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RESEARCH ARTICLE

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Generation and characterization of mice bearing null alleles of nradd/Nrh2

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Abstract

The *Neurotrophin receptor associated death domain* gene (*Nradd/Nrh2/Plaidd*) is a type I transmembrane protein with a unique and short N-terminal extracellular domain and a transmembrane and intracellular domain that bears high similarity to the p75 neurotrophin receptor (*p75NTR/Ngfr*). Initial studies suggested that NRADD regulates neurotrophin signaling but very little is known about its physiological roles. We have generated and characterized NRADD conditional and germ-line null mouse lines. These mice are viable and fertile and doṅt show evident abnormalities. However, NRADD deletion results in an increase in the proportion of dorsal root ganglion neurons expressing p75NTR. The NRADD conditional and complete knockout mouse lines generated are new and useful tools to study the physiological roles of NRADD.

KEYWORDS

P75NTR, PLAIDD, neurotrophin receptors, NRH2

1 | INTRODUCTION

Neurotrophins are trophic factors that modulate various aspects of neuronal biology through their interaction with Trk and/or p75NTR receptors (Bothwell, 2014; Ceni, Unsain, Zeinieh, & Barker, 2014; Kraemer, Yoon, & Carter, 2014). P75NTR (Ngfr/TNFR superfamily, member 16) was the first neurotrophin receptor discovered and the founding member of the tumor necrosis factor receptors superfamily (TNFR). P75NTR is a type I transmembrane protein characterized by an intracellular death domain (DD), a key structure for apoptotic signaling (Roux & Barker, 2002). The *Neurotrophin receptor associate death domain* gene (NRADD, also known as neurotrophin receptor homolog 2, NRH2 and p75-like apoptosis-inducing death domain, PLAIDD) is a type I transmembrane protein with a short N-terminal extracellular domain unrelated structurally to any other member of the TNFR family (Bredesen, Frankowski, Castro-Obregon, del Rio, & Rao, 2002; Wang et al., 2003). However, its transmembrane and intracellular domains share high similarity to those in p75NTR (Bredesen et al., 2002; Murray, Perez, Lee, Hempstead, & Chao, 2004). NRADD is broadly expressed embryonically, but its expression decreases in the adult (Bredesen et al., 2002). The protein is present in most intracellular compartments including the nucleus, and its relative enrichment in each compartment varies with cell type. NRADD interacts with p75NTR, TrkA, and sortilin (Hashimoto et al., 2004; Kim & Hempstead, 2009; Murray et al., 2004), suggesting that it might participate in neurotrophin signaling. NRADD, like p75NTR, is susceptible to regulated proteolysis to release an extracellular domain (ECD) and a soluble intracellular domain (ICD) by the sequential activity of alpha and gamma secretases (Gowrishankar, Zeidler, & Vincenz, 2004; Kanning et al., 2003). The small size of its unique extracellular region suggests that NRADD may not directly engage with ligands (Bredesen et al., 2002; Murray et al., 2004).

Overexpression studies in cell lines and primary neurons suggest that NRADD may play a role in apoptosis induction (Bredesen et al., 2002; Wang et al., 2003), likely through activation of the extrinsic apoptotic pathway involving caspase-8 (Wang et al., 2003). Also, overexpression of NRADD-ICD mimicked p75NTR-triggered NFkB signaling (Kanning et al., 2003). Acute knockdown of NRADD in cortical and DRG sensory neurons was shown to decrease the levels of total and plasma membrane sortilin and thus may have an impact on pro-neurotrophin signaling (Kim & Hempstead, 2009). Interestingly, NRADD biology might also be linked to neurodegenerative disease, in light of evidence from cell cultures showing NRADD participates in cell death induction by amyloid beta peptides (Hashimoto et al., 2004). Recent work using transgenic mice overexpressing NRADD suggested that the over-expression of NRADD (referred to as *p45*) reduced the

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proapoptotic signals induced by FasL, likely by interacting with FADD and blocking the recruitment of procaspase-8 (Sung et al., 2013). These mice showed decreased neuronal death *in vivo* after spinal cord injury.

