Rasal2 (nGAP)

1 Mouse generation

1.1 Mutation

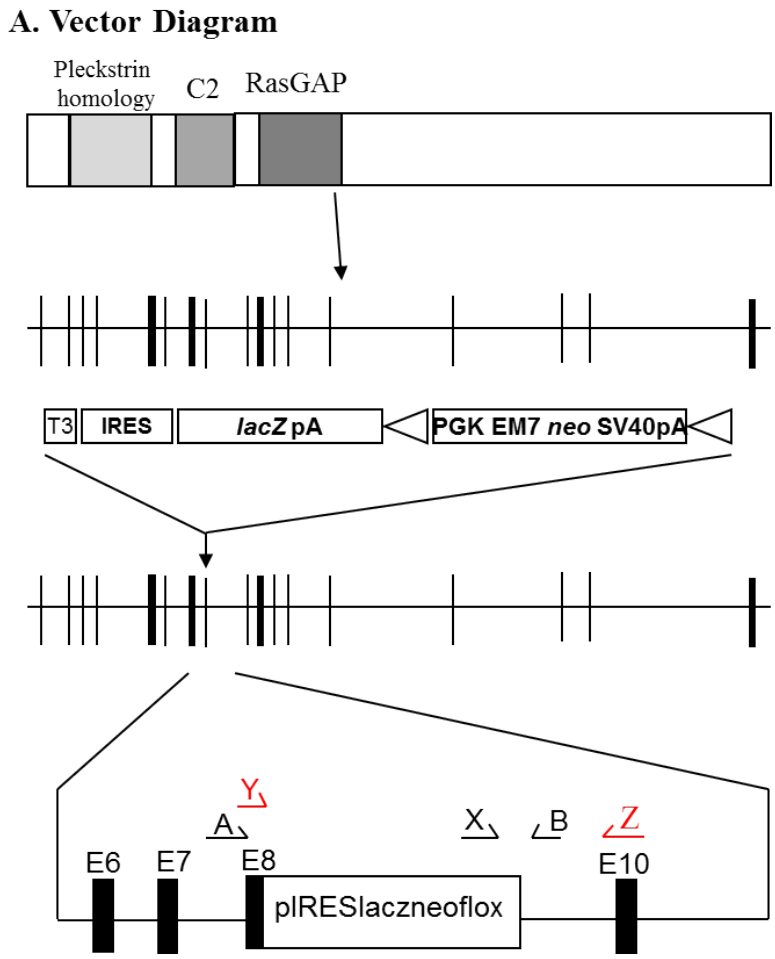


Figure 1: *Details of the mutation*

E14TG2a mouse embryonic stem (ES) cells were targeted with a vector containing 2.3kb and 2.8kb of flanking genomic DNA. This replaced 1.9kb of *Rasal2* genomic DNA (X157173891 to X157175814; Ensemble Build 75) with an IRES-lacZ-neo cassette. Genomic DNA was isolated from ES cells by Wizard SV 96 Genomic DNA purification system (Promega Cat A2371). Correctly targeted ES cells were identified by long range PCR using Expand Long Template PCR system (Roche Cat 11681842001). The correctly targeted ES cells were injected into C57BL/6 blastocysts to create chimeric mice, which were bred with 129S5 mice to generate heterozygous *Rasal2* mutant mice. Generation of *Rasal2* targeted mice *Rasal2* is an 18 exon gene that encodes a protein containing Pleckstrin homology, C2 and RasGAP domains (top). We replaced *Rasal2* exons 8-9 with a selection cassette in targeted mice and created a frameshift between exons 8 and 10. Primers used for genotyping(A,B, X) and RT-PCR (Y,Z).

1.2 Genotyping

Genotyping PCR consisted of a 2.7kb product amplified from the wild-type (wt) allele using a forward primer A (GACGAGCTGAAAATGTTCTCC) in the wt sequence upstream of the cassette and a reverse primer B (CAGCACTTTCTTCAAGGAACTC) downstream of the cassette. A 640bp product was amplified from the targeted allele using primer B with forward primer X (CATGTCTGGATCGATATCCC), within the selection cassette. After enzymatic amplification for 35 cycles (45 seconds at 94 degC, 45 seconds at 55 degC, and 1 minute at 72 degC), the PCR products were size-fractionated on a 1% agarose gel in 1x Tris borate-EDTA buffer (Image not shown).

