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

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***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 wild-type 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 showed 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 wild-type paternal allele contributes to learning and memory in neonatal mice.**

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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 CCCTC-binding factor (CTCF) binding and results in the inhibition of gene transcription from the maternal allele (Yoon *et al*. 2002, 2005). Hence, only the paternal allele is normally expressed in neonates. *Rasgrf1* is most highly expressed 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-mitogen-activated protein kinase (MAPK)-dependent memory consolidation and long-term plasticity in the amygdala (Brambilla *et al*. 1997) and Ras-extracellular signal-regulated kinase (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 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 wild-type 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 *et 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 embryonic stem 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 wild-type 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 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 wild-type (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 h 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 National Institutes of Health (NIH) guidelines.

Neonatal olfactory associative learning assay

Training

Mouse pups, 8 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 min. One of the diluted test odorants was then applied to all 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 conditioning odor 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 min and then were again separated from the dam for 120 min 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. 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 seconds 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 varied with respect to both odor identity and odor contingency between test trials; furthermore, the spatial orientation of the scented compartments 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 wild-type (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 (Q-PCR) in olfactory bulb, hippocampus and in the remainder of the brain (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 (Applied Biosystems, Carlsbad, CA, USA) specific for *Rasgrf1* and normalized to 18S rRNA levels. Alpha levels for statistical significance were Bonferroni corrected for 10 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 min, followed by 40 cycles of 95°C × 20 seconds, 58°C × 30 seconds, 72°C × 50 seconds, followed by 72°C × 5 min. PCR products were then digested with 10 U *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 wild-type (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 ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor tablets (Roche Applied Science, Indianapolis, IN, USA) in magnesium-containing buffer supplied by Millipore (Billerica, MA, USA) [#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 were then affinity purified using Ras and Rac activation assay kits (Millipore 17-218 and 17-283, respectively). Purified protein extracts were then sodium dodecyl sulphate–polyacrylamide gel electrophoresis 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 horseradish peroxidase-conjugated immunoglobulin G secondary antibody (Millipore 12-349). Blots were visualized using SuperSignal West Dura Substrate (Pierce 34075; Pierce Protein Research, Rockford, IL, USA), captured using a LAS-4000 imager's CCD camera and chemiluminescent detection function and quantified using MultiGauge v 3.0 software (Fujifilm Corp., Tokyo, Japan). The amounts of precipitated (active) protein were normalized to the amounts of input (total) protein for each structure and then normalized to wild-type 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 non-associative 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 in 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 h after training (short-term fear memory) or 24 h (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-h assessment. Day 4: 24-h assessment of fear conditioning. Detailed methods for behavioral tests other than learning and memory tests are provided in Supporting Information.

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, because 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 non-associative 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 on 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 seconds per trial with 120-second 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-second, 0.5-mA footshock was delivered. After an additional 10 seconds, the mouse was returned to its home cage. Half of the mice were then retested 30 min later (short-term fear memory), while the other half were tested 24 h 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 seconds).

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 seconds to explore (*Pre*). An 80-dB white noise auditory cue was then delivered for 30 seconds; during the last 2 seconds of cue presentation a 0.5-mA footshock was delivered. After an additional 60 seconds in the training enclosure, the mouse was removed to its home cage. Half of the mice began testing 30 min later (short-term memory), while the other half began testing 24 h later (long-term memory). For testing at either latency, mice were again placed in the training enclosure for 120 seconds during which freezing was measured (*Context*) and then returned to the home cage. After 1 h, 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 seconds (*Switch*), another 80-dB white noise cue was delivered for 30 seconds (with no shock), after which freezing was scored for an additional 120 seconds (*Cue*).

Statistical analysis

Neonatal behavior and most adult strength/motor and exploratory/sensory tests were analyzed with non-parametric tests (Mann–Whitney *U*-test for neonates, Kruskal–Wallis *H*-test 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 analysis of variance (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 Q-PCR analyses was Bonferroni corrected to account for multiple comparisons.

Results

Rasgrf1 imprinting affects olfactory associative learning in neonates

In wild-type mouse neonates, *Rasgrf1* expression is monoallelic, expressed only from the paternally inherited

allele. In addition to wild-types (MP), four mutant imprinting genotypes were generated that, as neonates, exhibited biallelic expression (B), were nominally null for *Rasgrf1* (N), expressed *Rasgrf1* solely from the maternal allele (MM) or 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 wild-type alleles, the biallelics (B, $z = 2.44$, $n = 59$, $P = 0.015$) and the wild types (MP, $z = 4.02$, $n = 68$, $P < 0.0001$; Fig. 1), showed a significant preference for the positively conditioned odor over the neutral odor. The other three genotypes (MM, +/*tm2*, N) did not show 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 wild-type (MP) performance further suggested that paternally inherited expression of the *tm2* allele did not fully restore wild-type expression.

