Brickley SG et al, Supplementary Material for:

TASK-3 two pore domain potassium channels enable sustained high frequency firing in cerebellar granule neurons. J. Neurosci.

Generation of TASK-3 knockout mice

We inactivated the TASK-3 gene by combined homologous and insertional recombination (Fig. S1 A-C). Our *intended* strategy consisted in flanking exon1 of the TASK-3 gene with loxP sites in the same orientation by homologous recombination, using the targeting vector shown in Fig. S1B. A neomycin resistance gene cassette, flanked by frt sites to allow later removal by Flp recombinase, was inserted as a positive selection marker for embryonic stem (ES) cell culture. However, the *actual* targeting event produced an unpredicted insertion of 4 copies of the neomycin cassette and inverted repeats of the TASK-3 exon 1 into the TASK-3 locus (Fig. S1C). Pulse field gel electrophoresis confirmed that all neo insertions were on one contiguous fragment (data not shown). In spite of the unusual targeting event, this was a *bona fide* knockout of the TASK-3 gene: the multiple (inverted) repeats of exon 1 and neomycin destroyed TASK-3 gene function.

Crossing the TASK-3 knockout mice with Flp enhanced deletor mice removed all the neo genes (not shown) and reactivated the TASK-3 gene as detected by *in situ* hybridization with a TASK-3 exon 1 probe (not shown); however, the loxP sites flanking exon 1 in this reactivated TASK-3 gene are inverted relative to each other, presumably because one of the tandem neo insertions was "back to front" with respect to the loxP site upstream of exon 1; thus it was not possible to use the "floxed" TASK-3 line as originally intended. We thus concentrated on analysing the TASK-3 KO line.

Examining expression of TASK-3 neighbouring genes on mouse chromosome 15

We and others have demonstrated that neomycin gene insertions can, but do not always, affect expression levels of neighboring genes (Olsen et al., 1996; Pham et al., 1996, Uusi-Oukari et al., 2000). Any "non-specific" effects of the neomycin gene must be considered case-by-case. Consulting the Ensembl server shows that no obvious gene candidates lie 3' of the TASK-3 gene; the nearest clear 3' gene occurs 561 Kb downstream and encodes a collagen alpha1 chain gene, collagen XXII, which is not expressed in brain (http://www.ensembl.org/Mus_musculus/geneview?gene=ENSMUSG00000045567; Koch et al 2004). On the 5' side of TASK-3, a large (466 Kb) gene, the ortholog of the human Tularik gene 1 (T1) (http://www.ensembl.org/Homo_sapiens/geneview?gene=ENSG00000167632; see Mu et al 2003; Nagase et al 2001; Okazaki et al 2004) is predicted to encode a protein originally termed T1/mKIAA1882 (also Riken clone 1810044A24; entry NM_180662 in the NCBI database) and also known as IBP (a novel regulator of rho GTPAses; Fanzo et al 2006) or NIBP (NIK and IKK(beta)-binding protein that enhances NF-(kappa)B activation – Hu et al 2005). The T1/mKIAA1882/IBP/NIBP gene is approximately 43 Kb upstream of the TASK-3 allele (http://www.ensembl.org/Mus_musculus/geneview?gene=ENSMUSG00000047921) (Fig. S2A). The T1/mKIAA1882/IBP/NIBP gene is expressed in many non-neuronal tissues such as kidney, heart, lung, skeletal muscle, tongue and T cells (Davies et al 2004; Hu et al 2005; Fanzo et al 2006). T1/mKIAA1882/IBP/NIBP function in mice is required for optimal T cell effector function, lymphocyte homeostasis and the prevention of systemic autoimmunity (Fanzo et al 2006); loss of T1/mIKIAA1882/IBP/NIBP produces a systemic autoimmunity phenotype (Fanzo et al 2006). However, the T1/mKIAA1882/IBP/NIBP gene is expressed in an overlapping region with the TASK-3 gene in brain (Davies et al 2004; Nagesse et al 2001; Hu et al 2005). Thus alterations in T1/mKIAA1882/IBP/NIBP expression could affect brain function (e.g. in modulating the NF-□B signalling cascade in neurons – Hu et al 2005).