Overexpression approaches or acute knockdown in cultured cells has provided clues on the role of the protein, but a more precise description of the role of the protein *in situ* would require the conditional deletion of the protein that could be temporally and spatially regulated. To that end, we sought to generate mouse lines bearing permanent or conditional deletion of the NRADD gene. Here we report the generation and characterization of a novel mouse line carrying conditional knockout alleles for *NRADD* (*NRADD*$^{flox/flox}$), as well as the complete knockout mice (*NRADD*$^{-/-}$). These lines will help elucidate the physiological and cell-specific functions of this intriguing and unique death domain-containing TNFR family member. We show that the conditional alleles are efficiently recombined by cre-recombinase in a cell-specific manner *in vivo* and that the complete KO mice are viable and fertile with no obvious malformations or defects. *NRADD* deletion results in an increase in the proportion of dorsal root ganglion sensory neurons expressing p75NTR, but does not affect their total number. Moreover, we show that in the adult, NRADD protein is highly expressed in the spleen.

2 | RESULTS

2.1 | Generation of mice carrying the floxed and knockout *nradd* alleles

To generate a conditional knockout allele for *NRADD*, our strategy was to flank exon2 (out of five exons), which contains the NRADD initiation codon, with loxP sites (Figure 1a). Embryonic stem (ES) cells were electroporated with the linearized targeting vector and positive clones were identified using a double selection strategy (Figure 1b). Clones with a normal karyotype were micro-injected into Bl6 blastocysts to create chimeras in an SV129 background. The chimeras were mated with C57BL6 wildtype (WT) animals and germline transmission was confirmed by PCR genotyping (Figure 1c,d). Heterozygous offspring were interbred to generate homozygous floxed mutant mice [*NRADD*-$^{flox/flox}$(Figure 1c)]. The PCR products from heterozygote tissue samples show different levels of the corresponding alleles that likely reflects suboptimal amplification of the longer floxed PCR product.

The NRADD floxed line was tested for its ability to undergo spatially-restricted recombination mediated by cre-recombinase. To delete NRADD specifically from neurons, *NRADD*$^{flox/flox}$ mice were bred to animals expressing cre-recombinase from the neuronal specific tubulin promoter, Tα1 (Tα1-Cre mouse). Genomic PCR genotyping demonstrated that the floxed NRADD allele was specifically recombined in neuronal tissue from mice bearing an allele of tα1 cre-recombinase (Figure 2a) and rtPCR showed that neuronal mRNA had the appropriate exon 2 excision (Figure 2b). The NRADD germline knockout mice was generated by crossing homozygous floxed mice to CMV-cre mice (Figure 1d). Mice bearing this knockout allele were backcrossed to C57BL6 mice for five generations and then maintained as heterozygotes in aC57BL6 background. Western blot analyses of different tissues showed that the protein is particularly abundant in spleen (Figure 2c). Figure 2d shows that heterozygous mice have a sharp decrease in NRADD protein levels in spleen and that homozygous null mice (*NRADD*$^{-/-}$) have no detectable NRADD expression in tissue.

Adult *NRADD*$^{-/-}$ mice have no obvious abnormalities and in appearance are indistinguishable from WT littermates (Figure 3a). Heterozygous mice breed normally, with normal litter sizes and expected Mendelian ratios (Figure 3b). Homozygous null mice are viable and fertile. Weight curves extending for up to 80 days of birth showed no differences between WT and null mice (Figure 3c).

2.2 | *Nradd* deletion increases p75NTR expression in dorsal root ganglion sensory neurons

NRADD has been shown to functionally interact with p75NTR. Since p75NTR null mice have a 50% decrease in DRG sensory neuron number (Lee et al., 1992), we speculated that *NRADD*$^{-/-}$ mice may have a similar defect. We therefore performed stereological counting to compare DRG sensory neuron numbers in WT and NRADD null littermates. Figure 4a,b shows that the number of neurons in L4 DRG is the same in WT and *NRADD*$^{-/-}$ mice. Interestingly, although neuronal number remained constant, we found that null mice displayed a 40% increase in the proportion of neurons that express p75NTR (Figure 4c,d), compared to their wildtype counterparts.

3 | DISCUSSION

Death domain-containing receptors that are members of the tumor necrosis factor receptor (TNFR) superfamily are capable of inducing cell death by engaging caspases through the extrinsic or intrinsic apoptotic pathways; however, they can also activate other signaling pathways such as NF-kB and MAP kinases. NRADD is an intriguing and understudied member of the DD-containing subfamily, and as a first step to elucidate its function *in vivo*, we have created and characterized conditional and knockout NRADD mouse lines. Mice bearing the complete deletion of *NRADD* are viable and fertile.

