mice.

The hippocampus is not thought to underlie learning in rodents until after weaning (Sullivan 2001). Interestingly, the effect of imprinting genotype on *Rasgrf1* expression levels in hippocampus differed from the pattern observed in olfactory bulb, and consequently did not correlate well with neonatal behavioral data. Specifically, biallelic neonates expressed significantly more *Rasgrf1* transcript than any other genotype (Fig. 2b; $p < 0.005$ for all pairwise comparisons; see Table S2 for complete statistics), and transcript levels between MP (wildtype) and MM neonates were similar (Table S2; $p > 0.05$). However, like the olfactory bulb pattern, the MM and +/*tm2* genotypes expressed similar levels of *Rasgrf1* (Table S2; $p > 0.05$), and null (N) animals expressed significantly lower *Rasgrf1* transcript levels than did any other genotype (Table S2; $p < 0.005$ for all pairwise comparisons).

In the remainder of the brain, *Rasgrf1* expression patterns were significantly different in all ten pairwise comparisons among genotypes (Fig. 2c; $p < 0.005$ for all pairwise comparisons; see Table S3 for complete statistics). Specifically, as observed in hippocampus, biallelic mice overexpressed, and null mice underexpressed, *Rasgrf1* transcript relative to wildtype (MP; Table S3; $p < 0.005$ for both comparisons). *Rasgrf1* expression levels in MM and +/*tm2* animals were intermediate between the MP animals and nulls, and significantly different

from both; moreover, *Rasgrf1* expression was significantly greater in MM than in +/*tm2* neonates (Table S3; $p < 0.005$ for all comparisons). Overall, these results support previous findings that early olfactory learning is dependent upon olfactory bulb.

Rasgrf1 expression and imprinting in null olfactory bulb

Because *Rasgrf1* expression has never before been detected in any brain structures in null mice, we performed two tests to verify the *Rasgrf1* expression in the olfactory bulbs of null mice observed in our Q-PCR studies. First, gel analysis of the PCR products confirmed the presence of *Rasgrf1* bands of identical size to those detected in mice carrying fully expressed alleles, indicating that the Q-PCR assays detected bona fide *Rasgrf1* transcript. (Fig. 3a). In a second test, we asked whether the *Rasgrf1* transcript detected in olfactory bulb was imprinted, or if imprinting mechanisms in this structure were behaving differently than in the remainder of the brain (see Methods). Reciprocal crosses between wildtype C57BL/6J and FVB/NJ animals were set up. Olfactory bulbs from P8 pups derived from these crosses were isolated, cDNAs were generated, and *Rasgrf1* transcript was assayed for parent-specific expression. The assay relied on an *Aci*I restriction site that produces a unique digestion pattern for each of the two strains. Progeny of C57BL/6J mothers and FVB/NJ fathers displayed 226/130 bp bands diagnostic of expression from the paternal FVB/NJ allele. From the reciprocal cross, progeny of FVB/NJ mothers and C57BL/6J fathers displayed the 210/146 bands diagnostic of expression from the paternal C57BL/6J allele (Fig. 3b). The restriction patterns verify amplification of *Rasgrf1* and demonstrate that expression in olfactory bulb is imprinted, with expression coming from the paternal allele, as is the case in the rest of the brain. Because the above crosses demonstrate that the maternal *Rasgrf1* allele is silent in olfactory bulb, it is likely that a level of expression from the paternal *tm1* allele is permitted in olfactory bulb and that olfactory bulb regulates *Rasgrf1* differently than other tissues.

Rasgrf1 mutation dysregulates Ras and Rac activation in neonates

RasGRF1 acts as a guanine-nucleotide exchange factor for both Ras and Rac proteins. Although prior work has indicated that RasGRF1 is not an active signaling intermediate in neonatal mouse cortical neurons (Tian *et al*. 2004), the differences in *Rasgrf1* expression that we observed among neonatal genotypes and brain structures, along with the differential effects of genotype on neonatal associative learning, suggest that this finding may not be general and that impaired expression of *Rasgrf1* could in fact affect Ras or Rac activation in neonates. To determine whether Ras or Rac activation was influenced by mutations at *Rasgrf1*, we quantified the levels of the active forms of both proteins in tissue samples from the olfactory bulb, hippocampus, and isocortex of P8 wildtype (MP) and null mutant (N) neonates.