We looked at brain expression of the T1/mKIAA1882/IBP/NIBP gene by *in situ* hybridization in wild-type littermates and homozygous TASK-3 KO brains (Fig. S2B). Oligonucleotides designed to detect the T1/mKIAA1882/IBP/NIBP transcripts were: mT1a**,** 5'-CCAACGTGTTTCCGCATGCGTCCTTGGCACCGCTTCTTGTAGTGT-3'; and mT1b**,** 5'-GGCCTGTGCCCCGAGGGCCCTCACATGCACGCTGGGTAAGCAAAC-3' We found that the T1/mKIAA1882/IBP/NIBP gene has a pan-cellular expression in wild-type brain (see also Davies et al 2004; Hu et al 2005), with the highest expression in cerebellum, hippocampus and olfactory bulb; expression is also likely in glial cells of white matter tracts (Fig. S2B). No change in expression of the T1/mKIAA1882/IBP/NIBP gene was observed in homozygous TASK-3 knockout brains compared with wild-type littermates (Fig. S2). Thus we established that the multiple neomycin gene insertions were confined to the targeted TASK-3 locus and that there were no consequences on neighbour genes.

Davies W, Smith RJ, Kelsey G, Wilkinson LS (2004) Expression patterns of the novel imprinted genes Nap115 and Peg13 and their non-imprinted host genes in the adult mouse brain. Gene Expression Patterns 4:741-747

Fanzo JC et al (2006) Loss of IRF-4-binding protein leads to the spontaneous development of systemic autoimmunity. J. Clin. Investigation 116:703-14

Hu, W. H., Pendergast, J. S., Mo, X. M., Brambilla, R., Bracchi-Ricard, V., Li, F., Walters, W. M., Blits, B., He, L., Schaal, S. M, Bethea, J. R., 2005. NIBP, a novel NIK and IKK(beta)-binding protein that enhances NF-(kappa)B activation. J Biol Chem. 280: 29233-29241.

Koch, M et a. (2004) A novel marker of tissue junctions, collagen XXII. J Biol Chem 279: 22514-22521.

Mu D et al 2003. Genomic amplification and oncogenic properties of the KCNK9 potassium channel gene. Cancer Cell 3: 297-302

Nagase T, Kikuno R, Ohara O (2001) Prediction of the coding sequences of unidentified human genes. XXI. The complete sequences of 60 new cDNA clones from brain which code for large proteins. DNA Res. 8: 179-187

Okazaki, N., R, F. K., Ohara, R., Inamoto, S., Koseki, H., Hiraoka, S., Saga, Y., Seino, S., Nishimura, M., Kaisho, T., Hoshino, K., Kitamura, H., Nagase, T., Ohara, O. and Koga, H., 2004. Prediction of the coding sequences of mouse homologues of KIAA gene: IV. The complete nucleotide sequences of 500 mouse KIAA-homologous cDNAs identified by screening of terminal sequences of cDNA clones randomly sampled from size-fractionated libraries. DNA Res. 11: 205-218.

Olsen EN, Arnold HH, Rigby PW, Wold B J (1996) Know your neighbors: three phenotypes in null mutants of myogenic bHLH gene MRF4. Cell 85: 1-4.

Pham CT, MacIvor DM, Hug BA, Heusel JW, Ley T (1996) Long-range disruption of gene expression by a selectable marker cassette. Proc Natl Acad Sci USA 93: 13090-13095.

Uusi-Oukari, M et al (2000) Long-range interactions in neuronal gene expression: evidence from gene targeting in the GABAA receptor beta2-alpha6-alpha1-gamma2 subunit gene cluster. Mol Cell Neurosci 16: 34-41.

