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Contribution of the Reelin signaling pathways to nociceptive processing

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Abstract

The *reeler* gene encodes Reelin, a secreted glycoprotein that binds to the very-low-density lipoprotein receptor (Vldlr) and apolipoprotein E receptor 2 (Apoer 2), and induces Src- and Fyn-mediated tyrosine phosphorylation of the intracellular adaptor protein Disabled-1 (Dab1). This Reelin–Dab1 signaling pathway regulates neuronal positioning during development. A second Reelin pathway acts through Apoer 2–exon 19 to modulate synaptic plasticity in adult mice. We recently reported positioning errors in *reeler* dorsal horn laminae I–II and V, and the lateral spinal nucleus. Behavioral correlates of these positioning errors include a decreased mechanical and increased thermal sensitivity in *reeler* mice. Here we examined mice with deletions or modifications of both the Reelin–Dab1 signaling pathway and the Reelin–Apoer 2–exon 19 pathway on a Vldlr-deficient background. We detected *reeler*-like dorsal horn positioning errors only in *Dab1* mutant and *Apoer2/Vldlr* double mutant mice. Although *Dab1* mutants, like *reeler*, showed decreased mechanical and increased thermal sensitivity, neither the single *Vldlr* or *Apoer2* knockouts, nor the *Apoer2–exon 19* mutants differed in their acute pain sensitivity from controls. However, despite the dramatic alterations in acute 'pain' processing in *reeler* and *Dab1* mutants, the exacerbation of pain processing after tissue injury (hindpaw carrageenan injection) was preserved. Finally, we recapitulated the *reeler* dorsal horn positioning errors by inhibiting Dab1 phosphorylation in organotypic cultures. We conclude that the Reelin–Dab1 pathway differentially contributes to acute and persistent pain, and that the plasticity associated with the Reelin–Apoer 2–exon 19 pathway is distinct from that which contributes to injury-induced enhancement of 'pain' processing.

Introduction

During development neurons follow a highly ordered pattern of migration to form laminated structures such as the cerebral cortex and cerebellum. This process of migration is disrupted by a mutation in the *reeler* gene, which encodes an extracellular matrix-type glycoprotein known as Reelin (Caviness, 1976; Goffinet, 1983; D'Arcangelo *et al*., 1995). Reelin binding to the very-low-density lipoprotein receptor (Vldlr) or apolipoprotein E receptor 2 (Apoer 2) leads to tyrosine phosphorylation of Disabled-1 (Dab1; D'Arcangelo *et al*., 1999; Hiesberger *et al*., 1999) by the Src family of non-receptor tyrosine kinases (Arnaud *et al*., 2003b; Bock & Herz, 2003). The downstream signaling events activated by Dab1 phosphorylation influence neuronal positioning during CNS development (Howell *et al*., 1997b; Sheldon *et al*., 1997). The neurons in *reeler* mice that manifest positioning errors are identified by the accumulation of high concentrations of Dab1; wild-type neurons contain low Dab1 levels as Reelin binding stimulates polyubiquitination and degradation of Dab1 (Rice *et al*., 1998; Arnaud *et al*., 2003a; Morimura *et al*., 2005).

An advantage of studying Reelin function in the spinal cord rather than cortical structures is that relatively few populations of spinal cord neurons are aberrantly positioned (Yip *et al*., 2000; Phelps *et al*., 2002; Villeda *et al*., 2006). The first positioning errors found in *reeler* spinal cord were the sympathetic and parasympathetic preganglionic neurons (Yip *et al*., 2000; Phelps *et al*., 2002). More recently, we reported incorrectly positioned Dab1-positive neurons in *reeler* dorsal horn and in the lateral spinal nucleus (LSN; Villeda *et al*., 2006), areas involved in the transmission of 'pain' messages (Burstein *et al*., 1990; Gauriau & Bernard, 2004; Olave & Maxwell, 2004). Importantly, there is a functional correlate of this positioning defect. Compared with controls, *reeler* mice exhibit a significant reduction in mechanical sensitivity and an increased thermal sensitivity/heat hyperalgesia (Villeda *et al*., 2006).

Beffert *et al*. (2005) and Chen *et al*. (2005) provided evidence in adult mice that Reelin is also a modulator of *N*-methyl-D-aspartate (NMDA) receptor function and hippocampal synaptic activity, and that a splice variant of exon 19 located within the cytoplasmic domain of Apoer 2 mediates this adult Reelin function. These authors further showed that mice that constitutively lack Apoer 2–exon 19 perform poorly in learning and memory tasks. Thus, to date two Reelin signaling pathways exist: the Reelin–Dab1 pathway signals through Dab1 phosphorylation and is involved in neuronal positioning during development (Howell *et al*., 1997b; Rice & Curran, 1999) and the exon 19 pathway signals through Apoer 2 and contributes to synaptic plasticity in adult animals (Beffert *et al*., 2005).

We previously reported positioning errors and sensory impairments in adult *reeler* mutants and attributed these defects to the loss of Reelin (Villeda *et al*., 2006). Now we ask if these sensory deficits result from a loss of the Reelin–Dab1 pathway during development or the Apoer 2–exon 19 pathway in adults. To this end, we evaluated thermal

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and mechanical nociceptive processing and dorsal horn positioning errors in mouse models missing or with a modification in either of the Reelin lipoprotein receptors or the Dab1 intracellular adaptor protein.

Materials and methods

Animals and tissue preparation

The *reeler* (B6C3Fe-*ala-Reln*^{rl}, Jackson Laboratory, Bar Harbor, ME, USA), *scrambler* (*A/A Dab1*^{scm}*/Dab1*^{scm}, Jackson Laboratory), *Dab1* (BALB/cByJ *Dab1*$^{-/-}$, generous gift from Dr J. Cooper, Seattle, WN, USA), *Vldlr* (B6; 129S7-*Vldlr*^{tm1Her}, Jackson Laboratory), *Apoer 2* (B6; 129S6-*Lrp8*^{tm1Her}, generous gift from Dr T. Curran) and *Apoer 2/Vldlr* (B6; 129S) double mutants and their corresponding controls were generated from breeding colonies maintained at UCLA. Mice with mutations that interrupt Reelin signaling through Apoer 2–exon 19 (*Apoer 2* Δ*ex19/Vldlr–/–* and *Apoer 2 Stop/Vldlr–/–*) or their control (*Apoer 2 ex19/Vldlr–/–*), as well as *Apoer 2/Vldlr* double mutants were generated in colonies maintained at UT Southwestern (Beffert *et al*., 2005, 2006a,b; Fig. 1). The *reeler*, *Dab1–/–*, *Vldlr–/–* and *Apoer 2–/–* mutant mice were genotyped using polymerase chain reaction screening as previously described by D'Arcangelo *et al*. (1995), Brich *et al*. (2003), Frykman *et al*. (1995) and Trommsdorff *et al*. (1999), respectively. The *scrambler* mutants were identified by their ataxic phenotype (Sweet *et al*., 1996). Sensory testing was performed on 3–10-month-old mice. After completion of sensory testing mice were deeply anesthetized with Avertin (0.3 mL/10 g body weight) or Nembutal (80 mg/kg), perfused through the heart with 4% paraformaldehyde and postfixed overnight. Spinal cords as well as L4–5 dorsal root ganglia (DRGs) were dissected for immunocytochemical experiments. Adult and embryonic tissue preparation followed the methods in Villeda *et al*. (2006).

Culture experiments

Male and female Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were caged together overnight, and the following morning vaginal smears were obtained to confirm insemination. The day a positive smear is detected is considered embryonic day 0 (E0). Pregnant rats were deeply anesthetized with ketamine (90 mg/kg) and xylazine (20 mg/mL) and killed by thoracotomy, and E13 pups were obtained through Cesarean section and decapitated. The slice culture procedures were similar to those detailed in Phelps *et al*. (1996) and Kubasak *et al*. (2004), except that Millicell inserts (Millipore, Billerica, MA, USA) with 3 mL of media were substituted for the collagen-coated coverslip method. A potent and selective inhibitor of the Src family of kinases, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2; 15 μM, Calbiochem, San Diego, CA, USA) or its inactive control 4-amino-7-phenylpyrazolo[3,4-*d*]pyrimidine (PP3; 15 μM, Calbiochem), were added to the defined media for the duration of the culture experiment to block Dab1 phosphorylation and the downstream function of Reelin (Arnaud *et al*., 2003b; Bock & Herz, 2003; Jossin *et al*., 2003). After 72 h all cultures were fixed with 2% periodate-lysine-paraformaldehyde for 2 h at 4 °C and rinsed with buffer. Slices were embedded in a gelatine–sucrose mixture (Phelps *et al*., 1996) and stored at −80 °C until sectioning. We immunostained 40-μm serial sections with a polyclonal Dab1 antiserum (2720, 1 : 10 000; Herz Laboratory, Dallas, TX, USA) following procedures detailed by Villeda *et al*. (2006), except sections were incubated for 48 h in primary antibody.

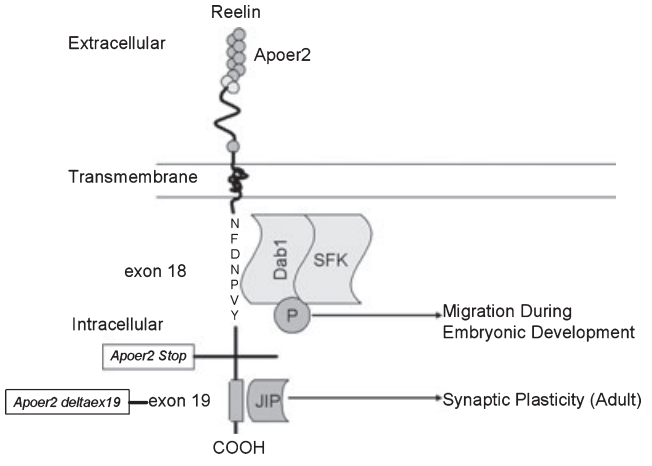

FIG. 1. Model of the two proposed Reelin signaling pathways. Boxes indicate mice with mutations in the intracellular domain of apolipoprotein E receptor 2 (Apoer2; Beffert *et al*., 2005, 2006b). Dab1, Disabled-1; JIP, Jun N-terminal Kinase interacting protein; SFK, Src family kinases.

Behavioral analyses

We evaluated thermal nociception using the Hargreaves paw withdrawal test (model 336G stimulator, IITC, Woodland Hills, CA, USA) on 14 (seven males and seven females) pairs of *Dab1* mutant and wild-type mice, 10 (five males and five females) pairs of *scrambler* and control animals, 12 (six males and six females) pairs of *Vldlr* mutant and wild-type animals, 11 (six males and five females) pairs of *Apoer 2* mutant and wild-type mice, six males each of *Apoer 2 ex19/Vldlr–/–, Apoer 2* Δ*ex19/Vldl–/–* and *Apoer 2 Stop/Vldlr–/–*. Unfortunately, the *Apoer 2/Vldlr* (B6; 129S) double-knockout mice die within 2–3 weeks after birth and therefore we were unable to test them.