Western blot analyses indicated significant differences between wildtype and null animals in the proportion of Ras and Rac protein that was activated (Fig. 4a,b). Among the three brain structures, extracts from olfactory bulb contained the lowest levels of activated Ras and Rac proteins, such that longer exposures were needed for their detection than that required for the other tissues. There was no significant difference in the amount of activated Ras protein between genotypes in olfactory bulb extracts ($t(6) = 1.09$, $p = 0.315$). Rac levels in olfactory bulb of null mice were too low to reliably measure the level of activation, so no ratio was calculated. In hippocampus, in contrast, extracts from null mice contained significantly lower proportions of activated Ras and Rac proteins than did wildtype animals (*Ras*: $t(6) = 3.44$, $p = 0.014$; *Rac*: $t(6) = 2.96$, $p = 0.0251$). Finally, in isocortex, null animals produced a significantly lower proportion of activated Ras ($t(6) = 3.18$, $p = 0.0191$) but a significantly *higher* proportion of activated Rac ($t(6) = 3.89$, $p = 0.0081$) than did wildtypes.

Together, these data indicate that *Rasgrf1* is expressed and functional in the neonatal brain, that it affects Ras and Rac activation in neonatal mice, and that there are multiple, region-specific differences in the activity of *Rasgrf1* signaling proteins. These effects of *Rasgrf1*, presumably in olfactory bulb, include the regulation of normal olfactory associative learning in neonates.

Body mass assays

The effects of *Rasgrf1* imprinting on body mass persist into adulthood, well after expression becomes biallelic at approximately P21 (Drake *et al.* 2009). Body mass was measured in wildtype and +/*tm2* mice from P8 through P63 for comparison with these published data, which do not include the +/*tm2* genotype. No significant differences in body mass were observed between wildtype and +/*tm2* males or females at any age measured, indicating that, similar to maternally-derived *tm2* expression, paternally-derived *tm2* expression is sufficient to produce a wildtype size phenotype (Fig. S2a,b). In contrast, mice that were null for *Rasgrf1* as neonates (+/*tm1*) remained significantly smaller than wildtypes even into adulthood (measured up to P63), whereas imprinted biallelics (*tm2*/+) were significantly heavier (Drake *et al.* 2009).

Adult behavioral assays

Because the effects of *Rasgrf1* imprinting on body mass persist into adulthood, (Drake *et al.* 2009), we asked whether the same was true of its effect on associative learning. Accordingly, we performed a battery of standard behavioral phenotyping tests on cohorts of adult mice drawn from the B, MM, MP (wildtype), and N genotypes. As mice of all genotypes exhibit biallelic expression of *Rasgrf1* as adults; the sole difference among the adult cohorts tested was a history of differential *Rasgrf1* expression profiles as neonates.

Strength and motor coordination—Adult mice first were tested on a series of strength and motor coordination tasks. First, escape latency from 11 mm and 5 mm balance beams was tested over three sequential trials per beam. Because there was a significant effect of sex on latency to escape for both beam diameters (Kruskal-Wallis test; 11 mm, $H(1) = 4.510$, $p = 0.034$; 5 mm, $H(1) = 13.551$, $p < 0.001$), the sexes were analyzed separately for this task. There were no significant differences among the four genotypes on escape latency from either diameter of beam for either sex (11 mm, females, $H(3) = 4.650$, $p = 0.199$; males, $H(3) = 3.587$, $p = 0.310$; 5 mm, females, $H(3) = 4.888$, $p = 0.180$; males, $H(3) = 4.157$, $p = 0.245$) (Fig. S3a). In other tests, there was no significant effect of sex (forelimb suspension *latency* $H(1) = 0.136$, $p = 0.713$, *hand-scoring* $H(1) = 0.007$, $p = 0.935$; vertical pole $H(1) = 0.048$, $p = 0.826$; hanging-wire grip test $H(1) = 0.384$, $p = 0.535$), so the sexes were combined for analysis. There was no effect of genotype on the latency to fall from a single wire forelimb suspension ($H(3) = 5.287$, $p = 0.152$), a vertical pole ($H(3) = 0.403$, $p = 0.940$), or a hanging-wire grip test using a modified cage lid ($H(3) = 2.929$, $p = 0.403$). Hand-scoring of the single-wire forelimb suspension test (see Methods) also revealed no significant differences among genotypes ($H(3) = 2.819$, $p = 0.420$) (Fig. S3b).

