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Supplemental Information

TRIM28 Represses Transcription of Endogenous Retroviruses in Neural Progenitor Cells

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Figure S1, Description of mapping strategy for ERVs. Related to Fig. 4

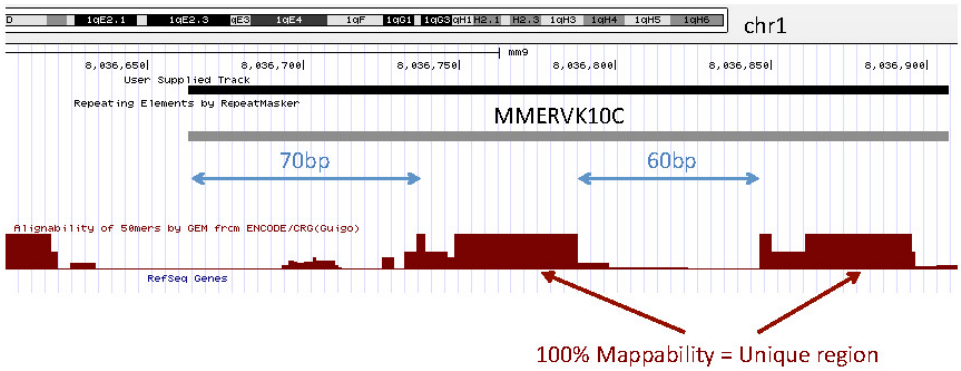

Figure S1. UCSC genome browser snapshot showing an example of mapability (50bp reads) of one MMERVK10C. This category of repeated elements contains parts with 100% mappabilty, i.e the 50bp reads map uniquely in this region and in no other part of the genome, thus making the mapping of those elements precise.

Figure S2, Additional example of an ERV-mediated mechanism that influences nearby gene expression. Related to Fig. 4