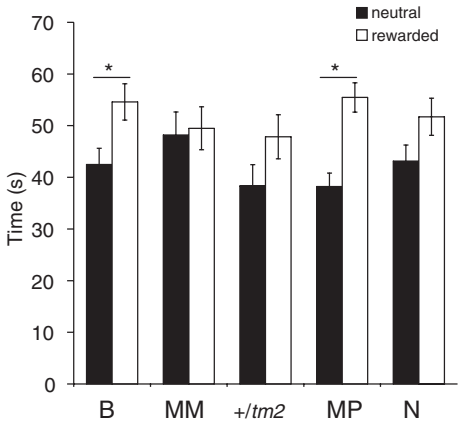

Figure 1: Associative olfactory learning in neonatal mice. Neonatal mice were tested for their ability to learn and remember an introduced maternal odor. Mice were tested for place preference by measuring time spent over either the neutral or the rewarded odor during a 120-second trial period (see *Materials and methods*). B, biallelic, $N = 59$; MM, monallelic maternal, $N = 31$; +/*tm2*, $N = 29$; MP, monoallelic paternal (wild-type), $N = 68$; N, null mutant, $N = 44$. Asterisks indicate $P < 0.01$. Error bars indicate SEM.

Quantification of Rasgrf1 transcript

The *tm2* allele essentially functioned as a null allele in the neonatal associative learning task, as neither the +/*tm2* nor the MM (*tm2*/*tm1*) genotypes differed from null mutants (+/*tm1*) in their performance and biallelic animals (*tm2*/+) did not differ from wild types. We therefore asked whether the level of *tm2*-derived *Rasgrf1* expression differed from wild-type allele expression. Using 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 wild-type (MP) mice expressed significantly elevated *Rasgrf1* transcript levels in olfactory bulb compared with 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 wild-type 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 (wild-type) 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 10 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 wild type (MP; Table S3; $P < 0.005$ for both comparisons). *Rasgrf1* expression levels in MM and +/*tm2* animals were intermediate between the MP animals and the 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 on olfactory bulb.

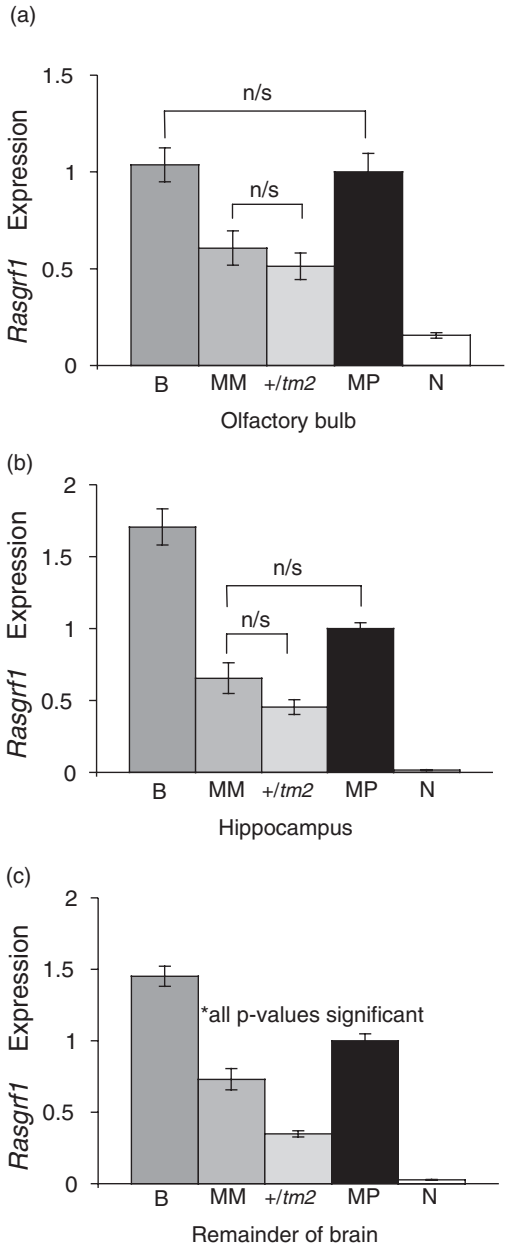

Figure 2: *Rasgrf1* transcript quantification in P8 brain regions. *Rasgrf1* transcript from P8 neonates was quantified in (a) olfactory bulb, (b) hippocampus and (c) the remainder of the brain (see *Materials and methods* for details). Data were normalized to 18S rRNA levels and then further normalized to the *Rasgrf1* expression level of the wild-type (MP) genotype. Except for those pairs depicted as non-significant (n/s), all pairwise comparisons were significantly different ($P < 0.005$, Bonferroni corrected for 10 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.