Exploratory behaviors—Mice were then tested to assess their relative mobility and exploratory tendencies. In a small open field test (18" square, 4×4 grid, 300 s trial period), there was no significant effect of genotype on either the number of line crossings (mobility; $H(3) = 0.723$, $p = 0.868$) or the proportion of time spent in the 12 edge squares of the open field (thigmotaxis; $H(3) = 3.151$, $p = 0.369$) (Fig. S3c). In the step-down visual cliff apparatus, nearly all (87%) of the mice across both sexes and all genotypes stepped down onto the opaque side. There was no effect of genotype on the latency to step down, irrespective of whether analysis included both sides ($H(3) = 1.309$, $p = 0.727$) or only the opaque side ($H(3) = 1.669$, $p = 0.644$) (Fig. S3d). The sexes were pooled for analysis in

these exploratory tests because pretests revealed no significant effects attributable to sex (open field mobility, $H(1) = 3.408$, $p = 0.065$; thigmotaxis, $H(1) = 0.242$, $p = 0.622$; step-down latency, $H(1) = 1.406$, $p = 0.236$, opaque side only, $H(1) = 0.721$, $p = 0.396$). In the social recognition task, the time spent investigating a newly introduced mouse was significantly greater than that spent investigating a cagemate, irrespective of sex or genotype (three-factor ANOVA; main effect of intruder familiarity, $F(1,84) = 25.864$, $p < 0.001$; for main effects of sex and genotype and all interactions, $p > 0.05$) (Fig. S3e). A discrimination index also was calculated for each subject as the difference between the two investigation times divided by their sum, such that an index of 0 indicated no distinction between cagemates and newly introduced mice, −1 indicated investigation only of the cagemate, and +1 indicated investigation only of the newly introduced mouse. Neither genotype nor sex, nor their interaction, significantly affected the discrimination index (two-factor ANOVA; main effect of genotype, $F(3,42) = 2.428$, p = 0.079; main effect of sex, $F(1,42) = 0.006$, $p = 0.938$; interaction, $F(3,42) = 0.988$, $p = 0.408$).

Learning and memory—There was no effect of genotype on the total number of arm entries ($H(3) = 2.493$, $p = 0.477$) or the proportion of alternations ($H(3) = 1.214$, $p = 0.750$) in a five-minute spontaneous alternation task (Fig. 5a). The sexes were pooled for analysis in this test because pretests revealed no significant effects attributable to sex (number of entries, $H(1) = 0.306$, $p = 0.580$; proportion of alternations, $H(1) = 1.050$, $p = 0.305$). Olfactory habituation significantly affected investigation times in all animals (Wilks' lambda; $F(3,41) = 9.072$, $p < 0.001$), but was not affected by genotype, sex, or their interaction (Wilks' lambda, interaction of habituation×genotype; $F(9,99.9) = 0.383$, $p = 0.941$; habituation × sex; $F(3,41) = 0.684$, $p = 0.567$; habituation × genotype × sex; $F(9,99.9) = 1.054$, $p = 0.403$) (Fig. 5b). That is, neither habituation to the first odor nor the degree of cross-habituation to the second, similar odor was significantly affected by genotype or sex.

In a light-dark inhibitory avoidance task, all animals exhibited strong one-trial fear learning (three-factor ANOVA; main effect of latency, $F(2,60) = 39.076$, $p < 0.001$) with latency to enter the dark side differing significantly between pretraining and both posttraining latencies (Tukey's HSD; $p < 0.001$ for both comparisons) but not between the 0.05 and 24 hour posttraining latencies ($p = 0.478$). Genotype was not a significant main effect ($F(3,60) = 0.298$, $p = 0.826$); it did not affect the latency to enter the dark compartment either before shock, 30 minutes after delivery of a shock in the dark compartment (short-term memory), or 24 hours after shock delivery (consolidated long-term memory (simple effects analysis; $p > 0.05$ in all cases; Fig. 5c). Sex also was not a significant main effect ($F(1,60) = 0.015$, $p = 0.904$), nor were any interactions significant ($p > 0.05$ in all cases).