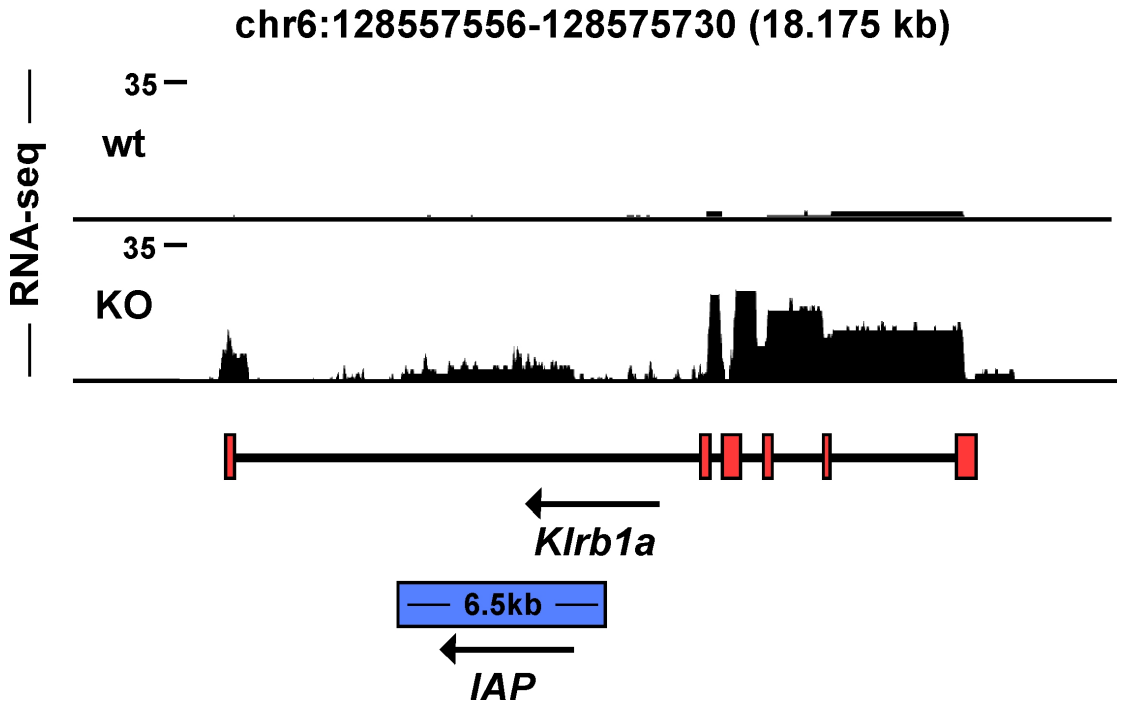

Figure S2. An IAP that is transcriptionally upregulated in TRIM28 -/- NPCs is located in an intron of Klrb1a. The expression of the IAP coincides with upregulated Klrb1a expression, possibly as a consequence of the loss of the TRIM28-silencing complex from the IAP.

Figure S3, Genotypic and phenotypic analysis of NestinCre-TRIM28flox animals. Related to Experimental Procedures