Rasgrf1 expression and imprinting in null olfactory bulb

Because *Rasgrf1* expression has never been detected before 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 *Materials and methods*). Reciprocal crosses between wild-type C57BL/6J and FVB/NJ animals were setup. 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 show 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 show 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 wild-type (MP) and null mutant (N) neonates.

Western blot analyses indicated significant differences between wild-type 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

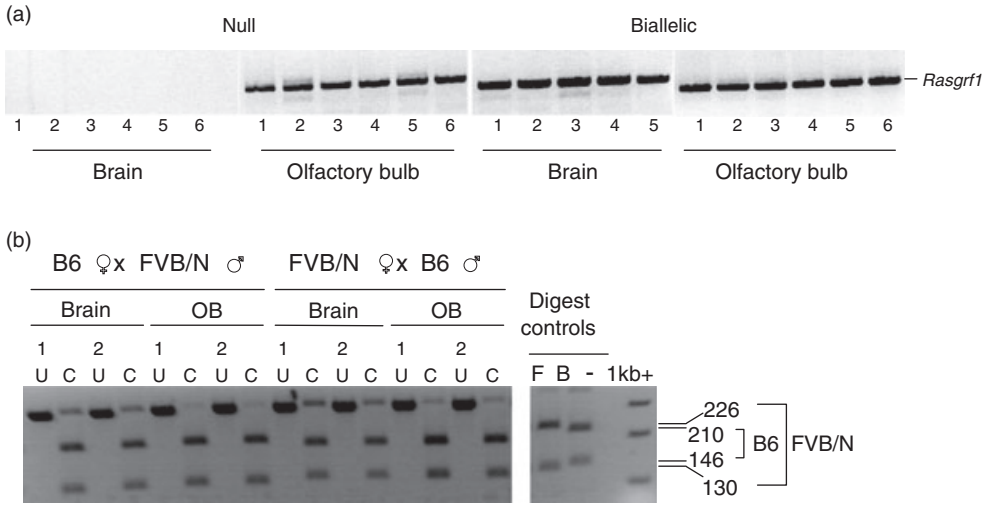

Figure 3: *Rasgrf1* is expressed and imprinted in neonatal null olfactory bulb. (a) *Rasgrf1* transcript was amplified by RT-PCR in the olfactory bulbs and the remainder of the brain in null and biallelic animals using RT-PCR. Six mice were used for each assay. (b) Imprinted expression was assayed by 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 226-bp and 130-bp bands; B6 expression generates 210-bp and 146-bp 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).

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 wild-type 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 wild types.

Together, these data indicate that *Rasgrf1* is expressed and functional in the neonatal brain, which 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 wild-type 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 wild-type 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 wild-type size phenotype (Fig. S2a,b). In contrast, mice that were null for *Rasgrf1* as neonates (+/*tm1*) remained significantly smaller than wild types 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 (wild-type) 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 of the sex (11 mm, females, $H(3) = 4.650$, $P = 0.199$; males, $H(3) = 3.587$, $P = 0.310$; 5 mm, females,