Finally, we ran an auditory cued/contextual conditioning task, in which an 80 dB white noise cue was paired with shock in a single conditioning trial, and freezing behavior was measured before training (*Pre*), when animals were placed back in the original context (contextual conditioning; *Context*), when animals were subsequently placed into a novel context (prior to testing cued conditioning; *Switch*) and finally in response to the auditory cue in this novel context (cued conditioning; *Cue*). There were no significant effects of genotype on freezing behavior either when tested 0.5 hours later (short-term fear memory; Wilks' lambda; $F(9,29.4) = 0.894$; $p = 0.542$) or 24 hours later (long-term fear memory; $F(9,39.1) = 0.543$; $p = 0.834$) (Fig. 5d,e). Sex also was not a significant main effect at either latency (0.5 hr, $F(3,12) = 2.742$, $p = 0.089$; 24 hr, $F(3,16) = 0.788$, $p = 0.518$), nor were any interactions significant ($p > 0.05$ in all cases).

DISCUSSION

Abnormal imprinted expression of *Rasgrf1* produces learning deficits in neonates

Several learning and neuronal phenotypes have been ascribed to G-protein signaling effects influenced by RasGRF1 (Brambilla *et al.* 1997; Farnsworth *et al.* 1995; Fasano *et al.* 2009; Giese *et al.* 2001; Kesavapany *et al.* 2006; Krapivinsky *et al.* 2003; Tonini *et al.* 2001; Yang & Mattingly 2006). We here demonstrate that neonatal learning and memory are dependent upon *Rasgrf1* expression levels in neonatal brain. Impaired *Rasgrf1* imprinting produced learning deficits in neonates, though this effect did not persist into adulthood, when *Rasgrf1* expression becomes uniformly biallelic. This contrasts with the body mass growth phenotypes associated with the manipulation of neonatal *Rasgrf1* expression, which persist beyond the age when expression becomes biallelic (Drake *et al.* 2009). Interestingly, in olfactory bulb, the location of neonatal olfactory preference learning (Sullivan 2001), the biallelic *Rasgrf1* genotype did not yield increased gene expression over wildtype (Fig. 2a), in contrast to its effect in other brain regions (Fig. 2b,c). That is, *Rasgrf1* transcript levels in olfactory bulb correlated directly with behavioral performance on the olfactory associative learning task, whereas other, non-olfactory phenotypes presumably depend on potentially dissimilar *Rasgrf1* expression patterns in other tissues. These results demonstrate tissue-specific regulation underlying a multiplicity of functions for *Rasgrf1*.

The *tm2* allele essentially behaved like a null allele in terms of its contribution to neonatal learning and memory performance, irrespective of whether it was maternally or paternally inherited; neither MM, +/*tm2*, or N animals demonstrated a learned preference for the rewarded odorant. Although these impairments were expected in the null animals based on previous assessments of *Rasgrf1* −/− knockout mice (Brambilla *et al.* 1997; Giese *et al.* 2001), it was a surprising result in the MM and +/*tm2* genotypes, which are phenotypically indistinguishable from MP animals in terms of body size and growth (Drake *et al.* 2009) (Fig. S2). No significant differences in weight were observed between wildtype and +/*tm2* males or females, indicating that, similar to maternally-derived *tm2* expression, paternally-derived *tm2* expression is sufficient to produce a wildtype size phenotype, but insufficient to restore wildtype learning and memory performance, as assessed by the olfactory odor learning paradigm. However, the persistence of *Rasgrf1* expression observed in the olfactory bulbs of null mutant animals may mitigate their olfactory learning deficit and conceal a greater dependence of olfactory learning on *Rasgrf1* than was observed; notably, while MM and +/tm2 mice expressed significantly more transcript than N mice, even in olfactory bulb, the nonzero expression level in N mice implies that we were unable to observe a neonatal olfactory learning phenotype for a mouse with zero *Rasgrf1* expression.

Rasgrf1-related learning and memory phenotypes in adult mice

Manipulation of *Rasgrf1* imprinting during the neonatal period had no persistent effect on learning and memory performance in adult mice. In contrast, adult *Rasgrf1* knockout animals exhibit clear learning and memory deficits, although the nature of these deficits is uncertain. Brambilla *et al.* (1997) found that mice lacking RasGRF1 were impaired in amygdala-dependent memory consolidation; retention of fear conditioning in inhibitory avoidance, cued conditioning, and contextual conditioning tests was normal when tested 0.5 hours after conditioning but severely impaired after 24 hours. In contrast, their performance on two hippocampus-dependent tasks (hidden-platform water maze, eight-arm radial maze) was unaffected across multiple days. Substantially different results were obtained by Giese *et al.* (2001), who found that RasGRF1-deficient mice were impaired on multiple hippocampus-dependent learning tasks (contextual discrimination, water maze with hidden platform, and social transmission of food preference) but not on inhibitory avoidance or contextual conditioning. All testing was performed at 21–24 hour post-conditioning