After mice were acclimated for 45 min, the heat source was focused onto the plantar surface of the right and left hindpaws for three trials each and the latency to withdraw from the heat source recorded in seconds (Hargreaves *et al*., 1988). The optimal intensity of the light source was determined for males and females in each strain by testing control animals at a range of intensities that elicit an 8–12 s response time. The heat source turns off automatically at 20 s to prevent injury. Data were analysed by gender, and the statistical significance of the mean values from both hindpaws was evaluated with an analysis of variance.

To assess mechanical nociception we used the up–down paradigm of Chaplan (Chaplan *et al*., 1994). Calibrated von Frey monofilaments (0.008–4.0 g) were applied to the plantar aspect of the animal's left hindpaw. If upon stimulation with the 0.4 g monofilament a paw withdrawal response was not observed, a stronger stimulation was applied or, if the withdrawal response was detected, a weaker stimulus was used. The responses were tabulated and the 50% response threshold determined. Statistical significance of the mean threshold values was evaluated with the Mann–Whitney test.

Carrageenan experiments

To assess responsiveness in a model of persistent pain associated with inflammation, we injected λ,κ-carrageenan (3%, in saline, 10 μL s.c.; Sigma) into the plantar surface of the left hindpaw (Benitz & Hall, 1959) in wild-type, *reeler*, *Apoer 2 ex19/Vldlr–/–* and *Apoer 2* Δ*ex19/Vldlr–/–* mice. We used the Hargreaves procedure to measure the latency to withdrawal from the thermal stimulus before and at several time points after injection of the carrageenan (Porreca *et al*., 2006). In addition, we evaluated mechanical nociception under inflammatory conditions by repeating the above von Frey protocol

before and at several time points after injection of carrageenan (Porreca *et al*., 2006).

Immunocytochemical procedures

We localized the distribution patterns of the components of the Reelin signaling pathway in thoracic and lumbar spinal cords in a minimum of three pairs of adult wild-type and *reeler* mutant mice. We used an anti-Reelin antibody (G10; Chemicon International, Temecula, CA, USA) to localize Reelin (Chin *et al*., 2007) and immunostained for Dab1 as described above. To identify the location of Apoer 2 and Vldlr we used polyclonal anti-Apoer 2 (2561, 1 : 5000; Beffert *et al*., 2006a) and anti-Vldlr (2897, 1 : 500; Herz Laboratory) on adult sections and followed the same protocol as detailed for Dab1 in Villeda *et al*. (2006), except that sections were blocked in 10% normal goat serum in PBST (0.1 M phosphate-buffered saline with 0.3% Triton X-100) for 1 h. For control experiments, spinal cord sections from *Apoer 2–/–* and *Vldlr–/–* mice were examined (data not shown).

We analysed non-adjacent cervical, thoracic and lumbar coronal sections from three–five pairs of adult age- and sex-matched mutant (*reeler*, *Dab1–/–*, *scrambler*, *Vldlr–/–* and *Apoer2–/–*) and control mice. To evaluate the nociceptor populations in DRG and their small diameter primary afferents in the dorsal horn we used the lectin IB4 (1 : 400; Vector Laboratories, Burlingame, CA, USA) to identify the non-peptidergic group (Snider & McMahon, 1998), calcitonin gene-related peptide (CGRP; 1 : 30 000; Chemicon International) to identify the peptidergic populations, and transient receptor potential vanilloid 1 antiserum (TRPV1; 1 : 12 000; gift of Dr D. Julius, San Francisco, CA, USA) to mark the heat-sensitive receptors (as described in Malmberg *et al*., 1997; Tominaga *et al*., 1998; Caterina *et al*., 2000; Villeda *et al*., 2006). To localize neurons targeted by substance P, we used anti-neurokinin-1 receptor antiserum (NK-1; 1 : 10 000; Novus, Littleton, CO, USA; Villeda *et al*., 2006). To assess the distribution pattern of interneurons in laminae I-II we localized calbindin (1 : 150 000; Swant; Bellinzona, Switzerland), calretinin (1 : 50 000; Swant) and parvalbumin (1 : 100 000; Swant) as reported (Villeda *et al*., 2006).

For fluorescent double-labeling experiments, we immunostained spinal cord sections from three–five pairs of wild-type and *Apoer 2/ Vldlr* double-knockout animals with guinea pig anti-NK-1 (1 : 1000; Chemicon) and rabbit anti-Dab1 (2720, 1 : 8000) antisera following a published protocol (Villeda *et al*., 2006). Alexa Fluor 488 goat anti-guinea pig and Alexa Fluor 594 goat anti-rabbit secondary antibodies were used for NK-1 and Dab-1 localization, respectively.

We evaluated the overall neuronal distribution in the spinal cord with a monoclonal antibody to the neuronal-specific protein (NeuN; 1 : 900; Chemicon; Houser & Esclapez, 2003). For the LSN cell counts, 40-μm-thick non-adjacent sections from three pairs of *Dab1*, *scrambler, Vldlr* and *Apoer 2* mutant and control mice at cervical, thoracic and lumbar levels were photographed, randomized, counted by three investigators blind to the genotypes and averaged. The number of cells per segmental level was averaged by animal and the overall means calculated. Statistical significance was evaluated with an analysis of variance.

Results

Dab1 *and* scrambler*, but not* Vldlr *or* Apoer 2 *mutants display thermal hyperalgesia*

In the absence of Reelin, Dab1, or both Vldlr and Apoer 2, mice display similar phenotypic abnormalities (Trommsdorff *et al*., 1999; Rice & Curran, 2001), while mice with a mutation in only one of the lipoprotein receptors do not display ataxia or other characteristic neurological impairments (Trommsdorff *et al*., 1999). Thus, once we determined that *reeler* mice exhibit thermal hyperalgesia (Villeda *et al*., 2006) we postulated that if this sensory deficit is caused by a deletion in the Reelin–Dab1 signaling pathway, then *Dab1* mutants will display similar impairments. In response to thermal stimulation applied during the Hargreaves paw withdrawal test, we found that *Dab1* mutants are indeed significantly more sensitive than their wild-type controls, with a 2.8 s difference between control and *Dab1* mutant males and a 3.0 s difference between control and mutant females ($P < 0.0001$ for males and $P = 0.001$ for females; Fig. 2A). Similar to the *Dab1–/–* animals, both male and female *scrambler* (hypomorph that contains ~5% of wild-type level of mDab1 protein; Sheldon *et al*., 1997) mutants have significantly shorter thermal response latencies than their gender-matched controls. Male *scrambler* mice differed on average by 3.2 s and females by 2.3 s compared with controls ($P = 0.015$ and $P = 0.01$, respectively; Fig. 2B). Thus, both mouse models with *Dab1* mutations display thermal hyperalgesia, with latencies comparable to those observed in *reeler* mice. In contrast to the sensory deficits observed in the *Dab1* and *scrambler* mutants, we did not detect thermal hyperalgesia in mice with a single deletion of either *Vldlr* or *Apoer 2*; this was true for both male and female single-knockout mice ($P = 0.111$ for all; Fig. 2C and D). Thus, when only one of the lipoprotein receptors is deleted, the response of mutants does not differ from the background control mice. As the double-receptor knockout animals of this strain die by 2–3 weeks of age, we could not test them.

Dab1 *and* scrambler *but not* Vldlr *or* Apoer 2 *mutants are remarkably less sensitive to noxious mechanical stimulation*

As we found a very significant increase in the mechanical nociceptive threshold in *reeler* mutants (Villeda *et al*., 2006), we next evaluated mechanical nociception in mice with deletions of other components of the Reelin–Dab1 signaling pathway (*Dab1*, *scrambler*, *Vldlr* and *Apoer 2*). Using von Frey monofilaments to test mechanical sensitivity, we found that the combined 50% threshold for *Dab1* wild-type and mutant mice was 0.4 g and 2.1 g, respectively (Fig. 3A). This illustrates a striking decrease in mechanical sensitivity in *Dab1* mutant mice ($P < 0.0001$). Mechanical nociceptive testing of *scrambler* mice also revealed significant increases in mechanical thresholds ($P = 0.0002$; Fig. 3B), comparable to that of the *Dab1* mutants. The combined average 50% threshold for male and female *scrambler* control and mutant mice was 0.5 g and 2.8 g, respectively (Fig. 3B). In contrast we report that the overall 50% threshold of *Vldlr* or *Apoer 2* single-knockout animals did not differ significantly from their wild-type controls (0.4 g for *Vldlr* mutant and 0.3 g for wild-type mice; $P= 0.34$; 0.26 g for *Apoer 2* mutant and 0.19 g for wild-type mice; $P = 0.24$; Fig. 3C and D). Thus, although *reeler*, *Dab1* and *scrambler* mutants are remarkably less sensitive to mechanical nociceptive stimulation, single lipoprotein mutants respond at wild-type levels.

Components of the Reelin–Dab1 signaling pathway in postnatal spinal cord

Reelin, but not Dab1, is highly expressed in the embryonic dorsal horn of wild-type mice, while Dab1, which is upregulated in the absence of Reelin, is readily detected in *reeler* mutants (Villeda *et al*., 2006), a pattern consistent with the importance of the Reelin–Dab1 signaling