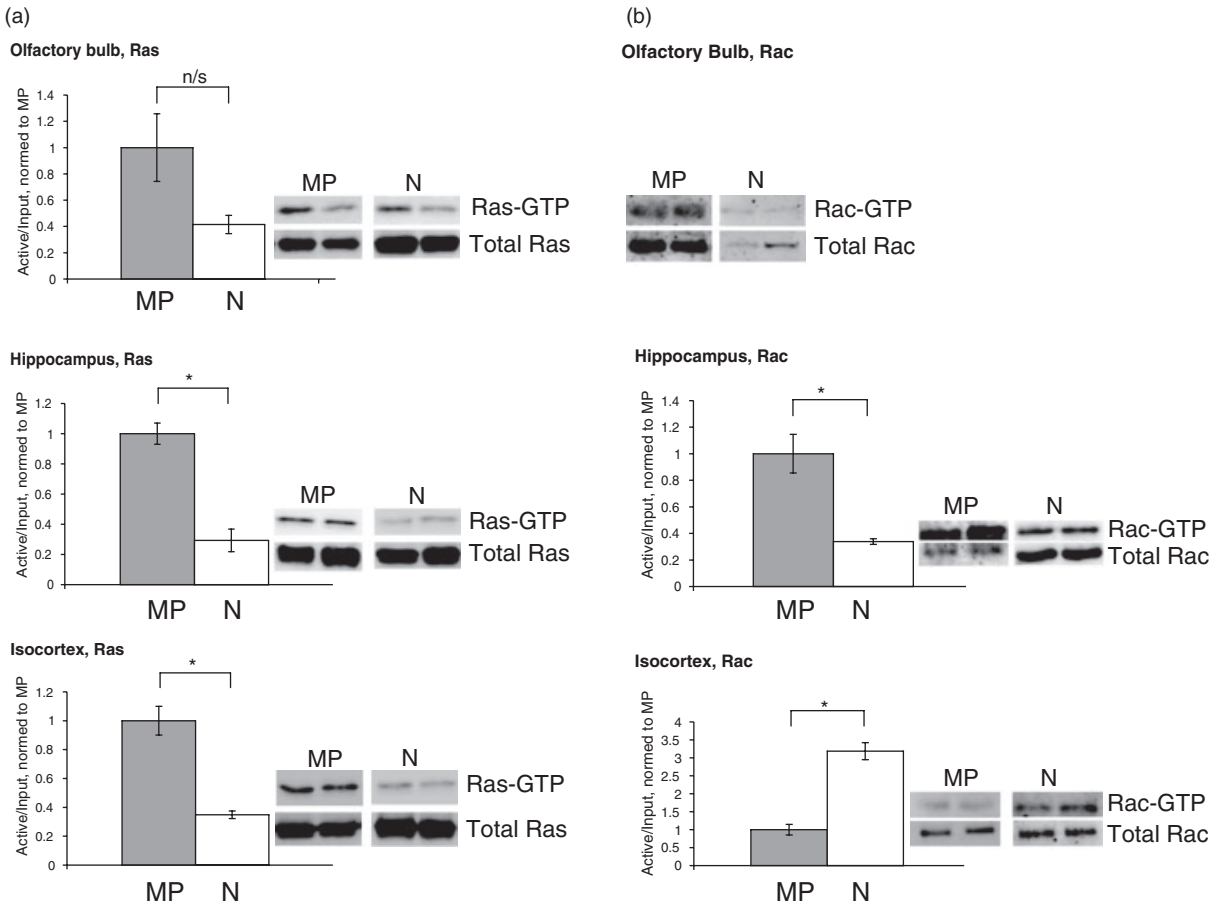

Figure 4: Relative expression levels of activated Ras and Rac proteins in null and wild-type brains regions depend on brain region. Levels of active and inactive Ras protein (a) and Rac protein (b) in the olfactory bulb, hippocampus and isocortex of wild-type (MP) and null mutant (N) neonates at P8. 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 wild-type 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.

$H(3) = 4.888$, $P = 0.180$; 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 *Materials and methods*) also showed 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-second 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 showed 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 was also 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 5-min spontaneous alternation task (Fig. 5a). The sexes were pooled for analysis in this test because pretests showed 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 h posttraining latencies ($P = 0.478$). Genotype was not a significant effect ($F_{3,60} = 0.298$, $P = 0.826$); it did not affect the latency to enter the dark compartment either before shock, 30 min after delivery of a shock in the dark compartment (short-term memory) or 24 h after shock delivery (consolidated long-term memory; simple effects analysis; $P > 0.05$ in all cases; Fig. 5c). Sex was also 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