latencies, except that the social transmission of food preference was also tested immediately after training, and RasGRF1-deficient mice were impaired at that latency as well. Finally, Fasano *et al.* (2009) replicated two of the results from Brambilla *et al.* (1997), showing that mice lacking RasGRF1 had impaired memory for inhibitory avoidance conditioning after 24 hours but not after 1 hour, and that they performed normally when tested in the water maze. The differences between these groups' findings presumably result from the different derivations of the mutant strains used, illustrating that genetic manipulations are not necessarily uniform in their phenotypic effects.

Possible molecular mechanisms underlying *Rasgrf1*-dependent olfactory learning

Early olfactory associative learning involves NMDA-receptor dependent processes (Lincoln *et al.* 1988; Weldon *et al.* 1997), and is mediated by CREB phosphorylation (Cui *et al.* 2007; McLean *et al.* 1999; Raineki *et al.* 2009; Yuan *et al.* 2003). In adults, NMDA receptors mediate olfactory learning at multiple levels in diverse species, including *Drosophila* (Hudson & Distel 1986), honeybees (Si *et al.* 2004), rats (Tronel & Sara 2003), and mice (Brennan 1994). RasGRF1 is known to directly associate with NMDA receptors via the NR2B subunit (Krapivinsky *et al.* 2003), which is striking given that certain olfactory memory paradigms are specifically NR2B-dependent (White & Youngentob 2004).

In the olfactory bulb, noradrenergic inputs from the locus coeruleus play a role in olfactory learning in neonatal rodent pups (Christie-Fougere *et al.* 2009; Moriceau & Sullivan 2004); specifically, activation of noradrenergic inputs to neonatal olfactory bulb during the presentation of an odor stimulus increases cAMP levels in mitral cells. Higher cAMP levels increase CREB phosphorylation, which mediates the formation of long-term odor preference memory (Cui *et al.* 2007; McLean *et al.* 1999). *Rasgrf1* has been shown to transduce signals arriving at the NMDA receptor and to activate the ERK/MAPK pathway in response (Krapivinsky *et al.* 2003; Tian *et al.* 2004), which lies upstream of CREB phosphorylation. While we did not identify a direct link between *Rasgrf1* and activation of the Ras or Rac pathways in olfactory bulb, we demonstrated that there are differences in the amounts of activated protein between wildtype and null mutant mice in other structures.

Our results also indicated that *Rasgrf1* expression in olfactory bulb may be subject to different epigenetic regulatory mechanisms than in other tissues. The paternally inherited null *tm1* allele reduced *Rasgrf1* expression to 1–2% of wild type levels in hippocampus and whole brain; expression in olfactory bulb was also reduced, but only to 20% of wildtype levels in P8 mice. Normal *Rasgrf1* expression in olfactory bulb at this age is exclusively paternally-derived, which indicates that the *tm1* repeat deletion is either failing to produce relevant hypomethylation, or that the transcription regulatory mechanisms controlling imprinted expression in the olfactory bulb differ from those used in other tissues, where imprinting also occurs.

Genomic Imprinting and the Conflict Hypothesis

It has been established that *Rasgrf1* expression influences postnatal growth, as *Rasgrf1* knock-out mice are smaller than their wild-type littermates (Clapcott *et al.* 2003; Itier *et al.* 1998). Moreover, proper imprinting in neonates is critical for maintaining normal growth into adulthood (Drake *et al.* 2009), an experiment made possible by the *tm1* and *tm2* alleles. Here, we demonstrated that growth phenotypes are not influenced by the parental allele *per se* from which *Rasgrf1* expression is derived, suggesting instead that growth is influenced by the overall level of *Rasgrf1* expression in the governing tissue. This result is consistent with the central but untested assumption of the "conflict hypothesis" describing the evolution of genomic imprinting (Moore & Haig 1991; Wilkins & Haig 2003). This hypothesis describes