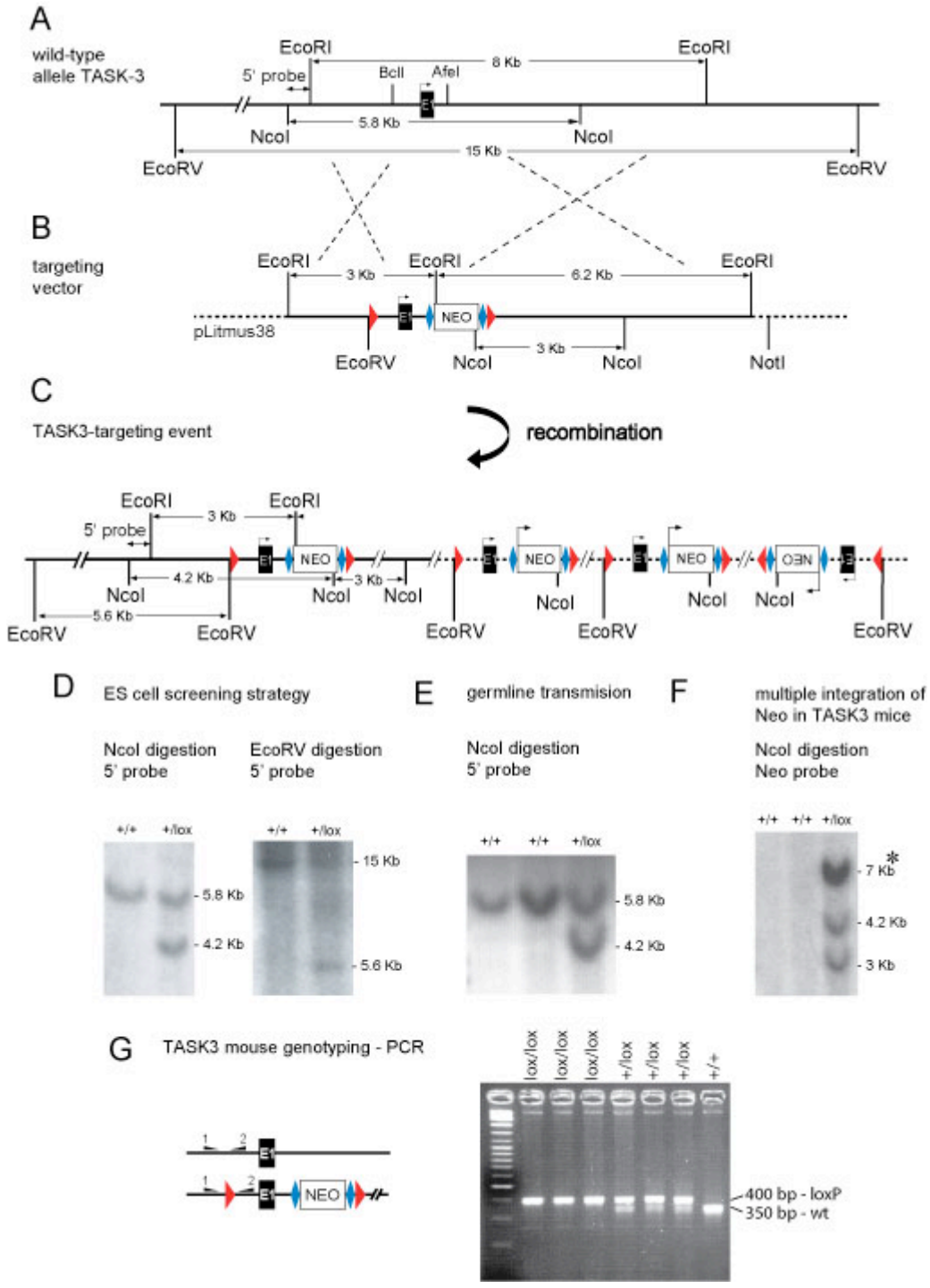
A
wild-type
allele TASK-3
EcoRI
8 Kb
EcoRI
5' probe
BclI
AfeI
5.8 Kb
NcoI
NcoI
15 Kb
EcoRV
EcoRV
B
targeting
vector
EcoRI
EcoRI
EcoRI
3 Kb
6.2 Kb
NEO
pLitmus38
3 Kb
EcoRV
NcoI
NcoI
NotI
C
TASK3-targeting event
recombination
EcoRI
EcoRI
3 Kb
5' probe
NEO
4.2 Kb
3 Kb
NcoI
NcoI
NcoI
5.6 Kb
EcoRV
EcoRV
EcoRV
NcoI
EcoRV
NcoI
NcoI
EcoRV
D
ES cell screening strategy
NcoI digestion
5' probe
EcoRV digestion
5' probe
+/+
+/lox
+/+
+/lox
5.8 Kb
4.2 Kb
15 Kb
5.6 Kb
E
germline transmision
NcoI digestion
5' probe
+/+
+/+
+/lox
5.8 Kb
4.2 Kb
F
multiple integration of
Neo in TASK3 mice
NcoI digestion
Neo probe
+/+
+/+
+/lox
7 Kb
4.2 Kb
3 Kb
G
TASK3 mouse genotyping - PCR
1
2
1
2
NEO
lox/lox
lox/lox
lox/lox
+/lox
+/lox
+/lox
+/+
400 bp - loxP
350 bp - wt