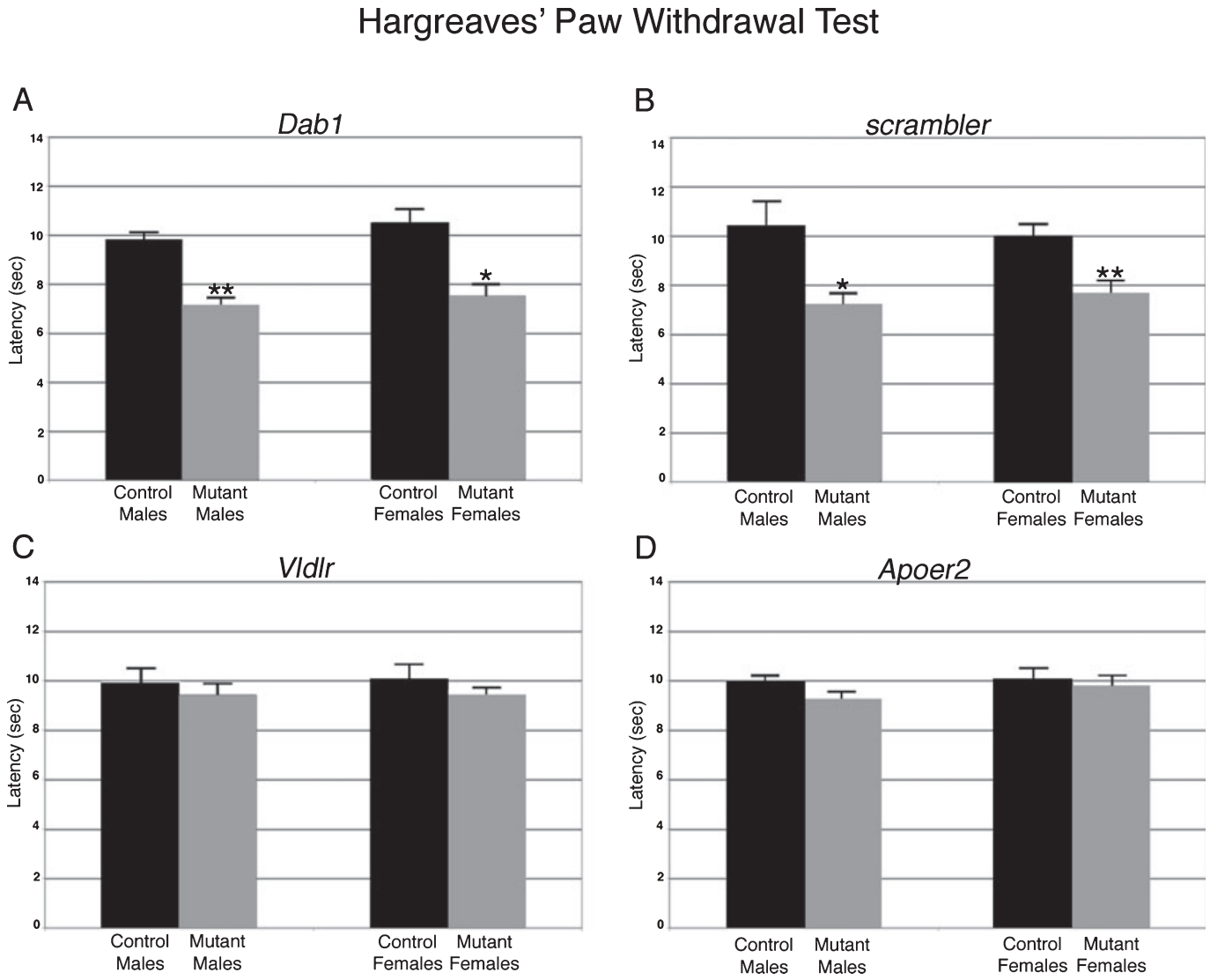

FIG. 2. Adult *Disabled-1* (*Dab1*) and *scrambler*, but not *very-low-density lipoprotein receptor* (*Vldlr*) or *apolipoprotein E receptor 2* (*Apoer2*) mutant mice display thermal hyperalgesia. (A) The average latency is 9.9 ± 0.27 s for wild-type males, 7.1 ± 0.28 s for *Dab1* mutant males, 10.5 ± 0.53 s for wild-type females and 7.5 ± 0.45 s for *Dab1* mutant females. Differences between the genotypes are significant for both males (**$P < 0.0001$, $n = 7$ pairs) and females (*$P = 0.001$, $n = 7$ pairs). (B) The average latency is 10.4 ± 0.96 s for control males, 7.2 ± 0.42 s for *scrambler* males, 10 ± 0.53 s for control females and 7.6 ± 0.46 s for *scrambler* females. Significant differences between the genotypes are found in male (*$P = 0.015$, $n = 5$ pairs) and female (**$P = 0.01$, $n = 5$ pairs) mice. (C) The average latency is 9.9 ± 0.65 s for wild-type males, 9.4 ± 0.47 s for *Vldlr* mutant males ($P = 0.531$, $n = 6$ pairs), 10.2 ± 0.63 s for wild-type females and 9.5 ± 0.33 s for *Vldlr* mutant females ($P = 0.327$, $n = 6$ pairs), differences that are not statistically significant. (D) *Apoer2* mutants and wild-type animals display similar withdrawal latencies. The average hindpaw withdrawal latency is 10 ± 0.21 s for wild-type males and 9.3 ± 0.33 s for *Apoer2* males ($P = 0.111$, $n = 6$ pairs), 10.1 ± 0.35 s for wild-type females and 9.8 ± 0.5 s for *Apoer2* mutant females ($P = 0.667$, $n = 5$ pairs).

pathway during development. Conceivably the 'pain' phenotypes observed in *reeler* mice are a manifestation of embryonic positioning errors. On the other hand, given the important contribution of NMDA-mediated processes to the development of persistent pain conditions, it is possible that changes in pain processing are a consequence of alterations in the Reelin signaling pathway that regulates synaptic plasticity in adults (Beffert *et al*., 2005). This is of particular importance as our sensory tests are, by necessity, performed on adult mice.

We addressed these questions in the next series of experiments. First, we asked whether the established components of the Reelin–Dab1 signaling pathway are present in adult superficial dorsal horn. Reelin is expressed in adult laminae I–II and V, and in the LSN in wild-type (Fig. 4A) mice, but not in *reeler* mutants (Fig. 4B). Because Dab1 is degraded in wild-type animals following Reelin signaling (Arnaud *et al*., 2003a), we used *reeler* spinal cords to assess Dab1 protein expression. Dab1 accumulates in neurons within laminae I–II, the deep dorsal horn and the LSN (Fig. 4D). Of the two lipoprotein receptors, Apoer 2 is strongly expressed in lamina II of control and *reeler* mice (Fig. 4E and F), but we were unable to detect Vldlr in the dorsal horn (data not shown) in either genotype despite its presence in sympathetic preganglionic neurons (unpublished data; Yip *et al*., 2004). Thus, the components of the Reelin–Dab1 signaling pathway are indeed found in the dorsal spinal cord of adult mice.

Deletion of Reelin or both of the lipoprotein receptors in embryonic mutant mice results in increased expression of Dab1 in cells that are

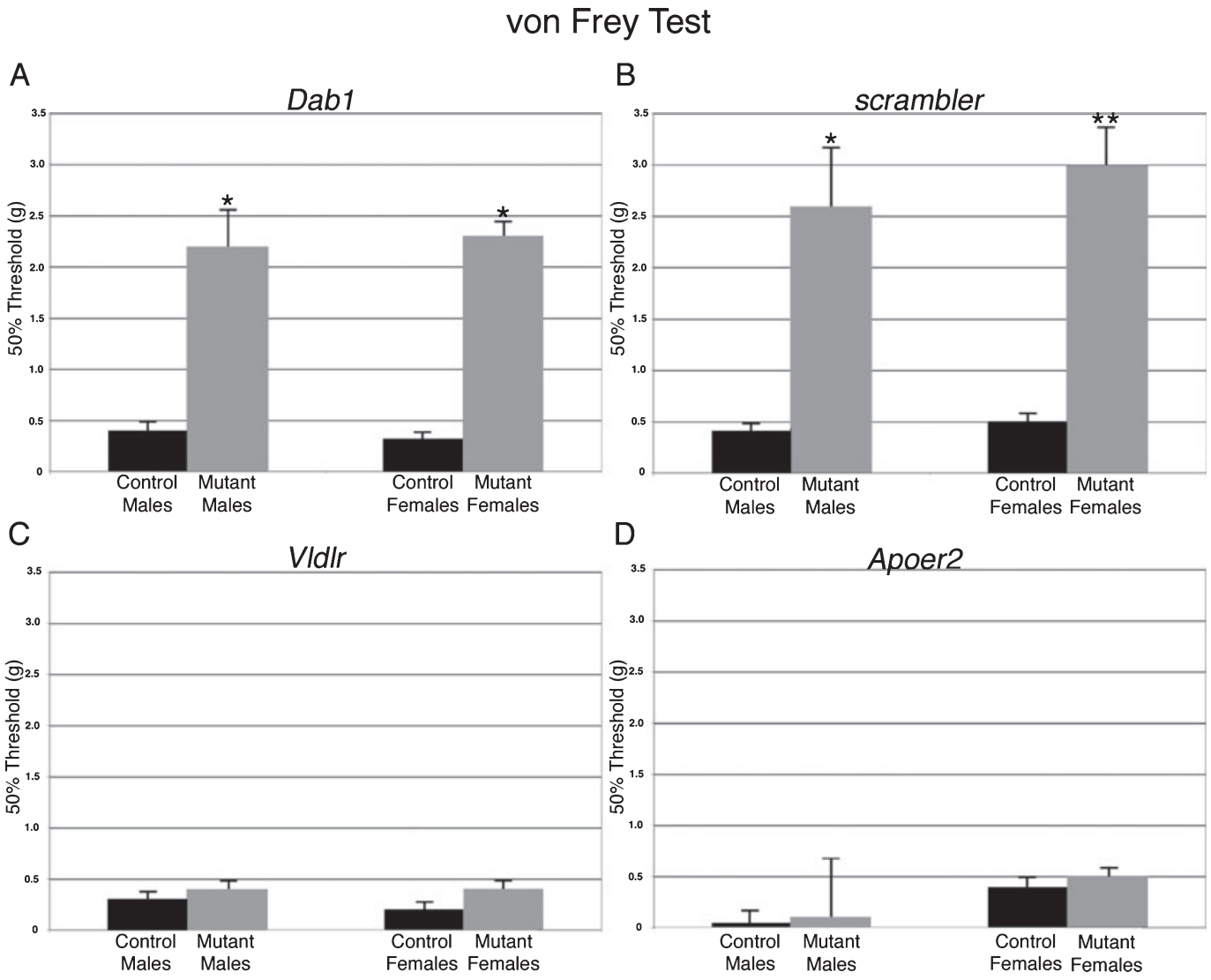

FIG. 3. Adult *Disabled-1* (*Dab1*) and *scrambler*, but not *very-low-density lipoprotein receptor* (*Vldlr*) or *apolipoprotein E receptor 2* (*Apoer2*) mutants are less sensitive to noxious mechanical stimuli. (A) The 50% threshold is 0.4 ± 0.09 g for wild-type males, 2.2 ± 0.41 g for *Dab1* mutant males, 0.3 ± 0.08 g for wild-type females and 2.3 ± 0.15 g for *Dab1* mutant female mice. Significant differences between genotypes are detected in males (*$P = 0.009$, $n = 7$ pairs) and females (*$P = 0.009$, $n = 7$ pairs). (B) The 50% threshold is 0.4 ± 0.09 g for control males, 2.6 ± 0.62 g for *scrambler* males, 0.5 ± 0.08 g for control females and 3.0 ± 0.44 g for *scrambler* females. Differences between the genotypes are statistically significant for both males (*$P = 0.006$, $n = 5$ pairs) and females (**$P = 0.002$, $n = 5$ pairs). (C) The 50% threshold is 0.3 ± 0.07 g for wild-type males, 0.4 ± 0.13 g for *Vldlr* mutant males ($P = 0.81$, $n = 6$ pairs), 0.2 ± 0.08 g for wild-type females and 0.4 ± 0.1 g for *Vldlr* mutant females ($P = 0.26$, $n = 6$ pairs). (D) Control and *Apoer2* mutant males have 50% thresholds of 0.04 ± 0.01 g and 0.1 ± 0.04 g, respectively ($P = 0.20$, $n = 6$ pairs). *Apoer2* wild-type and mutant females have 50% thresholds of 0.4 ± 0.14 g and 0.5 ± 0.1 g, respectively ($P = 0.40$, $n = 5$ pairs).

incorrectly positioned (Rice *et al*., 1998; Beffert *et al*., 2006a). We reasoned that if elevated levels of Dab1 persist in adult *reeler* or 2–3-week-old *Apoer2/Vldlr* double-knockout spinal cords, then the incorrectly positioned Dab1-labeled cells might contribute to the nociceptive defects detected in adults. We found occasional Dab1-labeled neurons in laminae I, II and V, and in the LSN of the adult *reeler* dorsal horn (arrows and arrowhead, Fig. 5B). Surprisingly, wild-type mice also contained Dab1-positive cells in lamina V and in the LSN (arrowhead, Fig. 5A). Although Dab1 is absent in *Dab1* mutant dorsal horn (Fig. 5D), adult wild-type mice have Dab1-immunoreactive cells in the LSN and medial dorsolateral funiculus (Fig. 5C, inset).

