Supplementary information, Data S1

Materials and Methods

Animals

All animals were housed under a 12-h light/dark cycle at 22-26 °C, with *ad libitum* access to water and chow. *Fxyd2*$^{-/-}$ mice is C57BL/6.Cg-*Fxyd2*^{tm1Kdr} (Jones et al., 2005), and the stock number is 011562-MU/H. In the mutant mice, a LacZ/neo-R cassette replaces most of exon 4 and a portion of intron 4. *Fxyd2*$^{-/-}$ mice were viable and had no observable developmental defects.

Real-time PCR

Total RNA from mouse DRGs and other tissues was extracted using the TRIzol reagent (Life Technologies). Total RNA (1 μg) was reverse transcribed using oligo dT primers. The PCR primers for amplifying FXYD2 mRNA were
5’-GGGGCGGTAAGAAACATAGGC-3’ and
5’-CAACTTGGAACAGGGAGTGGG-3’; for α1 subunit mRNA were
5’-TTTCAGAACGCCTACCTAGAGC-3’ and
5’-TGGAGATAAGACCCACGAAGC-3’; for β1 subunit mRNA were
5’-GCGACATCAATCACGAACGAG-3’ and
5’-GTATCCGCCCATCCCAAAGTA-3’; for FSTL1 mRNA were
5’-CCGAGCACGATGTGGAAACGA-3’ and
5’-ACAACTGGGCTGGCAGATGGA-3’. Primers for GAPDH mRNA were

5’-ATGGTGAAGGTCGGTGTGAACGGATTTGGC-3’ and 5’-TCTGGGTGGCAGTGATGGCATGGACTGTGG-3’. PCR was performed with equal amounts of cDNA in the 7500 Fast Real-Time PCR system (Applied Biosystems) using SYBR Premix Ex Taq^{TM} Perfect Real Time (Takara). Reactions (total 20 μl) were incubated for 5 min at 95 °C, followed by 40 cycles of 30 s at 95 °C and 30 s at 58 °C, and 1 min at 72 °C. Water controls were included to ensure specificity. The mRNA levels of the α1 and β1 subunits and of FSTL1, and FXYD2 were normalized to those of GAPDH. The level of FXYD2 mRNA in various tissues was normalized to that in the DRG.

***In situ* hybridization**

The probe for *Fxyd2* was the whole length of the cDNA; for *Atp1a1*, it was the 5’ region of the mRNA. The primers for the probes for FXYD2 mRNA were 5’-ATGGCTGGGGAAATATCAGATCTGT-3’ and 5’-TAGGCAGGTCAATGAAGATGAACTG-3’; for α1 subunit mRNA were 5’-GGAGCCTCGGCGGGAGGAGGCGGACACGTGGCAGC-3’ and 5’-CCATGTTCTGATACAGCTGCGGGCTCATACTTGTCT-3’; for β1 subunit mRNA were 5’-AAGGAGGAAGGCAGCTGGAA-3’ and 5’-GCTTCAAACCGAAGCCTCCCAA-3’; for FSTL1 mRNA were 5’-ATGTGGAAACGATGGCTGG-3’ and 5’-ATCTGGAATGATCTCAGCTTC-3’, for mRNA of MAS-related G-protein-coupled receptor, member B4 (MRGPRB4) were 5’-CCCTGGCCTGGAACATTAACAAC-3’ and

5’-GTTACAATGAGGAGAATATAAAA-3’; for mRNA of MRGPR, member D (MRGPRD) were 5’-CAAGACAATCCCTCATAGACACG-3’ and 5’-GAAAGGAGGGTGGTAAGGGTTAG-3’; for mRNA of tyrosine hydroxylase were 5’-AAATTGCTACCTGGAAGGAGGT-3’ and 5’-GGGTGGTACCCTATGCATTTAG-3’.

Plasmid construction

Full-length mouse cDNAs of FXYD2 was amplified with the primers 5’-ATGGCTGGGGAAATATCAGATCTGT-3’ and 5’-TAGGCAGGTCAATGAAGATGAACTG-3’ and was cloned into pcDNA3.1 Myc/His and pIRES-EGFP vector plasmids.

The α1 and β1 subunits of NKA were cloned into T-easy vector with primers for the α1 subunit (5’-ATGGGGAAGGGGGTTGGAC-3’ and 5’-CTAGTAGTAGGTTTCCTTCTCCAC-3’) and the β1 subunit (5’-ATGGCCCGCGGAAAAGC-3’ and 5’-TCAGCTCTTAACTTCAATTTTTACATC-3’). The α1 subunit was subcloned into pIRES-EGFP vector, and a FLAG tag was fused to the C-terminal. The primers for the α1 subunit-Flag were 5’-CGAGCTCATGGGGAAGGGGGTTGGACGAGACA-3’ and 5’-ACGTCGACTTACTTATCGTCGTCATCCTTGTAATCGTAGTAGGTTTCCTTCTCCACCCA-3’; the β1 subunit was subcloned into pcDNA3.1 (-) A. The primers for the β1 subunit-Myc were

5’-GCTCTAGAATGGCCCGCGGAAAAGCCAAGGAGG-3’ and

5’-GGGGTACCGCTCTTAACTTCAATTTTTACATCA-3’.

Behavioral tests

For behavioral testing, *Fxyd2*$^{-/-}$ mice and their littermates were habituated, and tests were performed blinded to genotype. The rotarod test was used to examine motor coordination in *Fxyd2*$^{-/-}$ mice. Mice were placed on the cylinder in a direction that required forward locomotion to avoid falling. Gradual acceleration occurred from a speed of 4 to 40 rpm in 5 min, and the rod was kept running for another 2 min at 40 rpm. The latencies to fall were recorded.

To examine heat sensitivity of the hindpaw, a mouse was placed in a plastic chamber on the surface of a glass plate, and the sensitivity of the hindpaw to heat stimuli was measured using a radiant heat stimulator (Ugo Basile, 37370, Plantar Test, Hargreaves Apparatus). The latency was determined as the duration from the beginning of the heat stimulus to the occurrence of a hindpaw withdrawal reflex. The cut-off time was 20 s.

The hotplate test was performed by using a hotplate (Ugo Basile, 35100, Hot/Cold plate) maintained at 50, 52 or 55 °C. The response latency was determined by observing the signs of nociception, such as jumping or licking of a paw. The cut-off times at 50, 52 and 55 °C were 70 s, 45 s and 30 s, respectively.

In the formalin-induced acute pain model, adult mice were injected with 0.5% formalin (20 μl) into the dorsal surface of the left hindpaw. The spontaneous

nociceptive response was determined by counting the licking and lifting time of the injected paw during each 5-min interval for 1 h [the first phase (0-9 min) and the second phase (10-60 min)].