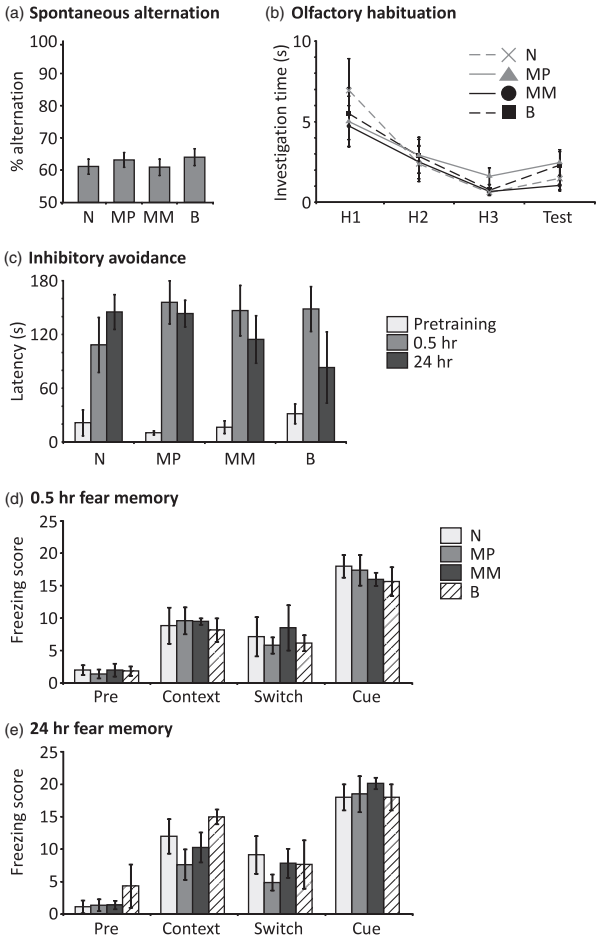

Figure 5: Learning and memory performance in adult mice. Adult mice all express *Rasgrf1* biallelically; genotype designations apply to each animal's history of neonatally imprinted expression. (a) Assessment of working memory in the spontaneous alternation task. The proportion of alternations was not affected by genotype. (b) Assessment of non-associative olfactory learning by habituation to odors. Neither habituation nor cross-habituation to a moderately different test odorant was 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 h (short-term memory) or 24 h (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 h 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- and 24-h latencies. Error bars indicate SEM.

tested 0.5 h later (short-term fear memory; Wilks' lambda; $F_{9,29.4} = 0.894$; $P = 0.542$) or 24 h 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 h, $F_{3,12} = 2.742$, $P = 0.089$; 24 h, $F_{3,16} = 0.788$, $P = 0.518$) 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 show that neonatal learning and memory are dependent on *Rasgrf1* expression levels in neonatal brain. Impaired *Rasgrf1* imprinting produced learning deficits in neonates, although 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 wild-types (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 show 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 showed 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 wild-type and +/*tm2* males or females, indicating that, similar to maternally derived *tm2* expression, paternally derived *tm2* expression is sufficient to produce a wild-type size phenotype, but insufficient to restore wild-type 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 non-zero 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 h after conditioning but severely impaired after 24 h. 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- to 24-h 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 h but not after 1 h 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 *N*-methyl-D-aspartate (NMDA)-receptor-dependent processes (Lincoln *et al*. 1988; Weldon *et al*. 1997) and is mediated by cAMP response element-binding protein (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 showed that there are differences in the amounts of activated protein between wild-type 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 wild-type 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* knockout 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 showed 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.

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Acknowledgments

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1: Combinations of *Rasgrf1* alleles used in this study. Mice carrying wild-type *Rasgrf1*^{tm1Pds} (Yoon *et al*. 2002) or *Rasgrf1*^{tm2Pds} (Yoon *et al*. 2005) mutations were bred to generate progeny with the combinations of alleles shown. Filled rectangles with rightward pointing arrows depict expressed alleles; absence of the arrows indicates gene silencing. Filled triangles are sequences needed for proper imprinting. These were deleted (open triangle) to generate the *Rasgrf1*^{tm1Pds} allele or replaced with enhancer containing sequences (enh) to produce the *Rasgrf1*^{tm2Pds} allele. The parental origin of each allele is indicated by a male (blue) or female (red) symbol on the left. The corresponding genotype designations are depicted on the right.

Figure S2: Body mass measurements of wild-type and +/*tm2* mice. (a) Females. No significant differences in growth were observed between genotypes. MP, $N = 37$; +/*tm2*, $N = 27$. (b) Males. No significant differences in growth were observed between genotypes. MP, $N = 32$; +/*tm2*, $N = 31$. For growth curves in other *Rasgrf1* imprinting genotypes, see Drake *et al*. (2009). Error bars indicate SEM.

Figure S3: Other behavioral phenotypes of adult mice. Adult mice all express *Rasgrf1* biallelically; genotype designations apply to each animal's history of neonatally imprinted expression. (a) Balance beam. Interestingly, there was a significant effect of sex on escape latency for both the 11 mm and 5 mm beams ($P < 0.05$, $P < 0.001$, respectively). These effects may be spurious, noting that no other related motor task suggested a significant effect of sex, but in any event there were no significant effects of genotype on escape latency for either sex. (b) Suspension tasks. Neither sex nor genotype significantly affected the latency to fall in a wire forelimb suspension test, a vertical pole test or a hanging wire grip test. (c) Open field tests. Neither sex nor genotype significantly affected either overall activity levels (line crossings) or thigmotaxis (proportion of time spent along the edges of the arena). (d) Visual cliff. Neither sex nor genotype significantly affected the step-down latency, whether measured only on the opaque ('shallow') side or irrespective of side. (e) Social recognition. The time spent investigating a newly introduced mouse was significantly greater than that spent investigating a cagemate, irrespective of sex or genotype. Error bars indicate SEM.