The small extracellular domain of NRADD suggests is incapable of binding a ligand and it is therefore expected to interact with other receptors to modulate signal transduction pathways. Given that p75NTR deletion results in a marked loss of DRG sensory neurons and given the high degree of similarity with the transmembrane and intracellular domains of p75NTR, we speculated that NRADD deletion might have an impact in DRG survival *in vivo*. To evaluate this possibility, we performed stereological estimations of DRG neuron numbers in WT and *NRADD*$^{-/-}$mice. Surprisingly, we found that *NRADD* deletion had no impact in neuron number.

The adult dorsal root ganglion is composed of a defined mixture of neuronal populations that detect distinct sensory modalities. These different neuronal subtypes can be characterized by their unique expression of certain combinations of neuropeptides and neurotrophin receptors. For example, approximately 30% of the neurons in DRG ganglia express the mRNA and protein for p75NTR (Lindsay & Harmar,

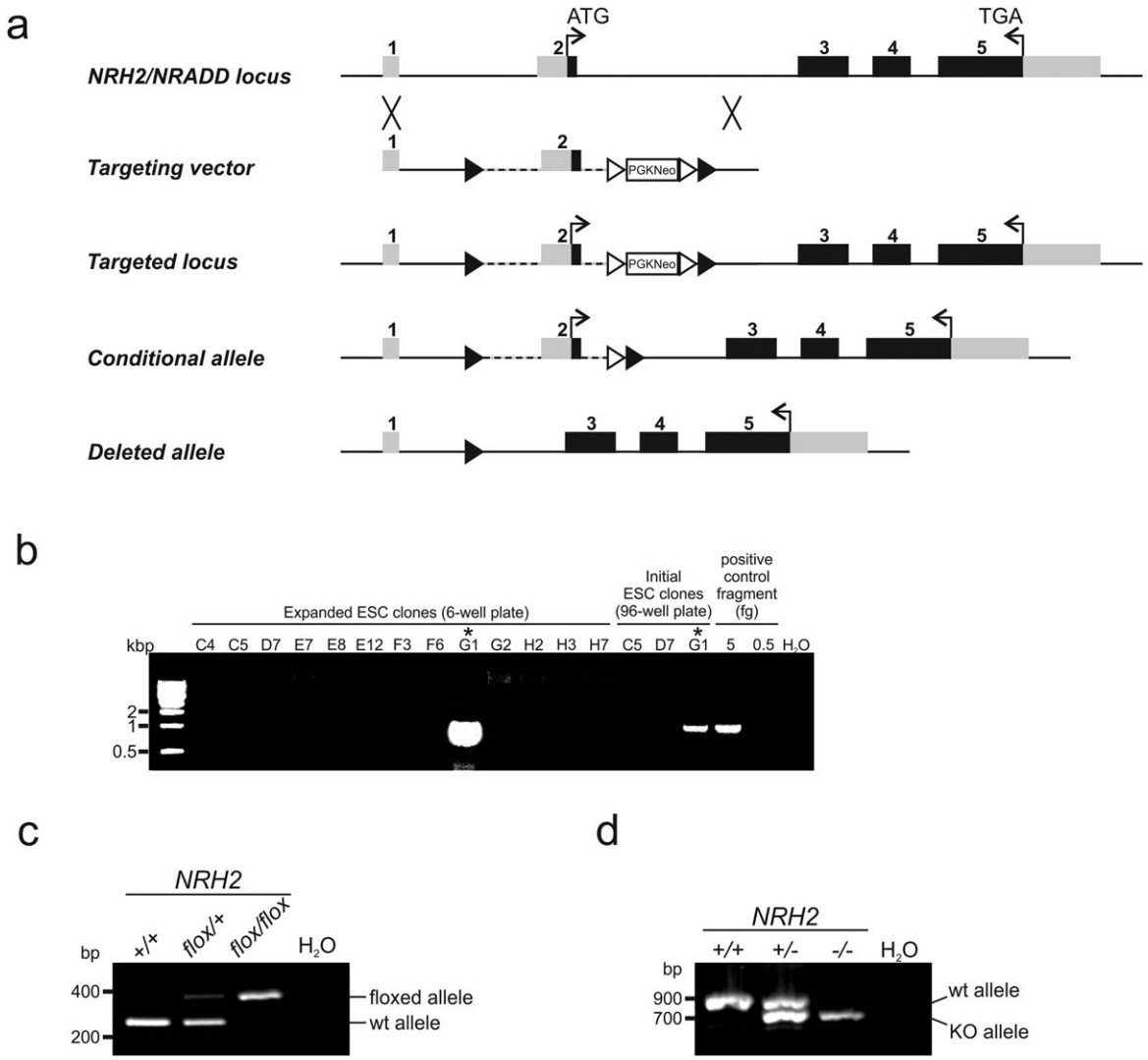

FIGURE 1 Generation of mice carrying the floxed and null NRADD alleles. (a) Schematics showing the NRADD locus in mice, the targeting vector and the targeted locus after homologous recombination. Arrows indicate the initiation and stop codons, black triangles are loxP sites and white triangles are FRT sites. The conditional allele is generated by excising the Neo-cassette. Then, the deleted allele was generated by recombining the loxP sites and thus removing exon 2 bearing the initiation codon. (b) PCR products of the screening of positive ESC clones. Clone G1 was used in subsequent steps. (c) Genotyping by PCR using genomic DNA for the presence of the floxed allele. (d) Genotyping by PCR using genomic DNA for the presence of the KO (deleted) allele

1989; Lindsay et al., 1990). Interestingly, we found that germline deletion of NRADD significantly alters that proportion. Given that the maturation of the different subpopulations within the dorsal root ganglia is modulated in part by NGF signaling (Luo et al., 2007; Tonra & Mendell, 1998), it is possible that the observed phenotype is a consequence of altered neurotrophin signaling in the absence of NRADD, as suggested by previous studies (Kanning et al., 2003; Kim & Hempstead, 2009). How the lack of NRADD affects signaling in this scenario needs further investigation. One possibility is that its absence affects the levels of neurotrophin receptors in the plasma membrane (Kim & Hempstead, 2009). Alternatively, since NRADD can associate with other neurotrophin receptors, it might affect their ability to recruit intracellular components for proper activation of downstream effectors (Hashimoto et al., 2004; Murray et al., 2004; Kim & Hempstead, 2009).

The physiological roles of NRADD are still unknown, and these mouse lines will greatly contribute to our understanding of its normal function.

4 | METHODS

4.1 | Generation of mice carrying the floxed and knockout *NRADD* alleles

NRADD locus was modified using the recombineering technique described by Liu and colleagues (Liu et al., 2003). A 10 kb fragment from *NRADD* locus was retrieved from a BAC (RP24-179LI9) and subcloned into a MC1TK-containing plasmid (pL253) by gap repair. Primers used to generate homology arms are described below. The region