Figure S1. **Eliminating TASK-3 gene function by homologous recombination** **A**, the area surrounding the first coding exon of the mouse TASK-3 gene; **B**, linearized targeting vector designed to introduce loxP sites that flank exon 1; **C**, schematic of the likely actual targeting event; the targeting vector was multiply integrated into intron 1 of the TASK-3 gene; **D**, embryonic stem cell (ES) cell screening strategy by Southern blot using the 5' external probe on ES cell genomic DNA, NcoI digestion gives a 5.8 Kb wild-type band and a 4.2 Kb mutant band; EcoRV digestion gives 15 Kb wild-type and 5.6 Kb mutant band; **E**, Southern blot confirming the germline transmission of the targeted TASK-3 allele (mouse tail DNA); **F**, Southern blot of NcoI digested mouse DNA hybridized with a neomycin probe; a 7 Kb band (marked with star) corresponds to the multiple integration of the targeting vector at the TASK-3 gene; **G**, PCR genotyping on genomic DNA using primers (1 & 2) flanking the 5' loxP site, the wild-type band is 350 bp whereas the targeted allele gives 400 bp. Shaded box, exon 1 (E1); NEO, neomycin resistance gene; red arrow heads, loxP sites; blue diamonds, frt sites; double-headed arrows indicate the expected size of restriction fragments for Southern analysis.

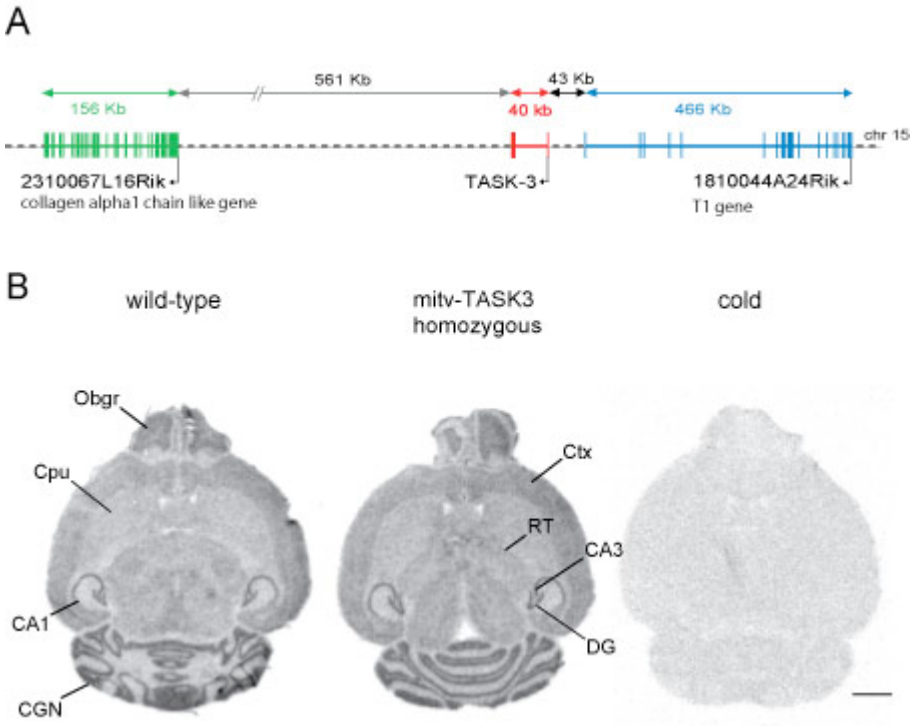

Figure S2. Genes flanking the mouse TASK-3 gene
A, map of the immediate genes flanking the TASK-3 gene (red) are 5' the T1 gene (blue) and 3' the collagen XXII gene (green) on mouse chromosome 15. **B**, *in situ* hybridization autoradiographs showing mRNA expression of the T1 gene in adult wild-type and TASK-3 knockout mouse brains. The expression level of T1 mRNA is not altered TASK3 brain. Cpu, caudate-putamen; Ctx, neocortex; DG, dentate granule cells; CGN, cerebellar granule cells; Obgr, olfactory bulb granule cells; RT, reticular thalamus; Scale bar, 2mm.