Table S1: Pairwise statistical analysis of *Rasgrf1* transcript levels in olfactory bulb. *Rasgrf1* transcript levels in P8 neonatal olfactory bulb were quantified for each genotype tested (see *Materials and methods*). Transcript levels found in each genotype were compared using Student's *t*-test; alpha levels were Bonferroni corrected for 10 multiple comparisons such that $P < 0.005$ indicates significance. The *t*-statistic and *P*-value for each pairwise comparison are depicted (df = 26 in all cases).

Table S2: Pairwise statistical analysis of *Rasgrf1* transcript levels in hippocampus. *Rasgrf1* transcript levels in P8 neonatal hippocampus were quantified for each genotype tested (see *Materials and methods*). Transcript levels found in each genotype were compared using Student's *t*-test; alpha levels were Bonferroni corrected for 10 multiple comparisons such that $P < 0.005$ indicates significance. The *t*-statistic and *P*-value for each pairwise comparison are depicted (df = 26 in all cases).

Table S3: Pairwise statistical analysis of *Rasgrf1* transcript levels in the remainder of the brain. *Rasgrf1* transcript levels in the remainder of the P8 neonatal brain (comprising the whole brain excepting olfactory bulbs, hippocampus, hypothalamus and pituitary gland; see *Materials and methods* for details) were quantified for each genotype tested (see *Materials and methods*). Transcript levels found in each genotype were compared using Student's *t*-test; alpha levels were Bonferroni corrected for 10 multiple comparisons such that $P < 0.005$ indicates significance. The *t*-statistic and *P*-value for each pairwise comparison are depicted (df = 26 in all cases).

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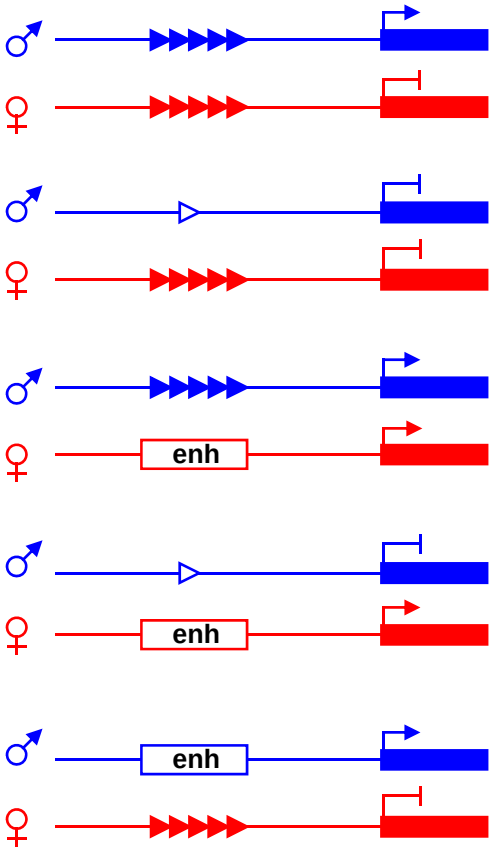
Figure S1



Allele genotype	Designation
wildtype wildtype	MP
Rasgrf1^{tm1Pds} wildtype	N
wildtype *Rasgrf1*^{tm2Pds}	B
Rasgrf1^{tm1Pds} *Rasgrf1*^{tm2Pds}	MM
Rasgrf1^{tm2Pds} wildtype	+/tm2