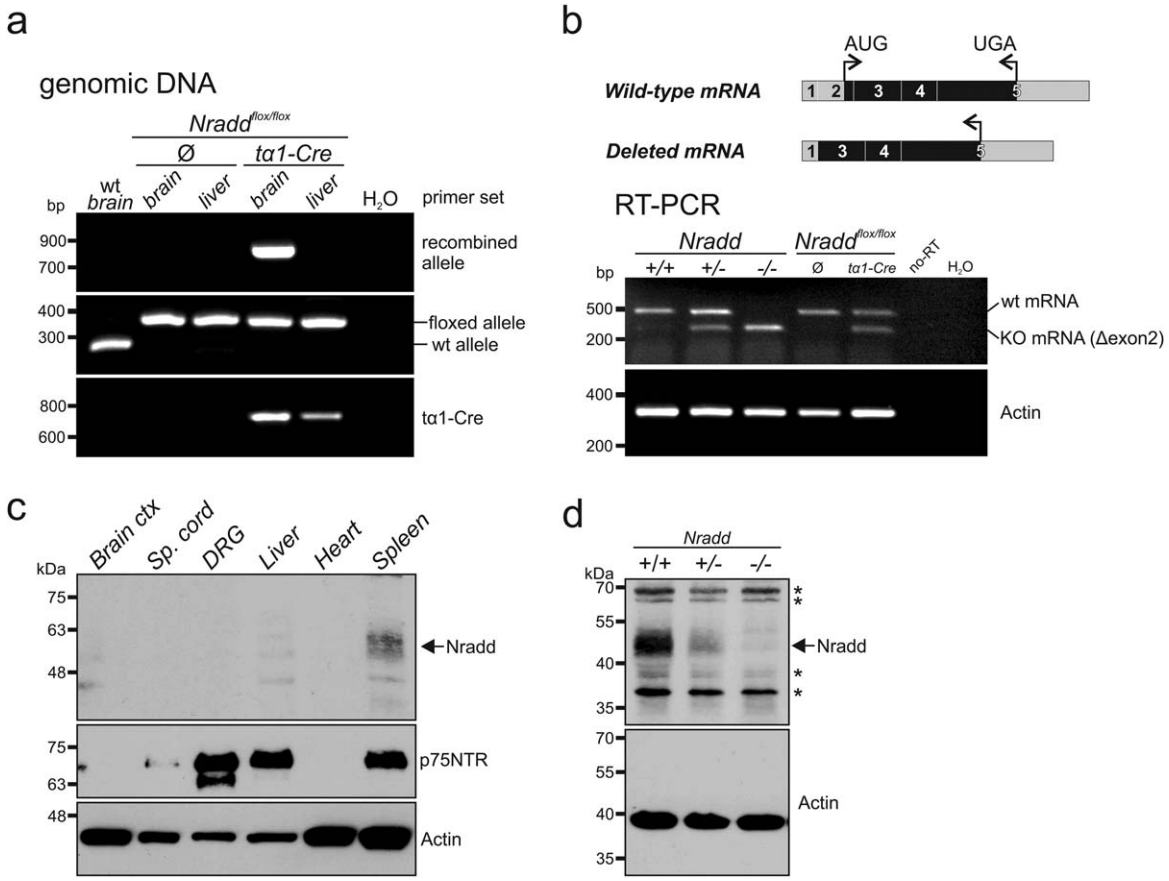

FIGURE 2 NRADD protein expression is lost in mutant mice. (a) Tissue-specific recombination of the conditional allele demonstrated by the activity of Cre-recombinase under control of the neuron-specific promoter for tubulin α1 isoform (Tα1). (b) RT-PCR product using primers flanking exon 2 shows that the recombined allele produces a truncated RNA that lacks the initiation codon and thus is not translated. (c) Western blot analyses of protein samples from different tissues from P90 mice to detect NRADD protein. (d) Protein samples from adult spleen from wildtype, heterozygous and null NRADD mice show that NRADD deletion results in complete protein depletion

retrieved includes the first base pair of exon1 to a location 10945bpdownstream of exon1. To generate the targeting vector, loxP sites were introduced to flank exon II of NRADD, in gap repaired pL253. First, a floxed neocassette was introduced via homologous recombination into the repaired plasmid. The neocassette was then removed by Cre recombination to leave a single loxP behind. To introduce a floxed Neo gene at the correct location, we generated homology arms to the targeting site (primer pair C–D and primer pair E–F). These arms were cloned into a pBluescript derived plasmid (pL452) on each side of a floxed neocassette. The fragment corresponding to the neogene and homology arms was excised from pL452 and coelectroporated along with the gap repaired plasmid into cells competent for Red recombination. Transformants were selected on kanamycin. The neomarker was deleted after electroporating the targeted plasmid into Cre expressing cells.

To introduce a second loxP site, a second set of homology arms was generated. The arms amplified from the intron 2/3 region were subcloned on both sides of a neogene flanked by a FRT site upstream and by FRT and loxP downstream. The selection cassette was introduced into targeting vector as described above. LoxP and FRT sites were tested by transforming targeting vector into cells that have Cre or Flpe gene under the control of an arabinose-inducible promoter.

Embryonic stem (ES) cells R1 (129 background) were electroporated with the linearized targeting vector using a BioRad Gene Pulser (settings were: capacitance EXT, 0.32 V, 250 μF, 50 μg of total DNA per two 10 cm plates of ES cells). After electroporation, the cells were plated on nineteen 10 cm dishes and 24 h after, they were put under selection with G418 (150 μg/ml) and ganciclovir (2 μmol/l). This double selection was maintained for 11 days. Three sets of 96 well plates (288) double-resistant colonies were picked, expanded, and analyzed for the presence of the right homologous recombination event by PCR. Fifteen positive clones were found. These clones were checked for karyotyping and the normal ones were microinjected into Bl6 blastocysts to create chimeras. The chimeras were mated with C57BL6 wildtype animals and germline transmission was confirmed by PCR genotyping. Heterozygous offspring were interbred to generate homozygousfloxed mutant mice. This NRADD colony is maintained in homozygosis (*NRADD*$^{flox/flox}$). TheNRADD knockout (*NRADD*$^{-/-}$) mice were generated by crossing homozygous floxed mice to CMV-cre mice [strain B6.C-Tg(CMV-cre)1Cgn/J, The Jackson Laboratories, ME, USA]. Heterozygous mice for the KO allele were backcrossed to C57CL6 mice for six generations and then maintained by crossing heterozygous mice.