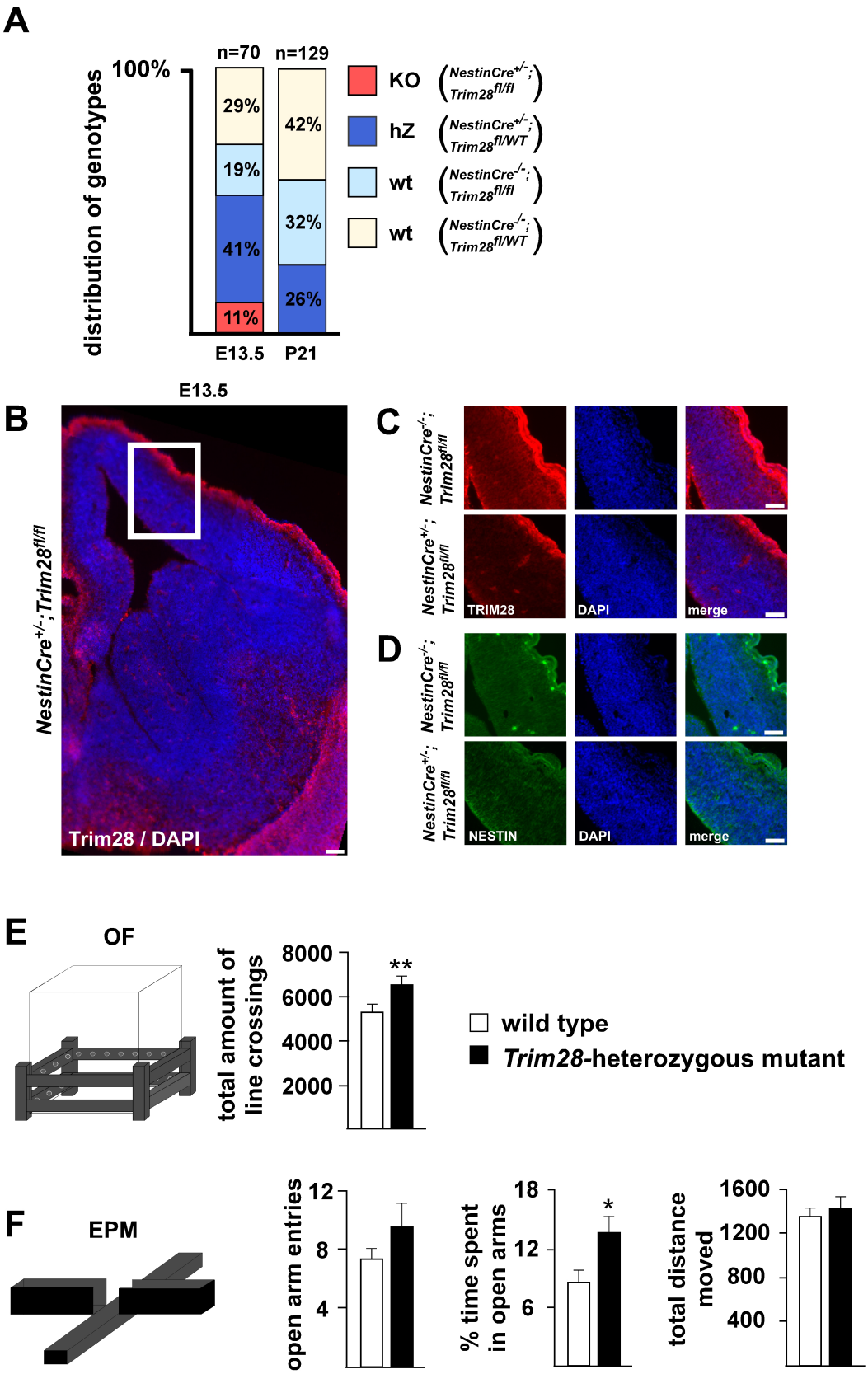

Figure S3. (A) Genotypic analysis of offspring from mating *NestinCre*$^{+/-}$;*Trim28*$^{fl/wt}$ males with *Trim28*$^{fl/fl}$ females at E13.5 and P21. *Trim28*$^{-/-}$ embryos were greatly underrepresented at E13.5, representing only 11% rather than the expected 25% of total litter size. The deletion of TRIM28 during brain development was ultimately lethal since the genotyping of three week old offspring demonstrated a complete absence of *NestinCre*$^{+/-}$;*Trim28*$^{fl/fl}$ mice**. (B-D)** Immunohistochemical analysis of E13.5 embryos deficient for *Trim28* and wild type control demonstrating normal gross morphology, lack of Trim28 expression and normal expression of the progenitor marker NESTIN in mutant mice. Scale bar represents 50mm. **(E&F)** Behavioral analysis of mice with a heterozygous conditional deletion of *Trim28* during brain development. We noted that in offspring from crossings between *NestinCre*$^{+/-}$;*Trim28*$^{fl/wt}$ males and *Trim28*$^{fl/fl}$ females, a subset of mice appeared to behave in a more active manner in comparison to other mice in the same cage. The mice were therefore tested for behavioral activity in the open field (OF) test and for anxiety-like behavior using the elevated plus maze (EPM). The OF test relies on the use of an open arena containing a grid of light beams with sensors, enabling detection and measurement of overall activity. We found that *NestinCre*$^{+/-}$;*Trim28*$^{fl/wt}$ mice, lacking one copy of *Trim28* in the brain, were significantly more active than control mice. The EPM test is an elevated four-arm maze with two walled arms (safe compartments) and two open arms (risky compartments) linked by a central platform. We found that *NestinCre*$^{+/-}$;*Trim28*$^{fl/wt}$ mice spent more time in open arms, indicative of decreased anxiety-like behaviors, when compared to wild-type littermates. Thus, these experiments demonstrate that heterozygous deletion of *Trim28* during brain development results in behavioral changes characterized by hyperactivity.

Table S1. Complete REPBASE counts from RNA-seq data of wt and TRIM28-KO NPCs. Table S1 is found as a separate excel-sheet. Related to Fig. 1

Table S2. Fold change KO/WT, REPBASE counts, RNA-seq NPCs. Table S2 is found as a separate excel-sheet. Related to Fig. 1

Table S3. Primer sequences. Related to experimental procedures.