Trommsdorff *et al*. (1999) reported that Dab1 protein levels are increased 13-fold in *Apoer2/Vldlr* double-knockout mice. In addition, they reported that Apoer2 has a higher affinity for Reelin and is distributed more ubiquitously in the brain than is Vldlr (Trommsdorff *et al*., 1999). We evaluated the pattern of Dab1 expression in the dorsal horn of both single- and double-receptor mutants, and found Dab1-labeled neurons in comparable positions within the LSN and lamina V of 2–3-week-old *Apoer2/Vldlr* (Fig. 5F), adult *Vldlr* (data not shown) and adult *Apoer2* (Fig. 5H) mutants and their wild-type controls (Fig. 5E and G). Dab1 staining is detected in laminae I–II and V of *Apoer2/Vldlr* double mutant spinal cords (arrows, Fig. 5F). Thus, Dab1 accumulates in restricted neuronal populations within laminae I–II and V of embryonic and early postnatal *reeler* and *Apoer2/Vldlr* mutants (Villeda *et al*., 2006). However, in the adult there is continued expression of Dab1 in neurons of lamina V and LSN in both control and mutant mice.

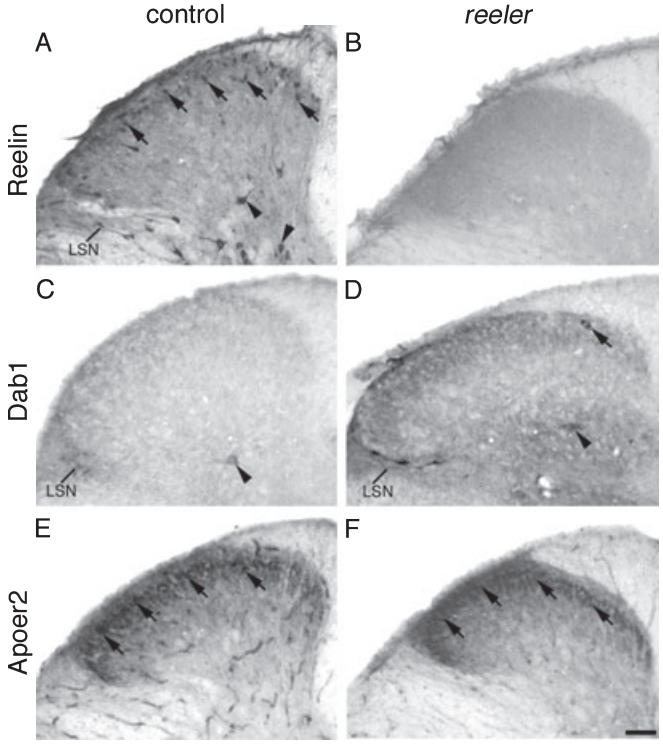

FIG. 4. Components of the Reelin signaling pathway are present in postnatal thoracic dorsal horn. (A and B) Reelin-labeled neurons are detected in laminae I–II (arrows), V (arrowheads) and in the lateral spinal nucleus (LSN) of P30 wild-type mice (A). Reelin is not detected in sections from *reeler* mutants (B). (C and D) Disabled-1 (Dab1) accumulates within neurons at the border between laminae I–II (arrow) of *reeler* mutants (D), and in lamina V (arrowhead) and the LSN of both control (C) and *reeler* spinal cords (D). (E and F) Apolipoprotein E receptor 2 (Apoer2) is most concentrated in lamina II (arrows) of wild-type (E) and *reeler* (F) mice. Scale bar: 50 μm (A–F).

Positioning errors occur in the dorsal horn of Dab1, scrambler *and* Apoer2/Vldlr *knockout mice, but not in* Vldlr *or* Apoer2 *single mutants*

The *reeler* mice display positioning errors in the superficial dorsal horn and in the LSN (Villeda *et al*., 2006), and Dab1 accumulates in neurons within these regions (Fig. 5B), even in adult mice. To investigate if similar positioning errors occur in other mutant mice of the Reelin–Dab1 signaling pathway, we examined the distribution of neurons in the superficial dorsal horn and LSN of adult *Dab1–/–*, *scrambler*, *Vldlr–/–*, *Apoer 2–/–* single and 2–3-week-old *Apoer 2/Vldlr* double mutants, and their respective controls. To assess the neuronal distribution patterns, we used NeuN immunocytochemistry, which selectively identifies neuronal cell bodies (Fig. 6A–F). In *Dab1*, *scrambler* and *Apoer 2/Vldlr* double-knockout mice we observed a neuronal compaction in lamina I and a neuron-sparse region in lamina II (Fig. 6B, C and F), just as we found in *reeler* mutants (Fig. 6D). However, in *Vldlr* and *Apoer 2* single mutants, neurons in the superficial dorsal horn are evenly distributed (Fig. 6E, data not shown), as they are in wild-type mice (Fig. 6A). Finally, to determine if neurochemically distinct subsets of interneurons of laminae I–II are affected by the above mutations, we used specific markers of several subsets, including calbindin, calretinin and parvalbumin (Ren & Ruda, 1994). That analysis did not reveal any apparent changes that were specific to subpopulations of interneurons in *Dab1* or *scrambler* mutant mice (data not shown).

Given the reduction in the number of LSN neurons in the *reeler* mice (Villeda *et al*., 2006), we quantified neurons in the LSN of mutants and controls from each of the four mouse lines and found a 40–50% reduction in the number of LSN neurons in *Dab1* and *scrambler* mutants compared with their controls (Fig. 6A–C; Table 1). In contrast, the number of LSN neurons in *Vldlr* and *Apoer 2* mutant spinal cords does not differ from those of wild-type mice (Fig. 6E; Table 1).

Substance P-containing primary afferents terminate on neurons bearing the NK-1 receptor (Brown *et al*., 1995) and together are important contributors to nociceptive processing (Cao *et al*., 1998; De Felipe *et al*., 1998). Previously we found Dab1-positive cells in laminae I–II of *reeler* mutants surrounded by NK-1-immunoreactivity, which we presume represents postsynaptic receptors (Villeda *et al*., 2006). As this association suggests that the positioning errors occur in elements of the 'pain' pathway, we next asked whether mice with deletions in the other elements of the Reelin–Dab1 signaling pathway have comparable aberrantly positioned neurons. Because Dab1 protein is undetectable in *Dab1* and *scrambler* mutants, we could only identify NK-1-positive neurons that are incorrectly located in lamina II or at the laminae II–III border in *Dab1* (arrow, Fig. 6H) and *scrambler* mice (arrows, Fig. 6I). These NK-1 receptor-immunoreactive lamina II neurons, which are not detected in wild-type mice, likely represent neuronal positioning errors similar to those in *reeler* mutants (arrow, Fig. 6J; Villeda *et al*., 2006). On the other hand we never found NK-1-expressing cells in a comparable position in the *Vldlr* (data not shown) or *Apoer 2* (Fig. 6K) mutants, or in their controls (data not shown). Figure 6K illustrates a NK-1-expressing neuron in lamina III of an *Apoer 2–/–* dorsal horn in a region that also contains such neurons in wild-type mice (Brown *et al*., 1995). Thus, in the single lipoprotein receptor mice the NK-1 receptor immunoreactivity is concentrated primarily in lamina I and in the LSN (Fig. 6K, data not shown).

Finally, we asked if incorrectly positioned NK-1-expressing neurons are detected in dorsal horns of *Apoer 2/Vldlr* double-knockout mice as reported in *reeler* mutants (Villeda *et al*., 2006). NK-1 receptor-immunoreactive neurons are found in lamina II (Fig. 6L) and at the laminae II–III border (Fig. 7B) in *Apoer 2/Vldlr* double-knockout mice. Furthermore, double-labeling experiments illustrate Dab1-immunoreactive neurons in laminae I–II and lamina V, and these mispositioned cells also bear the NK-1 receptors in double-receptor knockout mice (Fig. 7A–I). Specificity in these double-labeling experiments is revealed by a Dab1-positive soma in lamina V (small arrowhead, Fig. 7D) that does not express NK-1 receptors (Fig. 7E and F, small arrowhead) and a NK-1 receptor-immunoreactive neuron (Fig. 7E, large arrowhead) that does not contain Dab1 (Fig. 7F, large arrowhead).

Primary afferent nociceptors are normally distributed in dorsal horn laminae in all genotypes

Because of the profound impairments in nociceptive processing that we recorded in *Dab1* and *scrambler* mutants, we also examined the termination pattern of the small diameter primary afferents in the superficial dorsal horn in mice with various deletions of the Reelin–Dab1 signaling pathway. We found no significant differences in the distribution pattern of the non-peptidergic, IB4 (Supplementary material, Fig. 1G–L) or the peptidergic, CGRP (supplementary Fig. 1M–R) populations of C-fibers in any of the mice examined. We also found a comparable and normal distribution of the subset of nociceptors identified by immunostaining for the heat- and