1.3 Breeding

Birth of 3BTBM mice followed Mendelian ratios with 17% of offspring being homozygous knockouts. Genotypes of 3-week old pups from 3BTBM$^{+/-}$ intercrosses identified 86 wt, 121 3BTBM$^{+/-}$ and 42 3BTBM progeny (Chi-squared $p<0.001$). Male and female 3BTBM mice developed normally to adulthood, exhibited normal body size and no gross abnormalities. 3BTBM mice were maintained by backcrossing onto the 129S5/SvEvBrd background; heterozygous males and females were fertile and used to set up intercrosses to generate homozygous and wildtype mice to study.

2 Behaviour

2.1 Definitions

Table 1: *Definitions of Behaviour Variables*

Experiment	Variable	Units	Description
Elevated Plus Maze	EPM total distance	cm	Total distance (cm) travelled in any arm or central zone of the EPM
Elevated Plus Maze	EPM max speed	cm/s	Maximum speed (cm/s) travelled in any arm or central zone of the EPM
Elevated Plus Maze	EPM % time in open	percent	Percentage of time in the open or closed arms of the EPM spent in open arms
Elevated Plus Maze	EPM time in centre	s	Total time (s) spent in the central zone of the EPM
Elevated Plus Maze	EPM max speed, open vs closed	cm/s	Difference between the maximum speed (cm/s) observed in the open arms and the closed arms of the EPM
Open Field/Novel Object	OF, NOE total distance	log10 cm	Total distance travelled (log10 cm) during initial exposure to the open field and in presence of the novel object
Open Field/Novel Object	NOE vs OF distance travelled	cm	Difference in distance travelled (cm) in presence of the novel object and during initial exposure to open field
Rotarod	RR naive fall time	log10 s	Fall time on accelerating rotarod (log10 s), naive performance in session 1
Rotarod	RR learning	s/trial	Learning on rotarod, measured as increase in fall time per trial (s/trial) in session 1
Rotarod	RR memory	s	Memory on rotarod, measured as excess fall time at middle of session 2 relative to middle of session 1
Fear Training	Fear learning, trial effect	percent freezing	Fear learning, measured as extra % time freezing before third trial compared to % time freezing before first trial
Fear Training	Fear learning, tone effect	percent freezing	Fear learning, measured as increase in % time freezing due to third tone compared to increase in % time freezing due to first tone
Contextual Memory	Contextual memory, mean	percent freezing	Contextual memory, measured as difference in % time freezing during first 120 s re-exposure to the box compared to first 120 s in the box on previous day
Contextual Memory	Contextual memory, change	percent freezing	Contextual memory, measured as increase in % time spent freezing from first time bin of 30 s to fourth bin of 30 s during 120 s re-exposure to the box
Cued Memory	Cued memory, mean	percent freezing	Cued memory, measured as increase in % time spent freezing during 120 s of tone re-exposure compared to increase in % time spent freezing during initial tone on previous day
Cued Memory	Cued memory, change	percent freezing	Cued memory, measured as increase in % time spent freezing from first time bin of 30 s to fourth bin of 30 s during 120 s re-exposure to the tone

2.2 Behaviour Overview

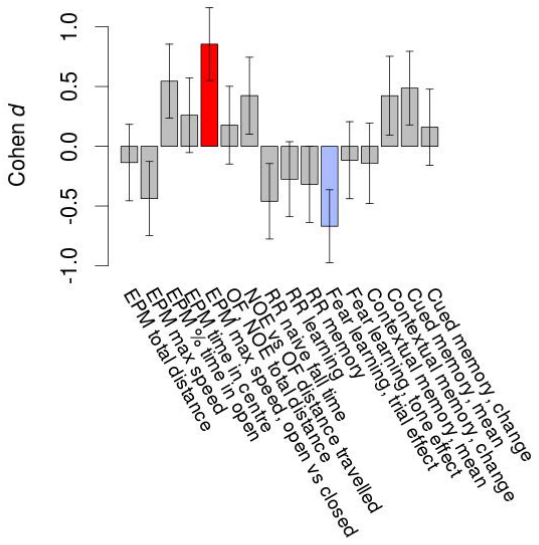


Figure 2: *Behaviour Overview. Alteration in a repertoire of eight innate and eight learned behaviour variables is shown. Cohen d effect sizes of the mutation are presented ± SE.*

Mutant mice showed little overall behavioural difference from wildtypes, with two of 16 behaviour variables significantly impacted by this mutation. In the elevated plus maze task, one behavioural variable, EPM max speed open vs closed, was significantly increased in mutants. In the fear learning task, one behavioural variable, fear learning trial effect, was significantly decreased in mutants. Open field/novel object exploration, rotarod, contextual memory and cued memory tasks were unaffected. Definitions of variables may be found below.