Primer	Sequence
Trim28 (genotyping)	GGAATGGTTGTTCATTGGTG ACCTTGGCCCATTTATTGATAAAG GCGAGCAGAATCAAGGTCAG
CRE-SYBR (genotyping)	GCCACCAGCTTGCATGATC GGAGCCGCGCGAGAAT
b-Actin	TAG GCA CCA GGG TGT GAT GG CAT GGC TGG GGT GTT GAA GG
GAPDH	TCC ATG ACA ACT TTG GCA TTG CAG TCT TCT GGG TGG CAG TGA
Trim28	GCCTCTGACTGAAGGTCCTG TCCAAGCCTGAGCTGGTACT
IAP	CGGGTCGCGGTAATAAAGGT ACTCTCGTTCCCCAGCTGAA
MerkLZ10	CAAATAGCCCTACCATATGTCAG GTATACTTTCTTCTTCAGGTCCAC
IAPLTR	TGTGCCAGGCAGTAAACAAG ACCAATCACCACAGGTCACA
Line1	TTTGGGACACAATGAAAGCA CTGCCGTCTACTCCTCTTGG
MusD	GATTGGTGGAAGTTTAGCTAGCAT TAGCATTCTCATAAGCCAATTGCAT
GAPDH (ChIP)	CCC ACT CCG CGA TTT TCA CTC TGC TCC TCC CTG TTC CA
ERVK10CLTR	GTGTGAGACACGCCTCTCCT GGGAGAGCTTGATTGCAGAG
ERVK10CGAG	TCAGGATCATGCTCAACAGC TGGCATTGTGAGCCAATCTA
ERVK10CPOL	GCCACCAGAGACATGGTTTT CGGGCTTCTTTTCTTGTGAG
ERVK10CENV	TATCGCCTCAGGGTTAATGC TGGATGCCACACAACTCATT
IAP1LTR	TGTGCCAGGCAGTAAACAAG ACCAATCACCACAGGTCACA
IAP1POL	TGGCCATACCCCAAAGATAA CCAGTTTACTGGGGCTGGTA
MMERVBiSulfite	ATAGTTTAATTTAAGATATGGGGTT ACAATAATCAATACCACTCTACAAC
2410018L13Rik	CCCACTGCCTCTAGCTTCAC TTCTTCCAGGGACATTTTGC
Fbxw19	TGTGTACGTGTGGGAGGAGA AGAAAGCAGGGAATGGGACT

Olfr1350	AGATATCCCTCCCAGCCTGT GGGCAAGGAGAAAGTGTTGA
Klrb1a	ACCATGAAACCCTGAGCAAC TGAGAGGCAGACAGCAGAGA
Zfp932	CAGGCTTGAATGGTCCCTTA TCAGCAAAGCCCATTCTTCT
MERVlnc-flank	GCAGTCAATGCTCTCCCAAT CCCATTCTTGAGGTTTTCTCTTT
IAPlnc-flank	GGATCTGGTTGTCCGAGTGT TCTGTTCCTGGCAATCCTTC
MERV3utr	AACTACAAAACAACAAACAAATAAGCA AAACTTGACTTCTTAAACCCATTCTT
MERVlncRNA	GTTTTGGAAGTTGCCTTGGA CCCAAGAACAGAAGCAGAGC
IAP3utr	TTGAAGCCAGGTGCAGTAAC TTCTGTTCCTGGCAATCCTT
IAPlncRNA	TCAGGATGTTGAGCCTGTTC GGGTTTCCTAGGTGCTGACA
IAP-Gene3UTR	GGTGAACTGCCTGGAAGAGA TGGGGTCCTAGTCACCTTTG
IAP-Gene3Inside	TCATGCCCACCATCTTGTAA CGTGTTGGCACCAGATTCTT

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Transgenic animals

All animal-related procedures were approved by and conducted in accordance with the committee for use of laboratory animals at Lund University. Generation and genotyping of mice with a floxable *Trim28* allele have been described previously (Weber et al., 2002). The previously generated *Nestin-Cre* mouse gives a broad pattern of recombination, primarily in the developing forebrain with limited recombination in other tissues (Tronche et al., 1999). The *Cre*-allele was genotyped according to standard procedures. For primer sequences see Table S3. Transgenic mice were back-crossed to a C57/Bl6-background for at least 8 generations.

Cell culture

Forebrain from embryonic day 13.5 (E13.5) was dissected, dissociated and cultured as neurospheres (500 000 cells/T25 flask (Nunc)) in mouse DMEM/F12 supplemented with Glucose, Glutamine (Sigma), penicillin/streptomycin (Gibco), Hormone mix (Apo-Transferrin Sigma-Aldrich, Insuline – bovine pancreas, Sigma-Aldrich), EGF (20ng/ml, R&D) and bFGF (10ng/ml, R&D) as previously described (Ahlenius and Kokaia, 2010).

Upon differentiation, neurospheres were plated on poly-L-lysin (Sigma P 6282) coated 4-well plates in differentiation medium (mNS-basic medium without EGF and bFGF and 1% fetal bovine serum (Gibco no.10106-78). Medium was applied to the cells every other day for a period of 10 days.