the evolution of genomic imprinting in mammals as a battle between the two parental genomes over optimum expression levels at imprinted loci, particularly those governing growth and resource consumption (Moore & Haig 1991). Previously untested was the assumption that equivalent amounts of expression derived from either parental allele would produce an equivalent phenotype, as we observed via the size phenotype at the ages assayed (Drake *et al*. 2009), (Fig. S2). However, the different patterns of imprinted *Rasgrf1* regulation observed in different tissues – even among three different regions of telencephalic cortex – indicate that such questions cannot be definitively answered without identifying the tissue or tissues within which *Rasgrf1* levels govern somatic growth, and suggest a yet-unappreciated complexity in the selective mechanisms underlying genomic imprinting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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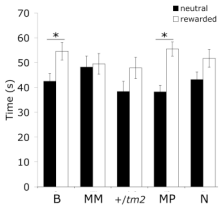

Figure 1. Associative olfactory learning in neonatal mice

Neonatal mice were tested for their ability to learn and remember an introduced maternal odor. Mice were tested for place preference by measuring time spent over either the neutral or rewarded odor during a 120-second trial period (see Methods). B, biallelic, *N*=59; MM, monallelic maternal, *N*=31; +/tm2, *N*=29; MP, monoallelic paternal (wildtype), *N*=68; N, null mutant, *N*=44. Asterisks indicate $p < 0.01$. Error bars indicate SEM.

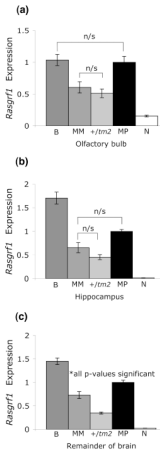

Figure 2. *Rasgrf1* transcript quantification in P8 brain regions

Rasgrf1 transcript from P8 neonates was quantified in **(a)** olfactory bulb, **(b)** hippocampus, and **(c)** the remainder of the brain (ROB; see Methods for details). Data were normalized to 18S rRNA levels and then further normalized to the *Rasgrf1* expression level of the wildtype (MP) genotype. Except for those pairs depicted as nonsignificant (n/s), all pairwise comparisons were significantly different ($p < 0.005$, Bonferroni-corrected for ten multiple comparisons; Tables S1–S3). Note that higher *Rasgrf1* expression levels tend to correspond to genotypes that are the best performers in the learned odor preference test, and that olfactory bulb expression patterns exhibit the best correlation with behavioral performance. Error bars indicate standard deviations.