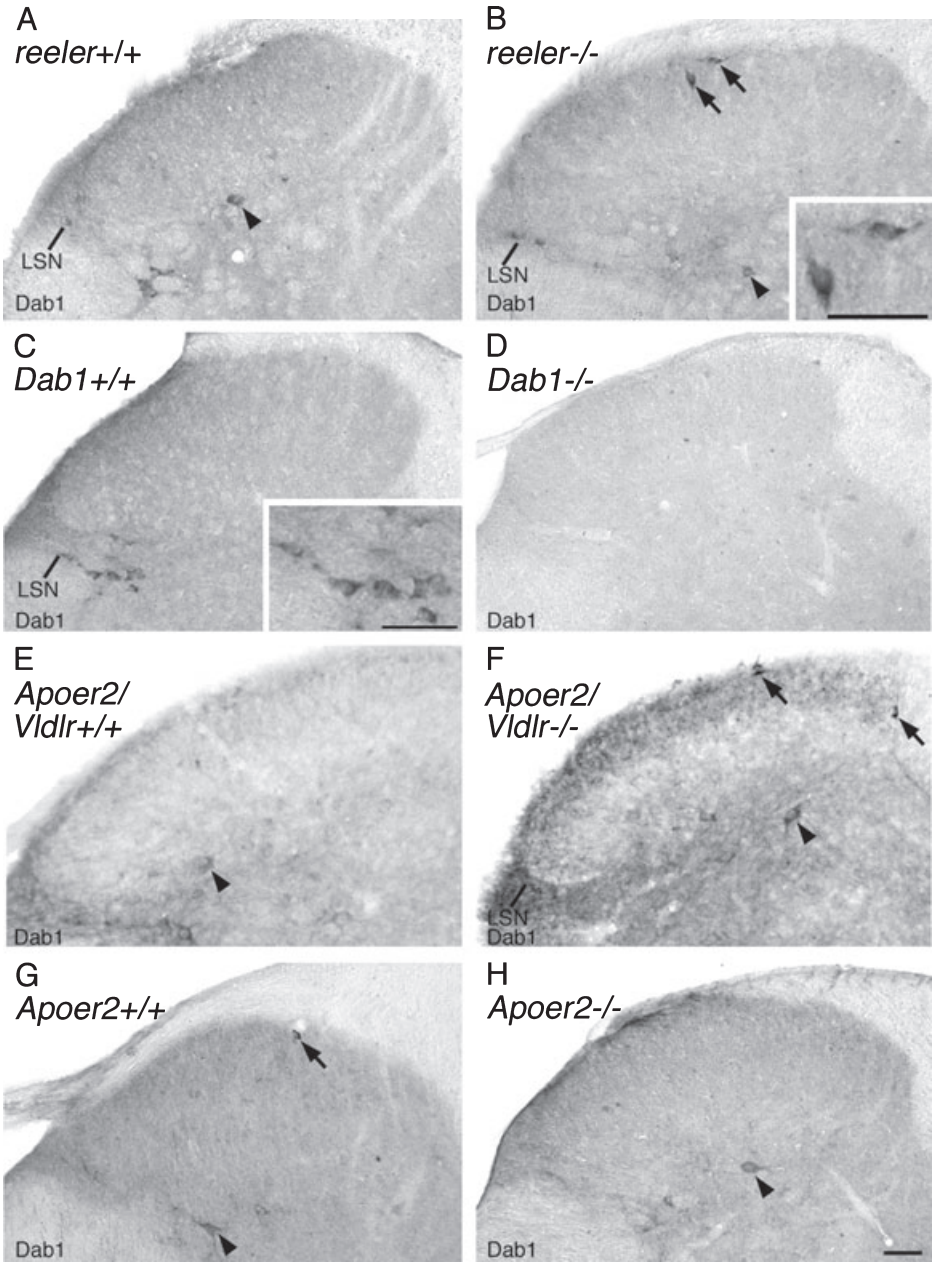

FIG. 5. Expression of Disabled-1 (Dab1) in lumbar dorsal horn of wild-type (A, C, E, G) and *reeler* (B), *Dab1* (D), *apolipoprotein E receptor 2* (*Apoer2*)/*very-low-density lipoprotein receptor* (*Vldlr*) double (F) and *Apoer2* (H) mutant mice. (A and B) Dab1-positive neurons are detected in laminae I–II of adult *reeler* (arrows, B; inset) and occasionally in the deep dorsal horn (arrowhead) and lateral spinal nucleus (LSN) of both wild-type (A) and *reeler* (B) mice. (C and D) Dab1-immunopositive neurons occupy the LSN and the medial dorsolateral funiculus (C; inset) of adult wild-type mice, while the *Dab1* null (D) is devoid of immunoreactivity. (E and F) Dab1 staining and immunoreactive somata (arrowheads) are localized in lamina I–II and in the deep dorsal horn of a postnatal day 14 (P14) *Apoer2*/*Vldlr* double mutant (F). Dab1-labeled neurons are also detected in the deep dorsal horn of a P14 wild-type mouse (arrowhead, E). (G and H) A Dab1-positive neuron is found in adult wild-type lamina I (arrow, G), and occasional labeled neurons are seen in lamina V of wild-type (G) and *Apoer2* mutant (H) mice. Scale bar: 50 μm (A–H); 50 μm (insets).

capsaicin-sensitive TRPV1 channel (supplementary Fig. 1A–F). These afferents terminated in lamina I and occasionally in medial lamina II in the lower segmental levels of lumbar dorsal horn of *Dab1*, *scrambler*, *reeler*, *Vldlr*, *Apoer 2* and *Apoer 2*/*Vldlr* double mutants and their controls (supplementary Fig. 1A–F). We conclude that the pattern of the central projection of primary afferent nociceptors is not altered in mice that contain any of the host of mutations in different parts of the Reelin–Dab1 signaling pathway. Furthermore, the cell size and distribution pattern of the peptidergic and non-peptidergic nociceptors in the DRG appeared similar in *Dab1*, *Vldlr* and *Apoer 2* animals (data not shown).

Reelin-dependent nociceptive processing is independent of Apoer 2–exon 19, but does require Dab1 binding

Beffert *et al*. (2005) reported that Reelin signals through Apoer 2 via two distinct intracellular pathways: the Dab1 pathway involves neuronal positioning during development, but signaling via the

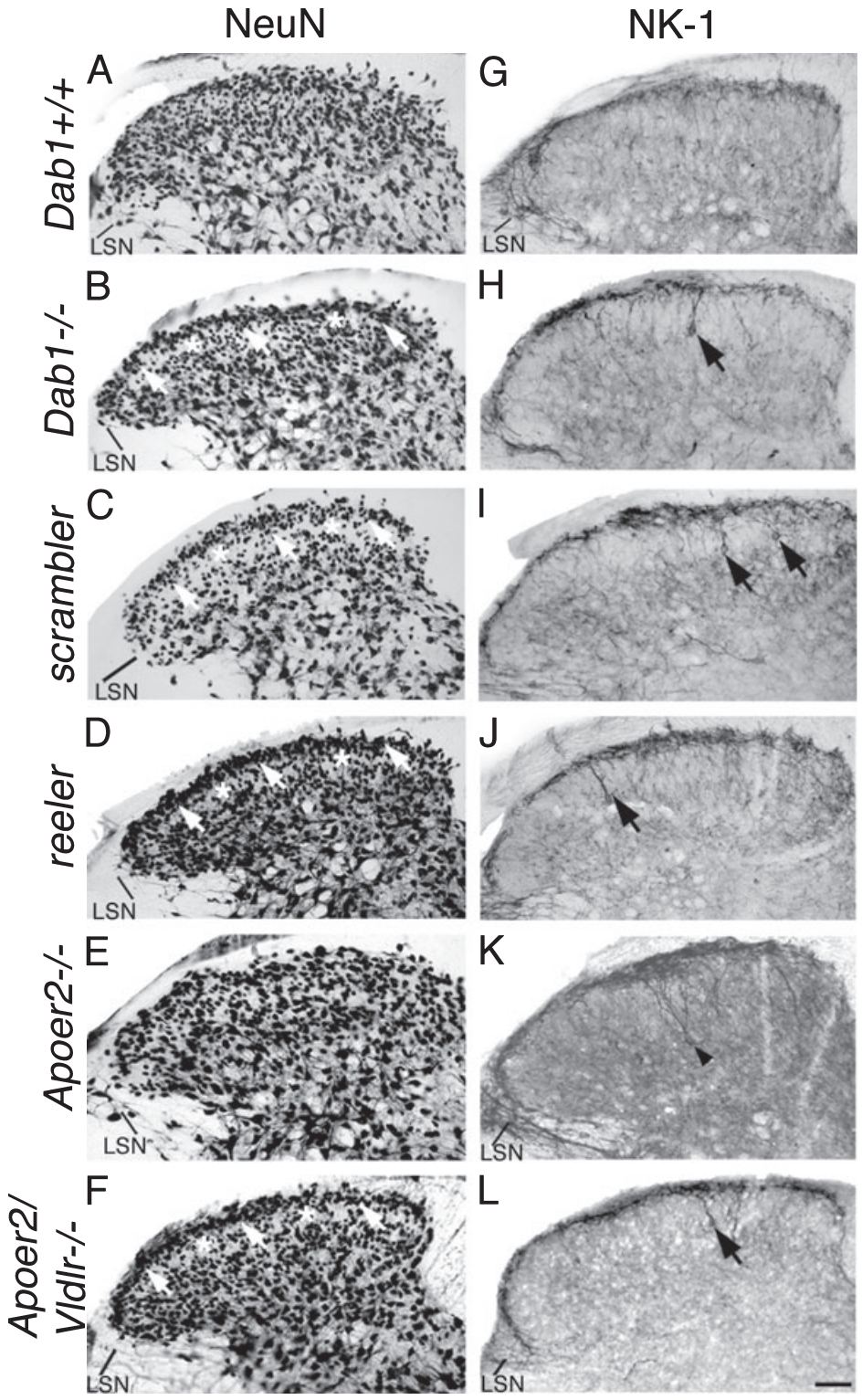

FIG. 6. Positioning errors exist in lumbar superficial dorsal horn of adult *Disabled-1* (*Dab1*) (B, H), *scrambler* (C, I), *reeler* (D, J) and postnatal P14 *apolipoprotein E receptor 2* (*Apoer2*)/*very-low-density lipoprotein receptor* (*Vldlr*) double (F, L) mutants, but not in *Apoer2* (E, K) single mutants. (A–F) Neuronal-specific protein (NeuN)-labeled neurons are distributed evenly in the superficial dorsal horn of wild-type (A) and *Apoer2–/–*(E) mice, but are compacted in lamina I of *Dab1* (white arrows, B), *scrambler* (white arrows, C), *reeler* (white arrows, D) and *Apoer2/Vldlr* double (white arrows, F) mutants. Lamina II contains neuron-sparse regions in *Dab1*, *scrambler*, *reeler* and *Apoer2/Vldlr* double mutants (white asterisks, B–D, F), but not in the wild-type (A) or *Apoer2* single mutant (E) dorsal horn. Fewer lateral spinal nucleus (LSN) neurons exist in mutants (B–D, F) than in wild-type (A) or *Apoer2–/–* (E) mice. (G–L) Expression of the neurokinin-1 (NK-1) receptor is concentrated in lamina I and the LSN of all genotypes (G–L), and NK-1-positive cells are occasionally detected in lamina III (arrowhead, K). NK-1-expressing neurons (arrows) are incorrectly positioned in lamina II or at the border of laminae II–III in *Dab1* (H), *scrambler* (I), *reeler* (J) and *Apoer2/Vldlr–/–* (L) dorsal horns. Scale bar: 50 μm (A–L).

TABLE 1. Lateral spinal nucleus neurons are reduced in *Dab1* and *scrambler*, but not in *Vldlr* or *Apoer2* mutant mice.

Animal and level	Cell Count	*P*-value*
Wild-type		
Cervical	7.4 ± 0.4	
Thoracic	6.5 ± 0.3	
Lumbar	7.9 ± 0.1	
Dab1–/–		
Cervical	3.5 ± 0.4†	0.002
Thoracic	2.5 ± 0.4†	0.001
Lumbar	3.3 ± 0.2†	<0.0001
Control		
Cervical	8.7 ± 0.9	
Thoracic	5.8 ± 0.4	
Lumbar	8.9 ± 0.6	
Scrambler		
Cervical	4.1 ± 0.1†	0.007
Thoracic	3.4 ± 0.1†	0.005
Lumbar	4.8 ± 0.4†	0.004
Wild-type		
Cervical	10.6 ± 0.9	
Thoracic	8.6 ± 0.3	
Lumbar	11.2 ± 0.2	
Vldlr–/–		
Cervical	9.4 ± 0.4	0.29
Thoracic	8.6 ± 0.3	0.96
Lumbar	9.3 ± 0.7	0.055
Wild-type		
Cervical	8.1 ± 1.0	
Thoracic	6.7 ± 1.1	
Lumbar	8.6 ± 1.7	
Apoer 2–/–		
Cervical	6.9 ± 0.3	0.12
Thoracic	5.3 ± 0.2	0.09
Lumbar	7.7 ± 0.9	0.49

Data (mean ± SEM) expressed per LSN from 3 pairs of control and mutant mice. *Compared with associated control/wild-type group. †Significantly different from control/wild type.