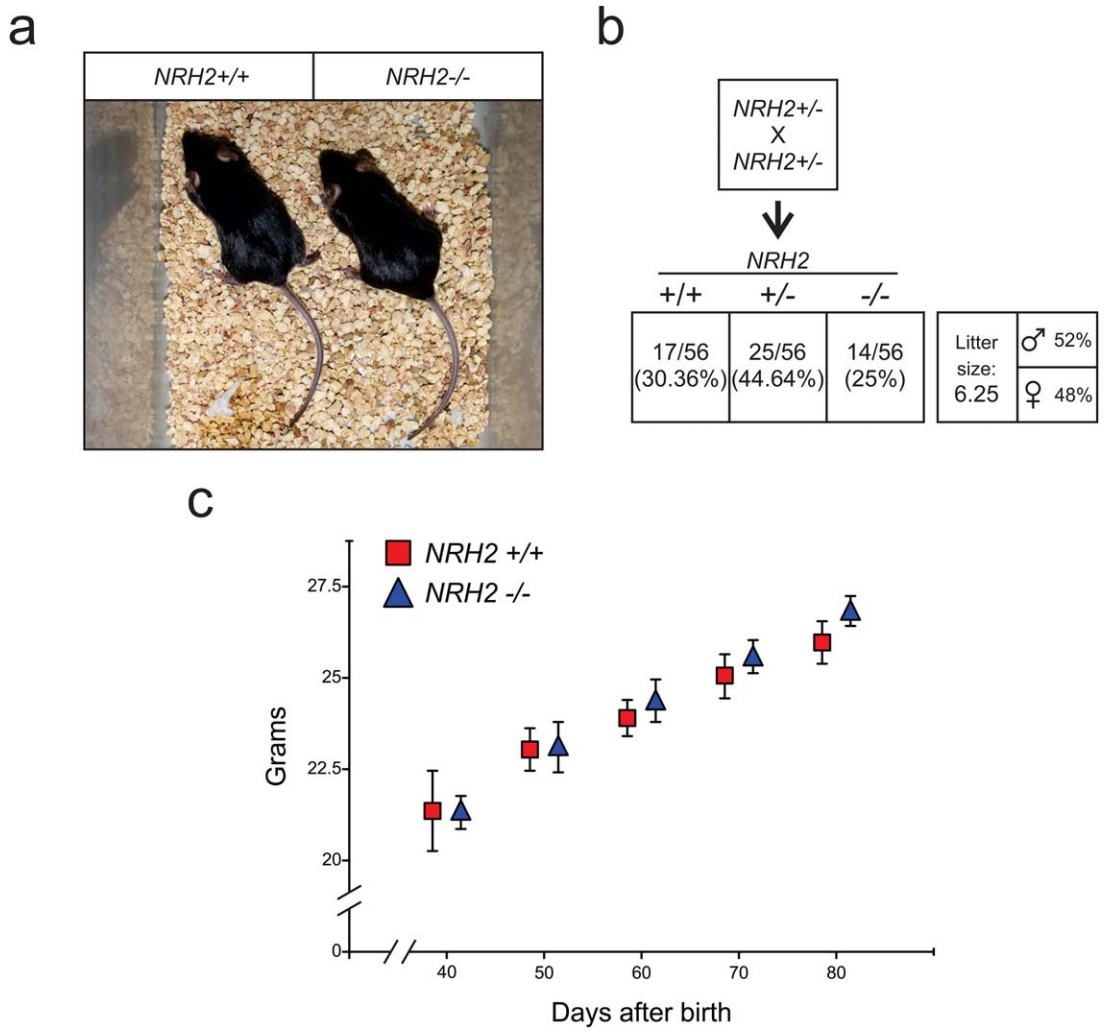

FIGURE 3 NRADDnull mice are viable and fertile. (a) Macroscopically, adult NRADD$^{-/-}$ mice are indistinguishable from their wildtype littermates. (b) Heterozygous NRADD mice (NRADD$^{+/-}$) breed normally, giving offspring of normal size, gender ratio, and genotypes with the expected Mendelian ratios. (c) Weight gain during the first 80 days of age is unaffected in NRADD$^{-/-}$animals

4.2 | Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated using RNeasy Mini kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). cDNA was generated from 600 ng of total RNA using the Omniscript RT kit (Qiagen) and random hexamers as primers. The PCR protocol (94°C for 1 min, 58°C for 1 min, and 72°C for 1 min) was performed using primers sets at 300 nM (see Table 1 for the sequence of the primers used). Fifteen microliters of the reaction were separated in a 1% agarose gel containing ethidium bromide, and PCR products were visualized under UV light and digitalized in a GelDoc^{TM} EZ System (Biorad, Hercules, CA, USA).