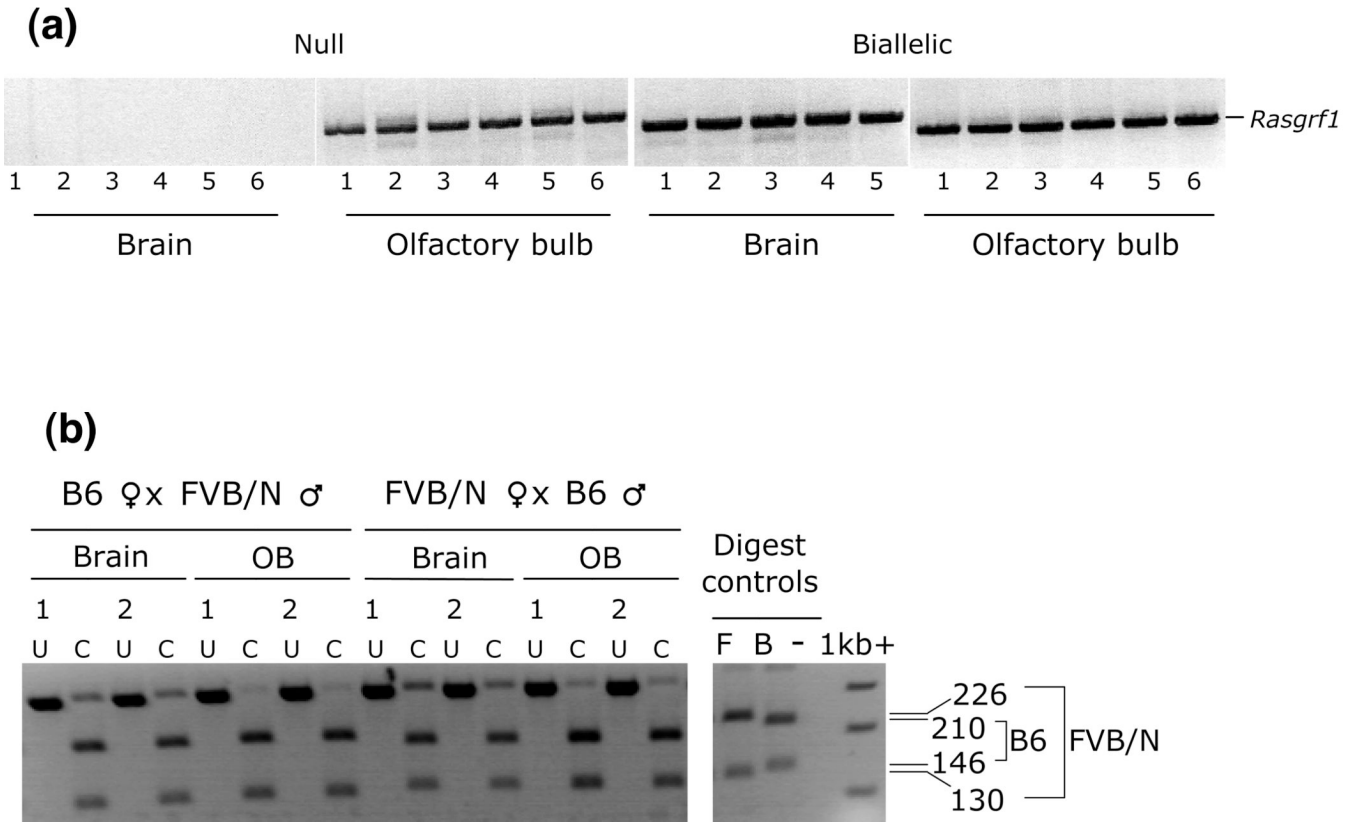

Figure 3. *Rasgrf1* is expressed and imprinted in neonatal null olfactory bulb

(**a**) *Rasgrf1* transcript was amplified by RT-PCR in the olfactory bulbs and the remainder of the brain in null and biallelic animals using RT-PCR. Six mice were used for each assay. (**b**) Imprinted expression was assayed by RT-PCR followed by *Aci*I digestion of the PCR products generated from tissues of progeny from reciprocal crosses between C57BL/6J (B6) and FVB/NJ (FVB) parents. Maternal strain is shown first. FVB expression generates 226bp and 130bp bands; B6 expression generates 210bp and 146bp bands. Digestion of amplicons (lanes labeled “C”) produced exclusively paternal banding patterns. Two to four mice were used for each test (lane pairs labeled “1” and “2” contain results from two different mice for each tissue type from each cross). *C*: cut/digested; *U*: undigested PCR products. Digest control lanes show transcript from each of the parent strains (*F*: FVB; *B*: B6).