Figure S2

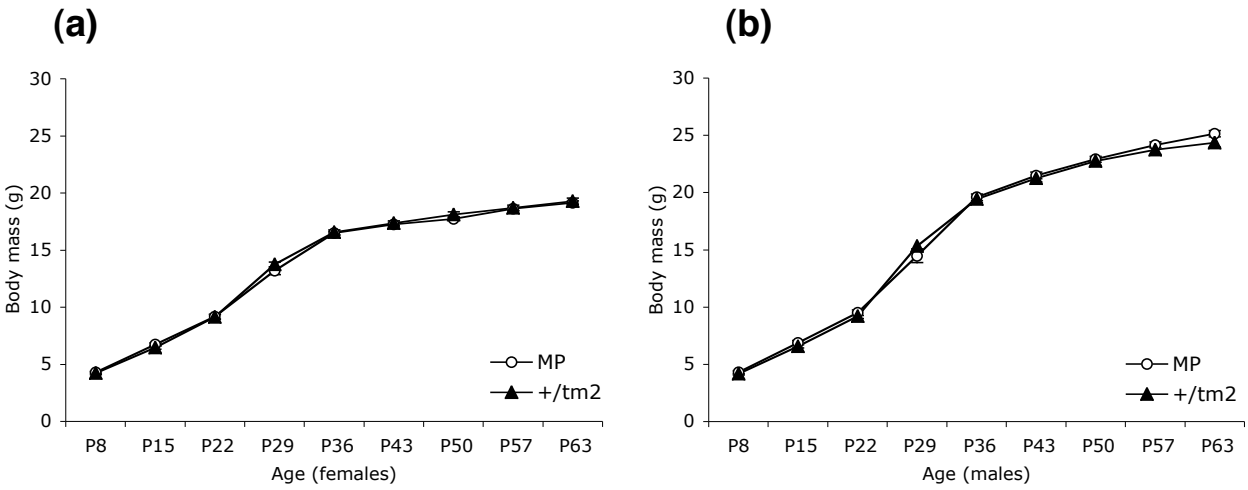

(a)
Body mass (g)
30
25
20
15
10
5
0
P8
P15
P22
P29
P36
P43
P50
P57
P63
Age (females)
MP
+/tm2
(b)
Body mass (g)
30
25
20
15
10
5
0
P8
P15
P22
P29
P36
P43
P50
P57
P63
Age (males)
MP
+/tm2

Figure S3

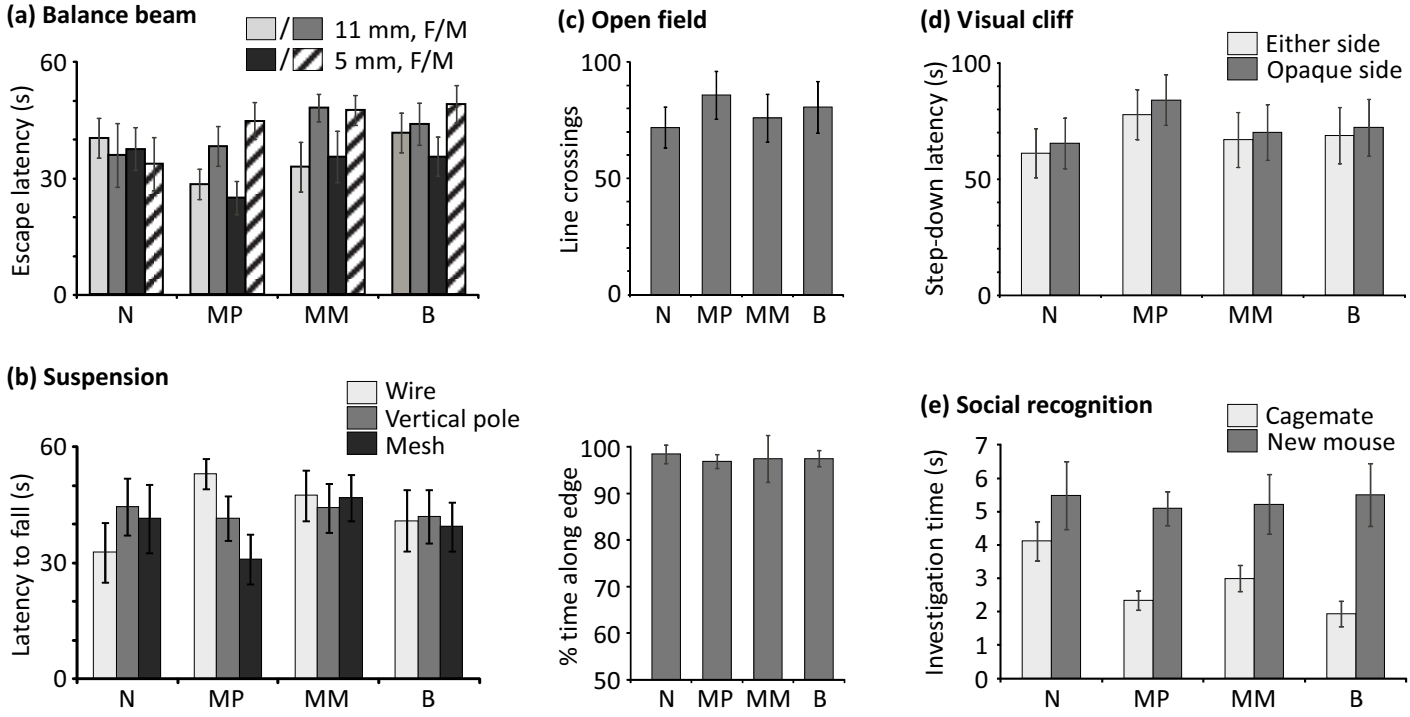

(a) Balance beam
11 mm, F/M
5 mm, F/M
Escape latency (s)
60
30
0
N
MP
MM
B
(b) Suspension
Wire
Vertical pole
Mesh
Latency to fall (s)
60
30
0
N
MP
MM
B
(c) Open field
Line crossings
100
50
0
N
MP
MM
B
% time along edge
100
90
80
70
60
50
N
MP
MM
B
(d) Visual cliff
Either side
Opaque side
Step-down latency (s)
100
50
0
N
MP
MM
B
(e) Social recognition
Cagemate
New mouse
Investigation time (s)
7
6
5
4
3
2
1
0
N
MP
MM
B