NPCs were established from neurospheres as previously described (Conti et al., 2005). In brief, neurospheres generated from E13.5 wild type and *Trim28*-deficient forebrain cells were cultured on 0.1% gelatin (Sigma G2500-100G) coated T25 flasks (Nunc) in mNSC-complete medium: (1µg/ml) EGF, bFGF (R&D) in mouse neural stem cell basic medium: (EUROmed-N medium (EuroClone), Glutamine (Sigma), 2.5 ml penicillin/streptomycin (Gibco), Hormone mix (Apo-Transferrin Sigma-Aldrich, Insuline – bovine pancreas, Sigma-Aldrich)). The cells were split every other day using accutase (PAA) and plated in a density of 60 000 cells/cm^2.

For differentiation of NPCs, cells were plated in a density of 30 000 cells/cm^2 on laminin (Invitrogen) coated 4-well plates using mouse neural stem cell basic medium. Medium was changed every other day for a period of 7 days.

Immunofluorescense

Embryos were collected at E13.5 and genotyped using tail-DNA. The embryos were kept at 4°C over night in 4% formaldehyde solution (Sigma no. F8775-250ml, Stock:36.5%) followed by incubation in 25% sucrose-solution for 36-48 hours. The embryos were fixed and frozen in Tissue-tek (Sakura O.C.T™ COMPOUND) and sectioned on the cryostat (MICROM HM500M) in 14µm coronal sections. Cell cultures were fixed using 4% formaldehyde solution for 10 min followed by 3 rinses in PBS.

Immunofluorescence was performed as previously described (Sachdeva et al., 2010; Thompson et al., 2005). In brief, fixed embryonic tissue and cells were blocked using 5% normal donkey serum, 0.25% triton-X in PBS. Primary antibodies: mouse anti-Trim28 (1:1000, Millipore MAB 3662), mouse anti-Nestin (1:200, BD556309), mouse anti-BIII-tubulin (1:1000, Promega G712A), rabbit anti-GFAP (1:1000,

DAKO Z0334) rabbit anti-IAPgag (1:2000, kind gift from B. Cullen). Secondary antibodies: goat anti mouse IgG: Alexa Fluor-568 and Alexa Fluor-488 (both Invitrogen; 1:500). All nuclei in sections and cells were counterstained with DAPI (Sigma D8417; 1:1000). Pictures were obtained using fluorescent microscopes (Leica DFC360TX, DMI 6000B) and a confocal microscope (Leica TCS SP8).

RNA studies

For the RNA-seq, total RNA was extracted from neurospheres (at passage 4) and attached NPCs using the RNeasy Mini Kit (Qiagen). RNA quality was verified using Agilent Bioanalyser prior to labeling. RNA-seq was performed as previously described (Rowe et al., 2010).

For qRT-PCR, 1.5 ug of RNA was used for the reverse transcription performed with random primers (Invitrogen) and SuperscriptII (Invitrogen) according to supplier's recommendations. SYBR green qRT-PCR was performed in triplicates as previously described. Data was quantified using the ΔΔCt-method and was normalized to GAPDH and β-actin expression. Primers were designed using Primer3 software (http://frodo.wi.mit.edu). The efficiency of all primer pairs was confirmed by performing reactions with serially diluted samples. Primer sequences are found in Table S3.

RNA-sequencing analysis

About 270 million reads were generated for three *NestinCre*$^{+/-}$;*Trim28*$^{fl/fl}$ knockout samples and three *NestinCre*$^{-/-}$;*Trim28*$^{fl/fl}$ wild type samples. The 50-base single end (neurospheres) and 50-base paired end (attached NPCs) reads were mapped onto the RepBase version 16.08 (Jurka et al., 2005) and to the mouse genome (mm9)

assembly. Mapping was done using the bowtie short read aligner (Langmead et al., 2009) and permitting up to three mismatches for the totality of the read. Differentially expressed genes and repeated elements were calculated using the Bioconducter/R package DESeq (Anders and Huber, 2010).