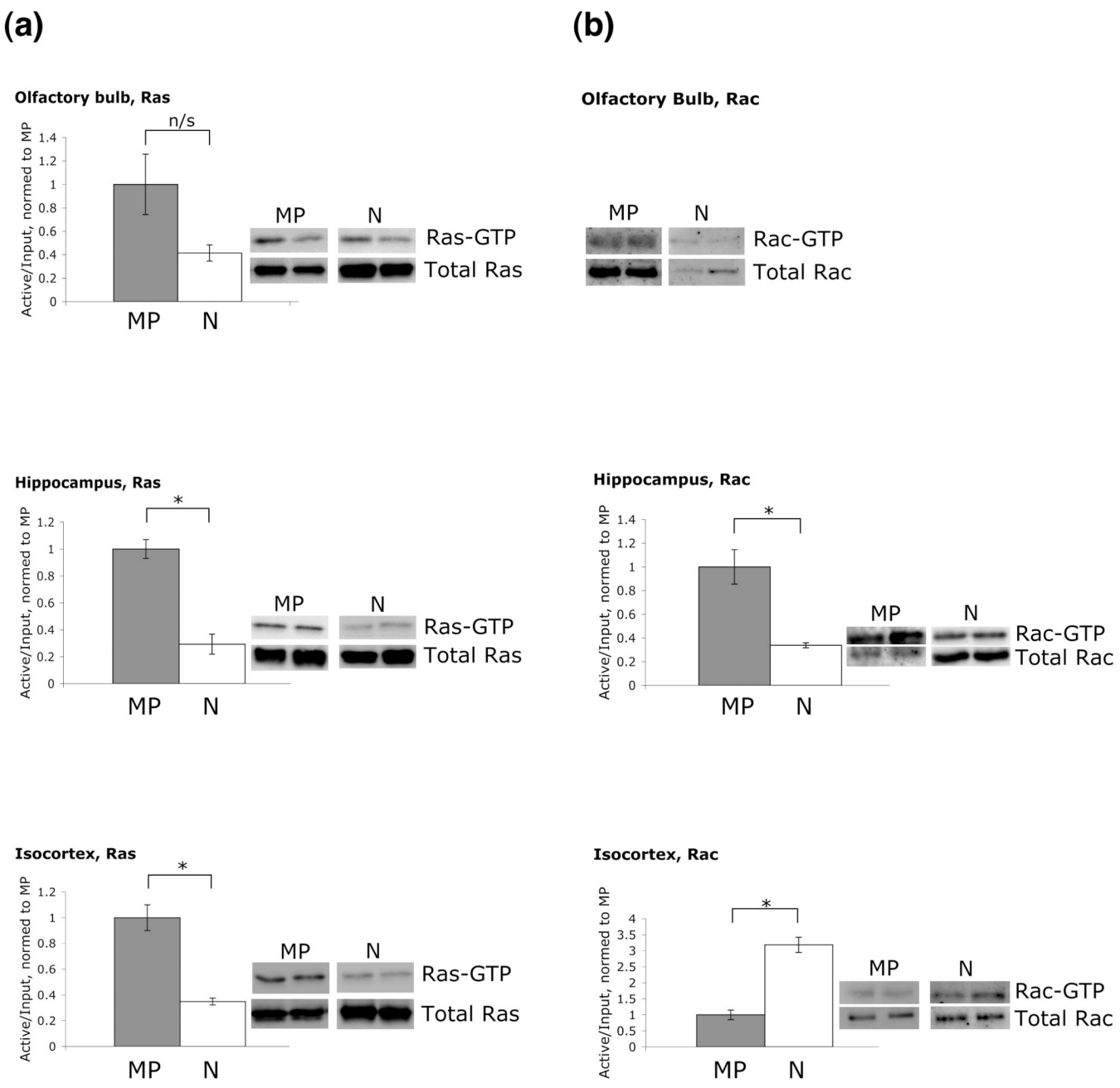

Figure 4. Relative expression levels of activated Ras and Rac proteins in null and wildtype brains regions depend on brain region

Levels of active and inactive Ras protein **(a)** and Rac protein **(b)** in the olfactory bulb, hippocampus, and isocortex of wildtype (MP) and null mutant (N) neonates at P8. Levels of Rac in olfactory bulb were too low to quantify. The amounts of precipitated (active) protein were normalized to the amounts of input (total) protein for each structure, and then normalized to wildtype levels. Representative blots are shown next to each graph. Three to four animals of each genotype were used in each test. Error bars indicate SEM.

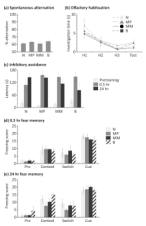

Figure 5. Learning and memory performance in adult mice

Adult mice all express *Rasgrf1* biallelically; genotype designations apply to each animal's history of neonatally imprinted expression. **(a)** Assessment of working memory in the spontaneous alternation task. The proportion of alternations was not affected by genotype. **(b)** Assessment of nonassociative olfactory learning by habituation to odors. Neither habituation nor cross-habituation to a moderately different test odorant were affected by genotype. **(c)** Light-dark inhibitory avoidance. All genotypes learned to associate dark entry with shock in one trial; learning and memory performance did not depend on genotype at either 0.5 hours (short-term memory) or 24 hours (long-term memory). **(d)** Short-term memory assessed by one-trial cued/contextual fear conditioning. There were no effects of genotype on the levels of freezing behavior scored. *Pre*, freezing in the training cage prior to tone-shock pairing; *Context*, freezing after being replaced in the training cage 0.5 hr after tone-shock pairing (contextual response); *Switch*, freezing after being placed in a novel test cage (60 min later; baseline for cued response); *Cue*, freezing in the novel test cage after presentation of tone CS (cued response). **(e)** Long-term memory assessed by one-trial cued/contextual fear conditioning. There were no effects of genotype on the levels of freezing behavior scored. Testing was performed identically to the short-term memory task. Separate cohorts were tested at 0.5 hr and 24 hr latencies. Error bars indicate SEM.