Apoer 2–exon 19 pathway influences learning and memory in adult animals (Fig. 1). To determine which signaling pathway is responsible for the profound changes in acute thermal and mechanical nociception that we observed in adult *reeler* mice, we repeated these analyses in several *Apoer 2* cytoplasmic tail mutants bred on a *Vldlr*-deficient background (*Apoer 2 ex19/Vldlr–/–* serve as a constitutively active knockin control, *Apoer 2* Δ*ex19/Vldlr–/–* and *Apoer 2 Stop/Vldlr–/–* are exon 19 mutants; Beffert *et al*., 2005, 2006b).

First we tested *Apoer 2 ex19/Vldlr–/–* and *Apoer 2* Δ*ex19/Vldlr–/–* that lack *Vldlr* and either constitutively express (ex19 control) or lack the alternatively spliced exon 19 (Δex19; Beffert *et al*., 2005). Then we compared the sensory testing results of control mice (*Apoer 2 ex19/Vldlr–/–*) with the *Apoer 2 Stop/Vldlr–/–* animals that contain a truncation between exon 18 and exon 19. Figure 8A shows that hindpaw withdrawal latencies in response to thermal stimulation are, in fact, similar in the three lines of mice (average latency of control *Apoer 2 ex19/Vldlr–/–* males is 9.3 s, compared with 8.0 s for *Apoer 2* Δ*ex19/Vldlr–/–* and 9.3 s for *Apoer 2 Stop/Vldlr–/–* males; $P = 0.09$ and $P = 0.87$, respectively). In addition, we found no differences when we tested mechanical sensitivity; the 50% thresholds among the three lines of mice were comparable (0.4 g for *Apoer 2 ex19/Vldlr–/–*, 0.4 g for *Apoer 2* Δ*ex19/Vldlr–/–* and 0.6 g for *Apoer 2 Stop/Vldlr–/–*; $P = 0.75$ and $P = 0.34$, respectively; Fig. 8B). These findings demonstrate that the exon 19 splice variant of Apoer 2 does not underlie the profound thermal and mechanical deficits of acute 'pain' processing that we observed in the *reeler* mice.

Neither Reelin signaling pathway is required for tissue injury-induced persistent pain

Although the exon 19 splice variant does not contribute to acute 'pain' processing, we next tested these mice in the setting of injury, where deficits associated with the exon 19 splice variant might be more pronounced. For example, when there is hindpaw inflammation, animals will withdraw the paw in response to normally innocuous stimuli. This drop in withdrawal threshold, called allodynia, results

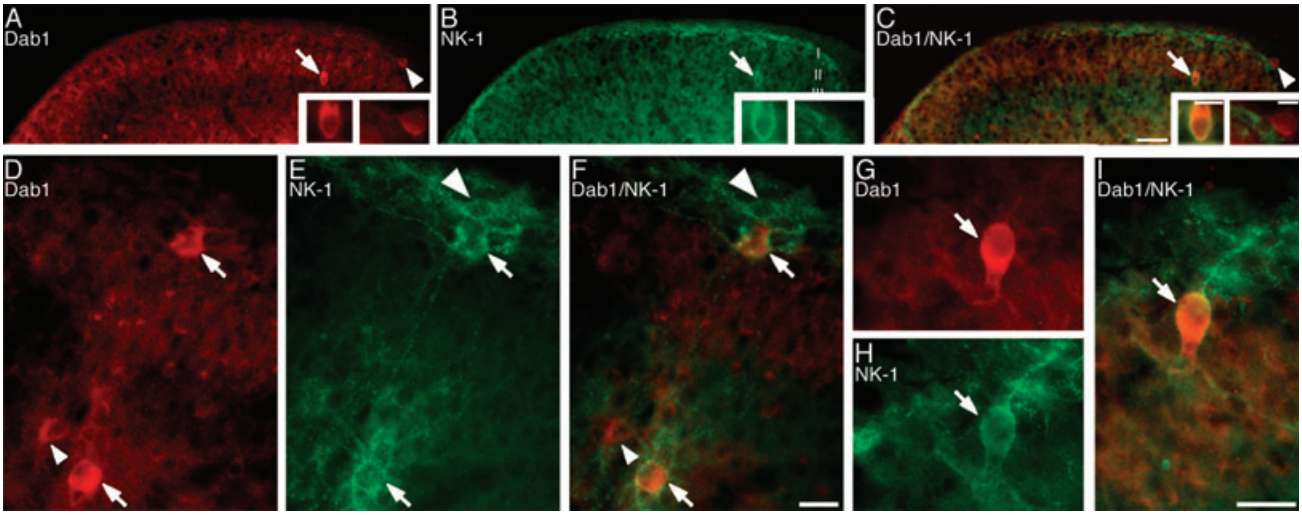

FIG. 7. Disabled-1 (Dab1)-positive neurons in postnatal P14 *Apoer2/Vldlr* double-knockout dorsal horn also express the Neurokinin-1 (NK-1) receptor. (A–C) A neuron on the border between laminae II and III is double-labeled with Dab1 (arrows, A, C, left insets) and NK-1 (arrows, B, C, left insets) antibodies. An incorrectly positioned Dab1-positive cell in lamina I (small arrowheads, A, C) is adjacent to an NK-1-labeled cell (B, C, right insets). (D–F) Dab1-immunoreactive neurons in laminae I and V (arrows, D, F) also express the NK-1 receptor (arrows, E, F). A neuron that only expresses Dab1 (small arrowheads, D, F) is detected in lamina V, and a single-labeled cell bearing NK-1 receptors (large arrowheads, E, F) is found in lamina I. (G–I) A neuron expressing both Dab1 (arrows, G, I) and NK-1 (arrows, H, I) is detected at the border between laminae I and II. Scale bar: 50 μm (A–C); 12 μm (insets A–C); 25 μm (D–F, G–I).

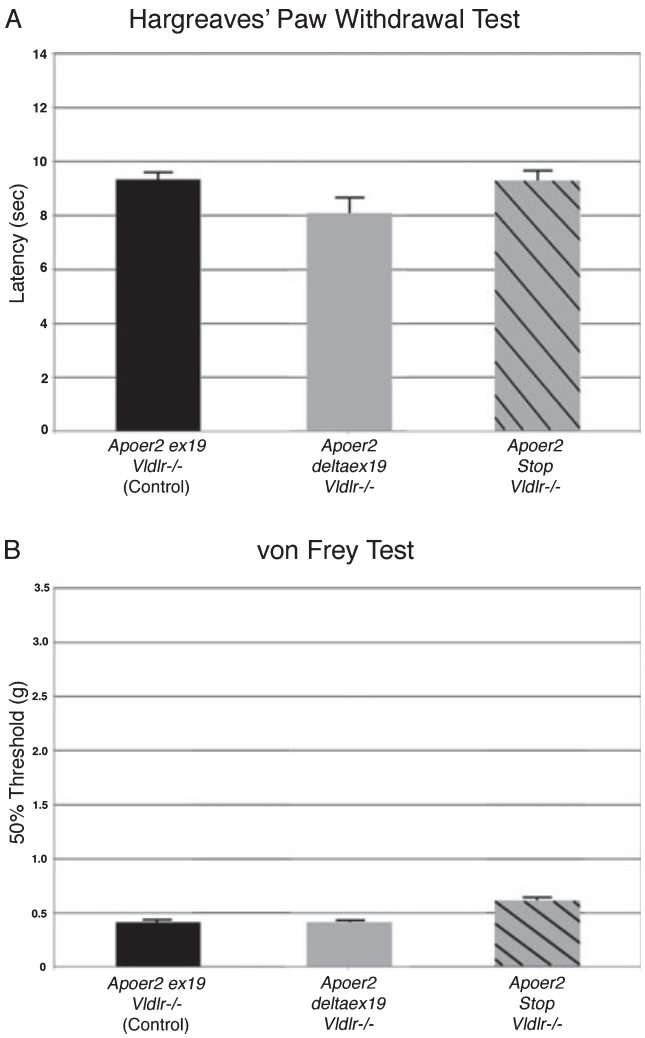

FIG. 8. Thermal and mechanical pain thresholds are normal in adult *apolipoprotein E receptor 2* (*Apoer2*) *exon 19* mutants on a *very-low-density lipoprotein receptor* (*Vldlr*)-deficient background. (A) The average hindpaw withdrawal latency is 9.3 ± 0.32 s for *Apoer2 ex19/Vldlr–/–*, 8.0 ± 0.62 s for *Apoer2* Δ*ex19/Vldlr–/–* and 9.3 ± 0.44 s for *Apoer2 Stop/Vldlr–/–* ($P = 0.09$; $P = 0.87$, $n = 6$ mice/genotype). (B) 50% threshold for *Apoer2 ex19/Vldlr–/–* is 0.4 ± 0.03 g, for *Apoer2* Δ*ex19/Vldlr–/–* is 0.4 ± 0.07 g and for *Apoer2 Stop/Vldlr–/–* is 0.6 ± 0.13 g ($P = 0.75$; $P = 0.34$, $n = 6$ mice/genotype).

both from a prostaglandin-mediated peripheral sensitization of nociceptor terminals and from a central sensitization process through which innocuous inputs carried by non-nociceptive primary afferents can access 'pain transmission' circuits, and through which the signals transmitted by nociceptors are amplified.

Central sensitization involves plastic changes in the dorsal horn that are comparable to the synaptic plasticity that occur in the hippocampus and that are altered in mice with the exon 19 splice variant of Apoer 2 (Beffert *et al*., 2005). Therefore, to test the hypothesis that central sensitization is also altered in these mice, we injected carrageenan into the hindpaw of *Apoer 2 ex19/Vldlr–/–* control and *Apoer 2* Δ*ex19/Vldlr–/–* mutant mice and monitored their withdrawal thresholds over time. Figure 9C and D illustrates that carrageenan indeed produced a substantial drop in both thermal and mechanical withdrawal threshold in these mice. Thus, the presence of the exon 19 splice variant did not prevent the sensitization from occurring (Fig. 9C and D). Neither the magnitude nor the duration of the thermal and mechanical allodynia are altered in mice that constitutively express or lack exon 19.

Somewhat surprisingly, perhaps, even in the *reeler* animals, in which baseline acute nociceptive processing is altered, we found that dramatic and prolonged thermal and mechanical allodynia still developed after carrageenan-induced tissue injury (Fig. 9A and B), and it did so to the same extent as in controls. This result illustrates that central sensitization is not influenced by either of the two established Reelin signaling pathways.