4.3 | Western blots

Tissue samples were homogenized with an electric pestle (Kontes, Vineland, NJ, USA) in icecold RIPA buffer: 50 mM Tris (pH7.4), 150 mM NaCl, 1% NP-40, 5 mM Na fluoride, 0.25% Na deoxycholate, and 2 nM $NaVO_3$, supplemented with a cocktail of protease inhibitors (Roche diagnostics, Indianapolis, IN, USA).Samples were boiled in Laemmli buffer for 5 min, separated in 7.5–15% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and transferred to 0.2 μm nitrocellulose membranes (Bio-Rad Life Science, Hercules, CA, USA). Membranes were blocked in TBS-T (10 mM Tris pH 8.0, 150 mM NaCl, 0.2% Tween 20, and 5% skim milk) for 1 h at room temperature. Membranes were then incubated in blocking buffer overnight at 4°C with the following primary antibodies: anti-NRADD (homegrown, purified sera; see below for more information about this antibody), anti-p75NTR (Majdan et al., 1997), anti-β-III tubulin (Tuj1 clone, Millipore) and antiactin (Fisher Scientific).The membranes were then washed and incubated with peroxidase-linked secondary antibodies. Blots were developed using chemiluminescence reagents followed by exposure of blots to autoradiographic X-OMAT-LS film (Kodak, Rochester, NY, USA). Densitometric analysis was done using ImageJSoftware (NIH, USA).Rabbit polyclonal antisera against NRADD were produced in our lab using recombinant mouse NRADD intracellular domain N-terminally fused to glutathione-*S*-transferase (GST) as antigen. Sera were purified by