For the repeated elements we have followed the mapping strategy as recommended for repetitive regions (Treangen and Salzberg, 2012)). We have used the recommended bowtie parameters (–best, -M 1 –strata). With these settings, reads that map to multiple positions are randomly attributed to one single position. This avoids inflating read-depth for repeated elements (by counting several time the same read) while keeping sensitivity (enough read-depth) for subsequent statistical analyses.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed using iDeal ChIP-seq kit (Diagenode) according to supplier's recommendations. The H3K9me3 antibody (Diagenode, pAb-056-050) was used at 2µg/reaction. Primer sequences are found in Table S3. SYBR green qPCR reactions were run in triplicates with Roche SYBR Green PCR Master Mix (Roche) using standard procedures. Negative control reactions without antibody or with control antibody were run for each sample and in all cases gave negligible values. To quantify the relative enrichment of each sequence, a ΔCt for each sample was determined (CtInput-CtSample). The relative enrichment was then calculated by raising 2 to the ΔCt power. Relative quantification between KO and WT samples was performed by calculating a ΔΔCt-value for each pair of samples that were run in parallel (KOΔCt – WTΔCt). The fold difference was then determined by raising 2 to

the ΔΔCt power. The fold difference amongst pairs was then normalized to GAPDH. All data are expressed as mean +/- SEM, based on the results of three independent experiments.

DNA-methylation analysis

Bisulfite sequencing was done using the EpiTect bisulfite kit (Qiagen) according to the supplier's recommendations. For each PCR, 1 µl of bisulfite-treated DNA was used. PCR conditions were as follows: 94 °C for 15 min, followed by 45 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. PCR-amplified products were cloned into a TOPO cloning vector (Invitrogen). Between 12 and 20 clones from each PCR were sent for sequencing. Sequence data were analyzed using QUMA: quantification tool for methylation analysis (Kumaki et al., 2008).

Behavioral testing

For the behavioral tests, *NestinCre*$^{+/-}$;*Trim28*$^{fl/wt}$ males were mated with *Trim28*$^{fl/fl}$ females. A total of 32 age-matched male and female offspring were used (10 heterozygous and 22 wild type). All animals, starting at 3 months of age, were initially exposed to the open field test (OF) followed by the elevated plus maze (EPM), leaving one week in between the tests. All behavioral testing took place during the light cycle. To eliminate odor cues, each apparatus was thoroughly cleaned with 70% ethanol and wiped dry after each animal.

Locomotion and reactivity to an open field was assessed in a white box (50x50x37 cm) under dim and dispersed light conditions. The OF test is a standard test that evaluates locomotor activity. It consists of a simple square box where two adjacent

walls contain 2 rows of light beams forming a coordinate system. Sensors in the walls of the box are connected to a computer, detecting every time an animal crosses the light beams. Each mouse was placed into the center of the field and allowed to move freely during 60 min. The total amount of light beam crossings of all four paws was captured throughout beam breaks (PASdata). Measures of the total amount of line crossings are used as an index of activity.

Anxiety was assessed using the EPM task, which is a standardized test for anxiety. The maze consists of two opposite open arms and two opposite closed arms (66 x 6 x 14 cm) arranged at right angles. The arms extended from a common central platform (6 x 6 cm) giving equal access to all arms. The maze was elevated 70cm above the floor under dim and dispersed light conditions. Mice were placed on the central platform and allowed to explore the maze for 5 min, while recording the total distance moved, time spent in the center, open and closed arms, number and latency of entries to the open and closed arms, by using video tracking software (Ethovision 3.1.16, Noldus). The total distance moved serves as indicator of spontaneous locomotor activity, while differences in the proportions of time spent in the open arms and in the center are used as a measurement of anxiety.

For the statistical analysis, data from the different wild-type genotypes (*NestinCre*$^{+/-}$;*Trim28*$^{wt/wt}$ *and NestinCre*$^{-/-}$;*Trim28*$^{wt/wt}$) were pooled since we never found behavioral differences when comparing these groups.

Statistical analysis

An unpaired t-test was performed in order to test for statistical significance. Data is presented as mean+/-SEM.

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