Embryonic rat spinal cord slices treated with PP2 recapitulate the migratory errors observed in the reeler dorsal horn

To directly test the hypothesis that the *reeler* dorsal horn positioning errors result from the loss of the Reelin–Dab1 signaling pathway, we used an organotypic slice preparation to ask if we could reproduce the migratory errors by blocking the Reelin–Dab1 pathway with PP2 (Arnaud *et al*., 2003b; Bock & Herz, 2003; Jossin *et al*., 2003). As a selective inhibitor of Src family kinases, PP2 added to the culture medium blocks Dab1 phosphorylation and thus the downstream action of Reelin. We previously reported that the migration errors in *reeler* dorsal horn occurred between E12.5 and 14.5 (Villeda *et al*., 2006), and that the pattern of accumulated Dab1 was evident in *reeler* laminae I, II and V (Fig. 10A). We prepared E13 slices of rat spinal cord, the developmental equivalent of an E11.5 wild-type mouse spinal cord, and grew them in defined media with either PP2 (15 μM) or the inactive compound (PP3, 15 μM) for 3 days *in vitro* (E13 + 3). As expected there is no Dab1 in E13 + 3 control slices treated with PP3 (Fig. 10B). However, Fig. 10C and D illustrate that substantial levels of Dab1 are detected in laminae I, II and V in slices treated with PP2. Furthermore, distinct Dab1-labeled neurons (arrows, Fig. 10C and D) are found in the E13 + 3 superficial dorsal horn. Thus, we can recapitulate the positioning errors seen in the superficial dorsal horn of *reeler* mice by inhibiting the Src family of kinases in wild-type rat spinal cord slices.

Discussion

Originally we identified a significant reduction in mechanical sensitivity and increased thermal sensitivity in *reeler* mice (Villeda *et al*., 2006), and now we show the same deficits in nociceptive processing when the intracellular adaptor protein of the Reelin signaling pathway is mutated in *Dab1* and *scrambler* mice. This unusual combination of sensory deficits, both hypo- and hypersensitivity to different painful stimuli, strongly implicates the involvement of the Reelin–Dab1 signaling pathway in processing diverse nociceptive messages. In contrast, sensory tests on single lipoprotein receptor mutants revealed normal behavior. The fact that the deficits occurred across different sensory modalities, the inputs of which are transmitted via distinct populations of primary afferents, implicates the circuitry in the dorsal horn as the locus of the deficit. Consistent with this hypothesis, we found no changes in the distribution of the major classes of nociceptors in the DRGs of *Dab1*, *Vldlr* and *Apoer 2* animals. On the other hand, we showed that positioning errors characteristic of the superficial dorsal horn and LSN of *reeler* mutants also exist in *Dab1*, *scrambler* and *Apoer 2/Vldlr* double mutants, but not in the *Vldlr* or *Apoer 2* single mutant mice. Somewhat surprisingly perhaps, we found that mice with mutations in the cytoplasmic tail of the *Apoer 2* (exon 19) that lack *Vldlr* respond to thermal and mechanical nociceptive tests as do control mice, consistent with the interpretation that the

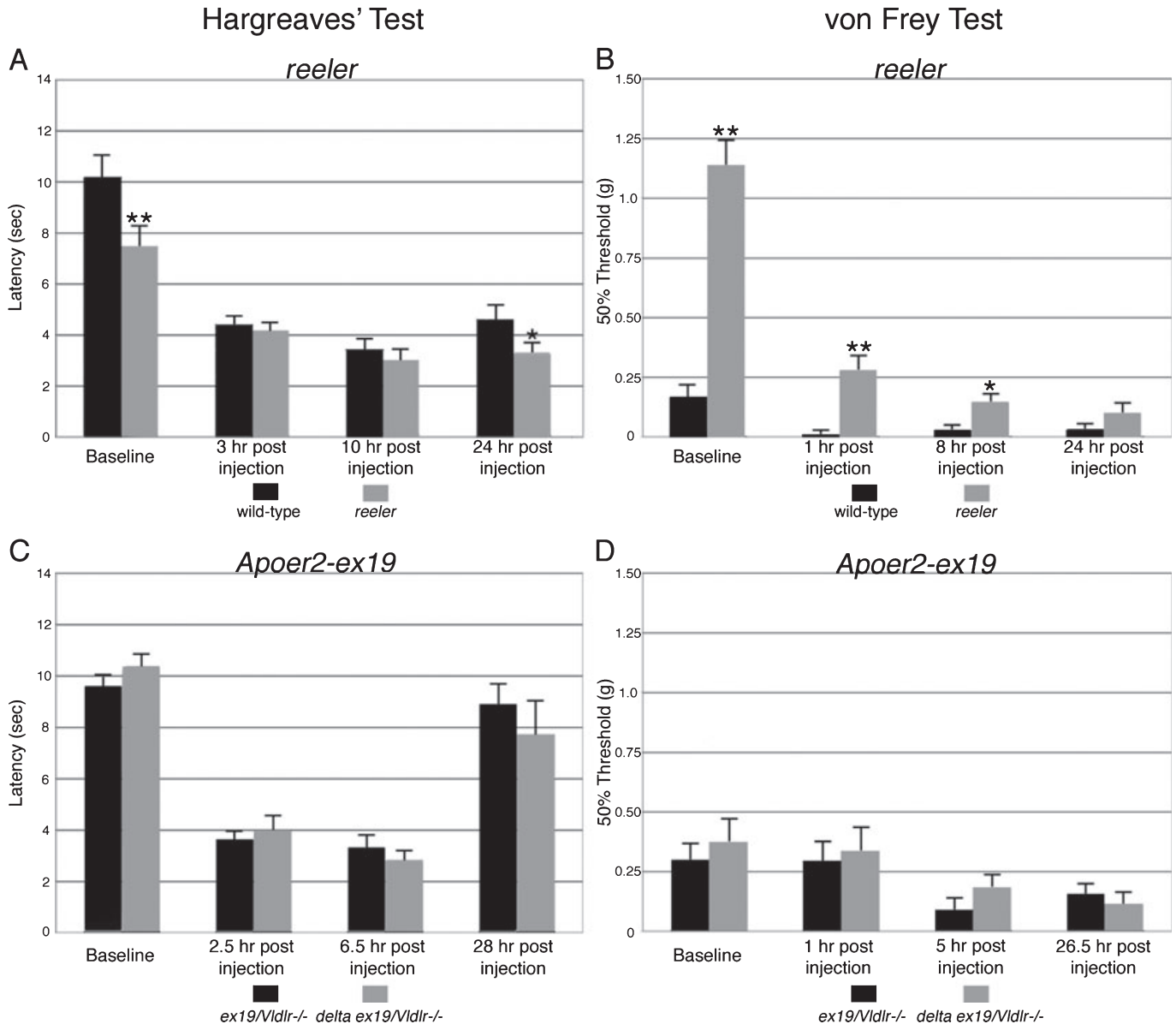

FIG. 9. Hindpaw inflammation produces prolonged thermal and mechanical hypersensitivity in adult *reeler* and *apolipoprotein E receptor 2* (*Apoer2*) Δ*ex19/very-low-density lipoprotein receptor* (*Vldlr*) mutant mice. (A) Both *reeler* and wild-type mice ($n = 6$ pairs) sensitize to thermal stimulation after carrageenan injection. The average baseline, 3 h, 10 h and 24 h post-injection latencies are 10.3 ± 0.84 s, 4.4 ± 0.27 s, 3.5 ± 0.33 s and 4.7 ± 0.51 s for wild-type, and 7.6 ± 0.76 s, 4.2 ± 0.26 s, 3 ± 0.38 s and 3.3 ± 0.32 s for *reeler* mutants, respectively. Significant differences between genotypes are detected at baseline (**$P = 0.002$) and 24 h after injection (*$P = 0.047$). (B) The 50% thresholds of *reeler* and wild-type controls ($n = 6$ pairs) are profoundly reduced for 24 h after injection of carrageenan. The 50% thresholds at baseline, 1 h, 8 h and 24 h after injection in wild-type mouse hindpaws are 0.17 ± 0.05 g, 0.02 ± 0.01 g, 0.03 ± 0.01 g and 0.04 ± 0.01 g, respectively, and for *reeler* mutants are 1.16 ± 0.1 g, 0.29 ± 0.05 g, 0.15 ± 0.03 g and 0.11 ± 0.03 g, respectively. Significant differences between genotypes are seen at baseline (**$P = 0.004$), 1 h (**$P = 0.004$) and 8 h (*$P = 0.01$) post-injection. (C) The *Apoer2* Δ*ex19/Vldlr* mutants and their controls ($n = 5$ pairs) both sensitize to thermal stimulation after carrageenan injection. The average baseline, 2.5 h, 6.5 h and 28 h post-injection latencies are 9.56 ± 0.33 s, 3.59 ± 0.27 s, 3.27 ± 0.43 s and 8.85 ± 0.75 s for *Apoer2 ex19/Vldlr–/–*, and 10.32 ± 0.46 s, 3.93 ± 0.52 s, 2.75 ± 0.36 s and 7.67 ± 1.35 s for *Apoer2* Δ*ex19/Vldlr–/–* mutants, respectively. There are no differences between genotypes. (D) The *Apoer2 ex19/Vldlr–/–* and *Apoer2* Δ*ex19/Vldlr–/–* mutant mice ($n = 5$ pairs) show a reduction in the 50% threshold by 5 h after carrageenan injection. The 50% thresholds at baseline, 1 h, 5 h and 26.5 h after injection of *Apoer2 ex19/Vldlr–/–* mice hindpaws are 0.3 ± 0.06 g, 0.29 ± 0.08 g, 0.09 ± 0.04 g and 0.15 ± 0.04 g, respectively, and for *Apoer2* Δ*ex19/Vldlr–/–* mutants are 0.37 ± 0.09 g, 0.34 ± 0.09 g, 0.18 ± 0.05 g and 0.11 ± 0.04 g, respectively. No significant differences are found between genotypes.

sensory defects result from a loss of the Reelin–Dab1 signaling pathway rather than the Apoer2–exon 19 pathway. Finally, by blocking the phosphorylation of Dab1 *in vitro* and replicating the migratory errors found in the *reeler* dorsal horn, we confirmed that the loss of the Reelin–Dab1 signaling pathway is involved in these migratory errors.

Reeler and Dab1 *mutant mice have similar sensory deficits and neuroanatomical abnormalities*

A host of studies of the cerebral cortex, hippocampal formation and cerebellum show that deletions of components of the Reelin–Dab1 signaling pathway generate identical regional defects and motor