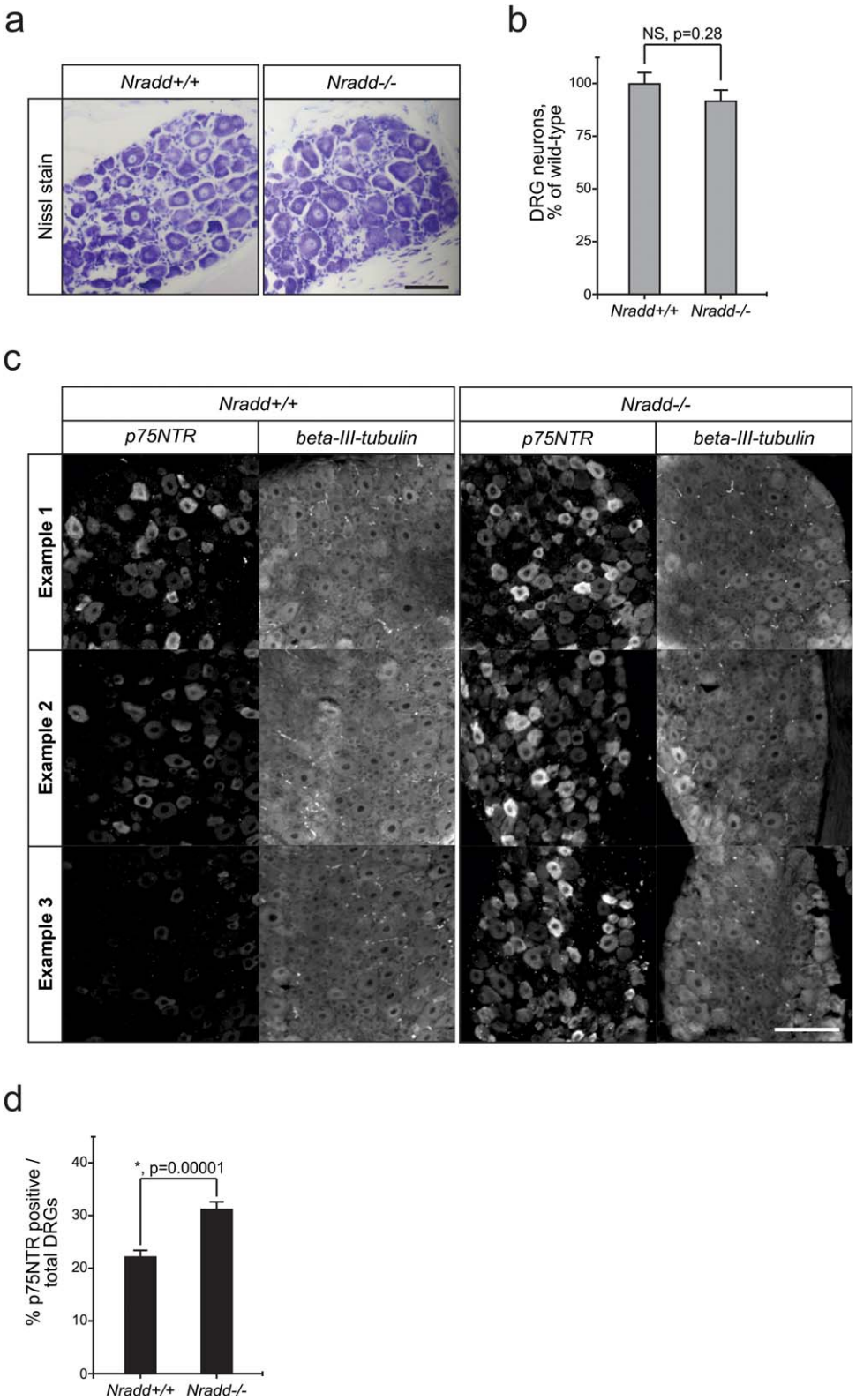

FIGURE 4 NRADD deletion increases p75NTR expression in dorsal root ganglion neurons. (a, b) The number of dorsal root ganglion sensory neurons is unaffected in NRADD null mice compared to wildtype littermates. Scale bar: 50 μm. (c, d) The proportion of neurons expressing p75NTR is increased in NRADD$^{-/-}$ DRGs compared to WT littermates. Scale bar: 100 μm

TABLE 1 Primers used for genotyping and detection of mRNAs by RT-PCR

Used to detect	Primer sequence		Figure
Transformed ESC clones	Forward	5′-ATGGCATCACCAGGAAGTCCCG-3′	Figure 1b
	Reverse	5′-GGTTATTGAATATGATCGGAATTGGGC-3′	
Genomic DNA, WT and floxed alleles	Forward	5′-CAGGGGCTGTTGAATTGTTT-3′	Figures 1c and 2a
	Reverse	5′-GCAGTTCGGTGCTGTAGTCA-3′	
Genomic DNA, WT and KO alleles	Forward	5′-CATGGGGTAACAGCTCAGGAT-3′	Figures 1d and 2a
	Reverse 1	5′-ACGCGTCGACTTCTCTGTCTCCCGCCTTTAGTGC-3′	
	Reverse 2	5′-AAGGAAAAAAGCGGCCGCCGTGCCTGCTAAGTGGTTACTG-3′	
mRNA, RT-PCR. WT and KO mRNA	Forward	5′-GCACCATGCTTTATAACGTCAGC-3′	Figure 2b
	Reverse	5′-TAAAGCTGGCAGCCAAGGTC-3′	

$(NH_4)_2SO_4$ precipitation, passed over GST beads, and purified by antigen affinity chromatography.

4.4 | Assessment of dorsal root ganglia neuron number

Sensory neurons within dorsal root ganglia were quantified in cross-sections of L4 ganglia dissected from P60-90 mice and fixed for 2 days in 4% paraformaldehyde. Tissue was embedded in paraffin and the entire DRGs were sectioned at 10 μm thickness. Every third section