Table S1. Pairwise statistical analysis of Rasgrf1 transcript levels in olfactory bulb.

Rasgrf1 transcript levels in P8 neonatal olfactory bulb were quantified for each genotype tested (see Methods). Transcript levels found in each genotype were compared using Students *t*-test; alpha levels were Bonferroni-corrected for ten multiple comparisons such that $p < 0.005$ indicates significance. The *t*-statistic and *p*-value for each pairwise comparison are depicted (df = 26 in all cases).

t statistic **p-value**	**B**	**MM**	**+/*tm2***	**MP**	**N**
B	--	$t = 3.60$ $\mathbf{p = 0.0013}$	$t = 4.74$ $\mathbf{p < 0.0001}$	$t = 0.25$ $\mathbf{p = 0.8007}$	$t = 12.73$ $\mathbf{p < 0.0001}$
MM		--	$t = 1.14$ $\mathbf{p = 0.2655}$	$t = 3.48$ $\mathbf{p = 0.0018}$	$t = 9.13$ $\mathbf{p < 0.0001}$
+/*tm2*			--	$t = 4.66$ $\mathbf{p < 0.0001}$	$t = 7.99$ $\mathbf{p < 0.0001}$
MP				--	$t = 12.96$ $\mathbf{p < 0.0001}$

Table S2. Pairwise statistical analysis of Rasgrf1 transcript levels in hippocampus.

Rasgrf1 transcript levels in P8 neonatal hippocampus were quantified for each genotype tested (see Methods). Transcript levels found in each genotype were compared using Students *t*-test; alpha levels were Bonferroni-corrected for ten multiple comparisons such that $p < 0.005$ indicates significance. The *t*-statistic and *p*-value for each pairwise comparison are depicted (df = 26 in all cases).

t statistic **p-value**	**B**	**MM**	***+/tm2***	**MP**	**N**
B	--	$t = 5.83$ $\mathbf{p < 0.0001}$	$t = 8.07$ $\mathbf{p < 0.0001}$	$t = 3.38$ $\mathbf{p = 0.0023}$	$t = 28.04$ $\mathbf{p < 0.0001}$
MM		--	$t = 2.24$ $\mathbf{p = 0.0338}$	$t = 2.67$ $\mathbf{p = 0.0128}$	$t = 22.21$ $\mathbf{p < 0.0001}$
+/tm2			--	$t = 5.00$ $\mathbf{p < 0.0001}$	$t = 19.97$ $\mathbf{p < 0.0001}$
MP				--	$t = 25.72$ $\mathbf{p < 0.0001}$

Table S3. Pairwise statistical analysis of Rasgrf1 transcript levels in the remainder of the brain. *Rasgrf1* transcript levels in the remainder of the P8 neonatal brain (ROB, comprising the whole brain excepting olfactory bulbs, hippocampus, hypothalamus and pituitary gland; see Methods for details) were quantified for each genotype tested (see Methods). Transcript levels found in each genotype were compared using Students *t*-test; alpha levels were Bonferroni-corrected for ten multiple comparisons such that $p < 0.005$ indicates significance. The *t*-statistic and *p*-value for each pairwise comparison are depicted (df = 26 in all cases).

t statistic **p-value**	**B**	**MM**	**+/*tm2***	**MP**	**N**
B	--	$t = 6.51$ $\mathbf{p < 0.0001}$	$t = 13.53$ $\mathbf{p < 0.0001}$	$t = 3.67$ $\mathbf{p = 0.0011}$	$t = 37.65$ $\mathbf{p < 0.0001}$
MM		--	$t = 7.02$ $\mathbf{p < 0.0001}$	$t = 3.09$ $\mathbf{p = 0.0047}$	$t = 31.14$ $\mathbf{p < 0.0001}$
+/*tm2*			--	$t = 10.37$ $\mathbf{p < 0.0001}$	$t = 24.12$ $\mathbf{p < 0.0001}$
MP				--	$t = 35.40$ $\mathbf{p < 0.0001}$