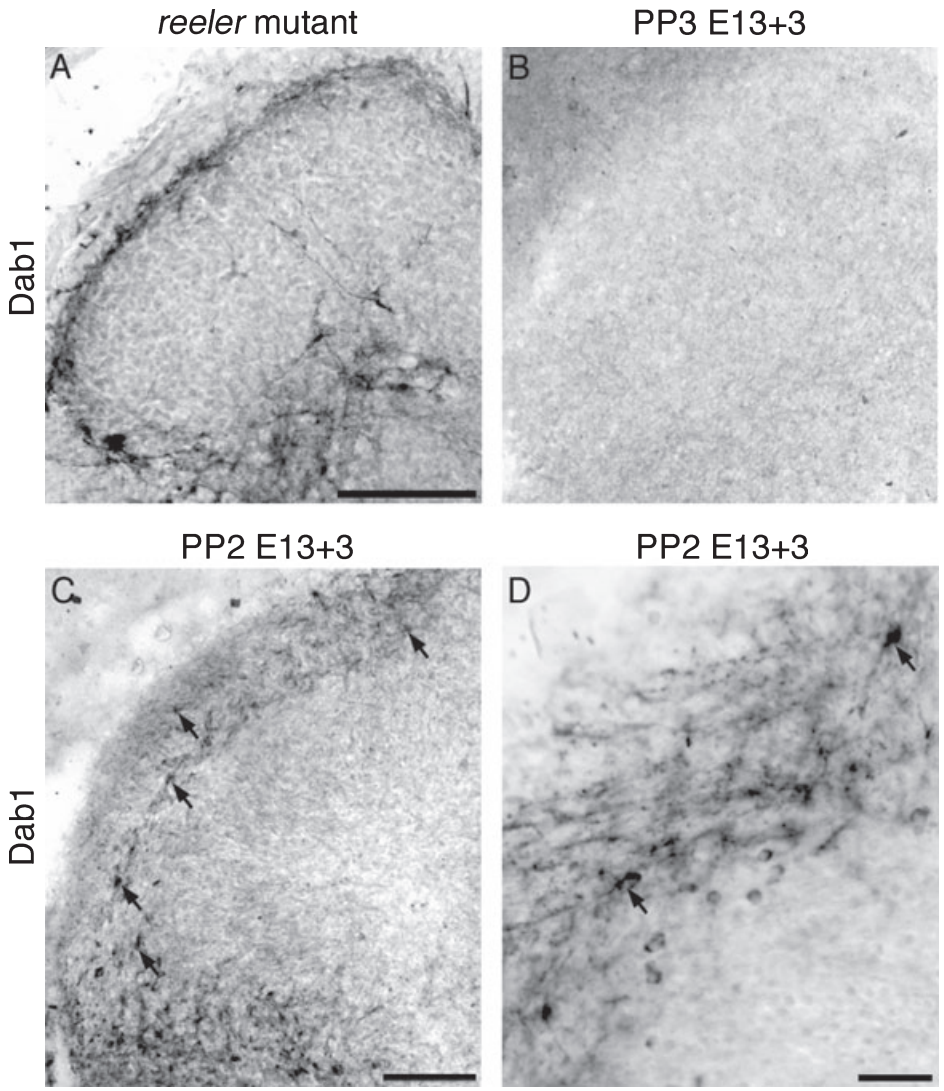

FIG. 10. Disabled-1 (Dab1) accumulates within aberrantly positioned neurons in the embryonic *reeler* dorsal horn and in embryonic rat spinal cord slices cultured with 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2). (A) Many Dab1-positive cells are localized in laminae I and V of an E14.5 *reeler* dorsal horn. (B) E13 rat spinal cord slice cultured for 3 days with the inactive control [4-amino-7-phenylpyrazolo[3,3-*d*]pyrimidine (PP3)] is devoid of Dab1 staining. Unilateral dorsal horn regions are illustrated in (B and C). (C and D) Rat E13 + 3 slices treated with PP2 display prominent Dab1-labeled neurons (arrows, C, D) concentrated within laminae I, II and V, in a pattern similar to that of *reeler* mutants (A). Scale bar: 100 μm (A); 100 μm (B and C); 25 μm (D).

phenotypes, but that single-receptor deletions display fewer behavioral and morphological differences (Caviness, 1976; Mariani *et al*., 1977; Goffinet, 1983, 1984; Sweet *et al*., 1996; Howell *et al*., 1997a,b; Trommsdorff *et al*., 1999; Rice & Curran, 2001). When we tested mice with *Dab1* or *reeler* mutations we found similar sensory deficits, thus implicating the involvement of the Reelin–Dab1 signaling pathway in the development of nociceptive processing. Importantly, the exaggerated response of mutants to thermal stimulation, despite their motor deficits, rules out the possibility that the sensory phenotypes result from motor impairments. In contrast to the *reeler* and *Dab1* mutants, the single lipoprotein mutants respond at levels comparable to their wild-type controls, findings in accordance with previous work showing no overt neurological deficits in single mutants compared with the double-receptor (*Apoer 2/Vldlr*) mutants (Trommsdorff *et al*., 1999).

The dorsal horn is a highly laminated region of the spinal cord gray matter and therefore a likely site for migratory errors to occur as a result of defects in the Reelin–Dab1 signaling pathway. *Reeler* mice have an aberrant neuronal compaction in lamina I, a 50% reduction in LSN neurons and positioning errors in laminae I–II neurons that bear the NK-1 receptor, which is targeted by the substance P population of nociceptors (Brown *et al*., 1995; Villeda *et al*., 2006). Here we show that similar anatomical abnormalities exist in the dorsal horn of *Dab1*, *scrambler* and *Apoer 2/Vldlr* double mutants. For example, lamina I is normally composed of a thin layer of neurons, some of which transmit nociceptive messages to the brainstem or thalamus. However, in *Dab1*, *scrambler* and *Apoer 2/Vldlr* double mutant mice lamina I is compacted and bordered by a neuron-sparse lamina II. These positioning defects likely contribute to the alterations that we detected in the behavioral assays. In contrast, lamina I of the *Vldlr* and *Apoer 2* single mutant mice resembles that of wild-type controls and, indeed, these mutant mice have normal 'pain' behavior. Thus, similar sensory deficits in conjunction with positioning errors in all three mouse models involved in the Reelin–Dab1 signaling pathway strongly implicate the involvement of this pathway in the development of nociceptive processing.

Do both Reelin signaling pathways contribute to the observed defects in nociceptive processing?

Here we show that components of the Reelin–Dab1 signaling pathway, i.e. Reelin, Apoer 2 and Dab1, are located in the adult dorsal horn and the LSN, areas involved in nociceptive processing. Their continued expression after the completion of neuronal migration indicates that Reelin may have an additional function in adult animals. Furthermore, Dab1 expression in wild-type spinal cord also varies developmentally; Dab1-positive cells are not detected in embryonic wild-type mice (Villeda *et al*., 2006), but are present in a few distinct cell types at adult ages. This change in Dab1 expression pattern in the wild-type spinal cord could reflect the lower levels of Reelin in adult mice or an alternate adult function of Reelin involving only a small number of neurons.

Evidence of a postnatal function of Reelin has been reported in both the retina and the hippocampus (Rice *et al*., 2001; Beffert *et al*., 2005; Chen *et al.*, 2005). Rice *et al*. (2001) localized the components of the Reelin signaling pathway in adult retina and demonstrated that this pathway modulates the structure and function of synaptic circuitry. The necessary components of both Reelin signaling pathways are also found in adult hippocampus (Niu *et al*., 2004), and Beffert *et al*. (2005, 2006a) showed that Reelin signaling through exon 18 and exon 19 of Apoer 2 has separate effects on learning and memory in adults. Beffert *et al*. (2006a) disrupted long-term potentiation (LTP) induction by site-directed mutagenesis of the Apoer 2–exon 18, and demonstrated abnormalities in learning and memory in adult animals. Additionally, they reported that after differential splicing of exon 19, Reelin signaling enhances LTP and thus modulates synaptic plasticity and memory (Beffert *et al*., 2005). This demonstrates that Reelin is capable of modulating NMDA receptor activity in the hippocampus. In the present study, we evaluated the contribution to acute nociceptive processing with or without this alternatively spliced variant by testing mice with or without a constitutively active exon 19 on a *Vldlr*-deficient background. Our results indicate that deletion of exon 19, either by mutation (*Apoer 2 Δex19/Vldlr–/–*) or premature termination (*Apoer 2 Stop/Vldlr–/–*), has absolutely no effect on the normal responses to noxious thermal and mechanical stimuli. Together these results indicate that Reelin signaling through Dab1 during development most likely gives rise to positioning errors that underlie the nociceptive impairments detected in *reeler*, *Dab1* and *scrambler* mutant mice.

As central sensitization in the spinal cord involves changes in the dorsal horn that share features underlying the synaptic plasticity that occurs in the hippocampus (Ji *et al*., 2003), we tested mice that constitutively express or lack the Apoer 2–exon 19 splice variant in a persistent pain model. Although the carrageenan produced a prolonged drop in the thermal and mechanical withdrawal threshold in both control and mutant mice, neither the magnitude nor the duration varied between genotypes. Thus, the presence or absence of exon 19 did not prevent the occurrence of sensitization. Remarkably, post-tissue injury thermal and mechanical allodynia did not differ between *reeler* mutants and their wild-type controls, despite the hyperalgesia and decreased sensitivity to mechanical stimulation observed in tests of acute pain. These findings suggest that central sensitization in the spinal cord dorsal horn is not influenced by either of the Reelin signaling pathways.

A possible explanation for the differences in the hippocampus and spinal cord is that Dab1-mediated tyrosine phosphorylation in the hippocampus predominates on the NR2B subunit of the NMDA receptor (Beffert *et al*., 2005). By contrast, the majority of studies of NMDA receptor-mediated central sensitization in the spinal cord focus on the NR1 subunit (South *et al*., 2003), and most report that NR1 is phosphorylated following injury (Brenner *et al*., 2004; Ultenius *et al*., 2006); in fact, even though selective blockade of the NR2B subunit inhibits mechanical and thermal hypersensitivity in the setting of injury (Malmberg *et al*., 2003), there is no evidence for phosphorylation of the NR2B subunit in the dorsal horn (Caudle *et al*., 2005). Thus, despite some commonalities in the processes of synaptic plasticity that occur in the hippocampus and spinal cord (e.g. NMDA dependence), they are not identical. Finally, the differential contribution of the Dab1 signaling pathways to acute and injury-induced persistent pain emphasizes that persistent pain is not merely prolonged acute pain, but rather involves distinct signaling mechanisms.

In vitro *recapitulation of the embryonic* reeler *dorsal horn migratory errors*

During embryonic development Dab1 is tyrosine phosphorylated by the Src family kinases (Howell *et al*., 1997a; Arnaud *et al*., 2003a), and one previous study reported that a specific chemical inhibitor of Src family kinases, PP2, generated cortical malformations similar to, but less severe than, those of *reeler* mutants (Jossin *et al*., 2003). In addition, *Fyn/Src* double mutants exhibit *reeler*-like phenotypes (Kuo *et al*., 2005). By blocking the phosphorylation of Dab1 with PP2 in early embryonic wild-type rat spinal cords we were able to detect accumulated Dab1 in neurons within the superficial dorsal horn and lamina V, areas comparable to those positioning errors detected in the *reeler* dorsal horn. By recapitulating the *reeler* migratory errors in an organotypic slice, we provide further evidence that the disruption of the Reelin–Dab1 signaling pathway causes the positioning defects seen in the *reeler*, *Apoer 2/Vldlr* and *Dab1* mutant dorsal horn.

Supplementary material

The following supplementary material may be found on http://www.blackwell-synergy.com

Fig. S1. Primary afferent distribution appears normal in adult wild-type, *Diasbled-1, scrambler, reeler, apolipoprotein E receptor 2* (*Apoer2*) and P14 *Apoer 2/very-low-density lipoprotein receptor* double-knockout lumbar superficial dorsal horn.

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Abbreviations

Apoer 2, apolipoprotein E receptor 2; CGRP, calcitonin gene-related peptide; Dab1, Disabled-1; DRG, dorsal root ganglion; E, embryonic day; LSN, lateral spinal nucleus; LTP, long-term potentiation; NeuN, neuronal-specific protein; NK-1, neurokinin-1; NMDA, *N*-methyl-D-aspartate; P14, postnatal day 14; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; PP3, 4-amino-7-phenylpyrazolo[3,3-*d*]pyrimidine; TRPV1, transient receptor potential V1 (vanilloid receptor); Vldlr, very-low-density lipoprotein receptor.

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