was Nissl-stained and neurons were counted and analyzed using the "optical fractionator" principle (West, Slomianka, & Gundersen, 1991). The systematic random sampling and counting procedure were performed using StereoInvestigator software (MBF Bioscience, Williston, VT) connected to a Nikon Eclipse E800 microscope (Nikon Instruments, Melville, NY) equipped with an *x*/*y*/*z*-motorized stage and a color CCD camera (Optronics, Microfire S99808). Eight DRGs were analyzed per genotype.

4.5 | Immunohistochemistry

L4 DRGs were dissected from adult mice and drop-fixed in 4% paraformaldehyde for 2 days. Ganglia derived from each of the experimental groups were embedded in the same paraffin block and sectioned with a microtome (10 μm thickness). Mounted sections were hydrated in decreasing ethanol concentrations and then blocked with normal goat serum 5% and Triton-X 0.03%. Primary antibodies against p75NTR (Majdan et al., 1997) and β-III tubulin (Tuj1 clone, Millipore) were used overnight. After washes in PBS, the slides were incubated at room temperature with Alexa-conjugated secondary antibodies (Jackson Laboratory) for 1 h. After washing the secondary antibodies, a coverslip was placed using fluorescent mounting medium (Dako) and examined on a wide-field fluorescent microscope.

4.6 | Statistical analysis

All data are presented as mean ± SEM. The Student's *t* test (two-tailed) was used for statistical tests.

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