2.3 Elevated Plus Maze - Innate/Instinctive Behaviour

Table 2: *Analysis of Innate or Instinctive Variables: mean(SEM) of behaviour scores for wildtype and mutant males and females, with p-values of differences*

Variable	Units	Wildtype M (n=12)	Wildtype F (n=10)	Mutant M (n=11)	Mutant F (n=10)	P(sex x mutation)	P(mutation)
EPM total distance	cm	954 (67)	952 (87)	881 (115)	945 (119)	0.74	0.67
EPM max speed	cm/s	20.9 (0.9)	18.8 (0.8)	18 (1.1)	18.7 (1.5)	0.22	0.17
EPM percent time in open	%	5.6 (2.6)	14.4 (8.7)	18.1 (4.6)	23.4 (8.8)	0.79	0.096
EPM time in centre	s	99 (12)	116 (20)	114 (20)	131 (23)	0.99	0.43
EPM max speed, open vs closed	cm/s	-13.3 (2.3)	-9 (2.5)	-7.3 (1.8)	-2.9 (2.1)	0.98	0.009 **

EPM total distance: Total distance (cm) travelled in any arm or central zone of the EPM
EPM max speed: Maximum speed (cm/s) travelled in any arm or central zone of the EPM
EPM percent time in open: Percentage of time in the open or closed arms of the EPM spent in open arms
EPM time in centre: Total time (s) spent in the central zone of the EPM
EPM max speed, open vs closed: Difference between the maximum speed (cm/s) observed in the open arms and the closed arms of the EPM

2.4 Open Field & Novel Object Exploration - Innate/Instinctive Behaviour

Table 3: *Analysis of Innate or Instinctive Variables: mean(SEM) of behaviour scores for wildtype and mutant males and females, with p-values of differences*

Variable	Units	Wildtype M (n=12)	Wildtype F (n=10)	Mutant M (n=11)	Mutant F (n=10)	P(sex x mutation)	P(mutation)
OF, NOE total distance	log10 cm	3.08 (0.09)	3.07 (0.07)	3.11 (0.09)	3.14 (0.11)	0.82	0.59
NOE vs OF distance travelled	cm	-475 (134)	-416 (135)	-308 (85)	-260 (132)	0.96	0.2

OF, NOE total distance: Total distance travelled (log10 cm) during initial exposure to the open field and in presence of the novel object
NOE vs OF distance travelled: Difference in distance travelled (cm) in presence of the novel object and during initial exposure to open field

2.5 Motor Behaviour - Innate/Instinctive Ability, Learning & Memory

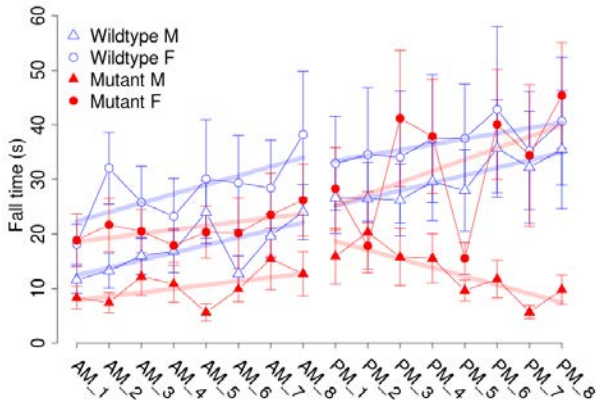


Figure 3: *RR histories. Mutant and wildtype (WT) males and females as indicated. Data represent mean ± SEM.*

Table 4: *RR analysis: mean(SEM) of behaviour scores for wildtype and mutant males and females, with p-values of differences*

Variable	Units	Wildtype M (n=12)	Wildtype F (n=10)	Mutant M (n=11)	Mutant F (n=10)	P(sex x mutation)	P(mutation)
RR naive fall time	log10 s	1.05 (0.09)	1.31 (0.08)	0.81 (0.11)	1.24 (0.07)	0.37	0.084
RR learning	s/trial	1.4 (0.7)	1.7 (1.4)	0.7 (0.4)	0.7 (1)	0.89	0.39
RR memory	s	12.8 (4.7)	8.7 (4.4)	2.7 (1.8)	11.4 (4.1)	0.12	0.3

RR naive fall time: Fall time on accelerating rotarod (log10 s), naive performance in session 1
RR learning: Learning on rotarod, measured as increase in fall time per trial (s/trial) in session 1
RR memory: Memory on rotarod, measured as excess fall time at middle of session 2 relative to middle of session 1

2.6 Fear Conditioning - Learning & Memory

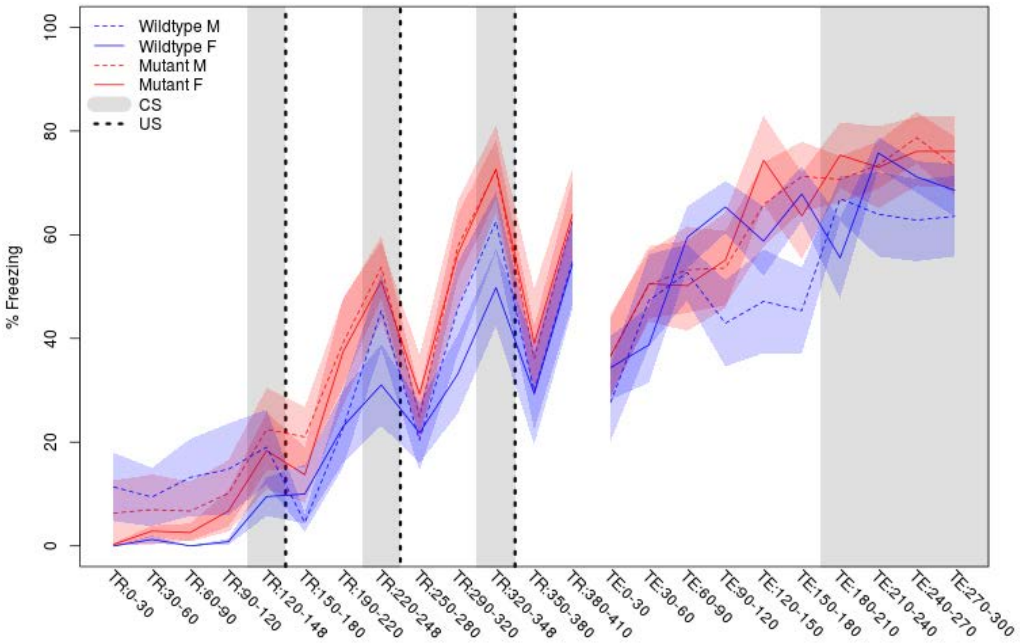


Figure 4: *FC histories. Percent freezing presented as mean ± SEM. TR:N-n, percent time spent freezing during time interval $N < t \leq n$ seconds on training day. TE:N-n, same on testing day.*

Table 5: *FC analysis: mean(SEM) of behaviour scores for wildtype and mutant males and females, with p-values of differences*

Variable	Units	Wildtype M (n=12)	Wildtype F (n=10)	Mutant M (n=11)	Mutant F (n=10)	P(sex x mutation)	P(mutation)
Fear learning, trial effect	% freezing	47.6 (7.6)	49.6 (8.8)	31 (8.2)	32.3 (7.8)	0.97	0.043 *
Fear learning, tone effect	% freezing	2.8 (8.8)	6.3 (8.1)	3.6 (4.8)	-0.2 (5.4)	0.62	0.72
Contextual memory, mean	% freezing	40.9 (9.5)	45 (7.6)	30.5 (6.1)	49 (5.8)	0.35	0.63
Contextual memory, change	% freezing	13.1 (6.4)	12.1 (5.4)	11.9 (6)	30.2 (4.1)	0.096	0.18
Cued memory, mean	% freezing	-8 (7.7)	-5.3 (6.9)	8.9 (8.4)	-0.6 (4.9)	0.41	0.13
Cued memory, change	% freezing	-7.1 (3.4)	-8.9 (3.4)	-12.5 (6.8)	3.4 (8.5)	0.14	0.62

Fear learning, trial effect: Fear learning, measured as extra percent time freezing before third trial compared to percent time freezing before first trial
Fear learning, tone effect: Fear learning, measured as increase in percent time freezing due to third tone compared to increase in percent time freezing due to first tone
Contextual memory, mean: Contextual memory, measured as difference in percent time freezing during first 120 s re-exposure to the box compared to first 120 s in the box on previous day
Contextual memory, change: Contextual memory, measured as increase in percent time spent freezing from first time bin of 30 s to fourth bin of 30 s during 120 s re-exposure to the box
Cued memory, mean: Cued memory, measured as increase in percent time spent freezing during 120 s of tone re-exposure compared to increase in percent time spent freezing during initial tone on previous day
Cued memory, change: Cued memory, measured as increase in percent time spent freezing from first time bin of 30 s to fourth bin of 30 s during 120 s re-exposure to the tone