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Nedd4-2 (NEDD4L) controls intracellular Na^+-mediated activity of voltage-gated sodium channels in primary cortical neurons

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Short title: Nedd4-2 regulates Na_Vs in cortical neurons

Abbreviations: CNS, central nervous system; Na_Vs, voltage-gated sodium channels; ENaC, epithelial sodium channel; DRG, dorsal root ganglia; WT, wild-type; Nedd4-2$^{-/-}$, Nedd4-2-deficient; KO, knockout (mice).

Abstract

Nedd4-2, a HECT-type ubiquitin-protein ligase, has been implicated in regulating several ion channels, including voltage-gated sodium (Na_v) channels. In *Xenopus* oocytes Nedd4-2 strongly inhibits the activity of multiple Na_vs. However, the conditions under which Nedd4-2 mediates native Na_v regulation remain uncharacterised. Using Nedd4-2 deficient mice, we demonstrate here that in foetal cortical neurons Nedd4-2 regulates Na_vs specifically in response to elevated intracellular Na^+, but does not affect steady-state Na_v activity. In dorsal root ganglia neurons from the same mice, however, Nedd4-2 does not control Na_v activities. Our results provide the first physiological evidence for an essential function of Nedd4-2 in regulating Na_vs in the central nervous system.

Keywords

Ubiquitin protein ligases, sodium transport, neurons, patch-clamp techniques

Summary Statement:

The study shows for the first time that the ubiquitin ligase Nedd4-2 regulates voltage-gated sodium channel activity in cortical neurons, specifically in response to elevated intracellular Na^+ concentration.

Introduction

The voltage-gated sodium channels (Na_vs) are responsible for Na^+ influx following local depolarizing stimuli to regulate excitation, secretion and contraction in excitable cells [1]. Na_vs are comprised of a large multi-membrane spanning, pore-forming α-subunit and one or more auxiliary β-subunits [1]. In mammals, nine Na_v channel α-subunits have been identified with dynamic spatially and temporally regulated expression and electrophysiological properties [2].

Many ion channels and transporters are regulated by ubiquitination-dependent endocytosis, trafficking, degradation and recycling. Nedd4-2, a member of the Nedd4 family of ubiquitin ligases is a key regulator of ion channels, including Na_vs [3,4]. The best characterised target of Nedd4-2 is the epithelial sodium channel (ENaC), and it has been shown that the WW domains of Nedd4-2 bind to specific regions of the ENaC subunits called PY motifs (PPxY) [5]. Electrophysiological studies have demonstrated that intracellular Na^+ concentration regulates cell surface expression of ENaC subunits and that this is via Nedd4-2 mediated ubiquitination which leads to channel endocytosis and degradation [3, 5-8]. Additionally, loss of Nedd4-2 in mice leads to elevated expression and activity of ENaC, resulting in perinatal lethality caused by early fluid clearance and an inability to inflate lungs [9].

Much less is known about the regulation of Na_vs by Nedd4 family members. In a previous study we demonstrated that 7 of the 9 Na_v channels contain a highly

conserved PY motif in the carboxyl termini of their α subunits and that all PY motif containing Na_vs can bind two of the WW domains of Nedd4 and Nedd4-2 *in vitro* [4]. Furthermore, two representative Na_v channels (Na_v1.2 and Na_v1.7) tested were found to be ubiquitinated by Nedd4-2 and the activities of all three channels tested (Na_v1.2 and Na_v1.7 and Na_v1.8) were inhibited by Nedd4-2 in *Xenopus* oocytes [4]. In other studies Nedd4-2 was shown to bind, ubiquitinate and downregulate Na_v1.2, Na_v1.3, Na_v1.5 and Na_v1.6 [10-12]. Only one study demonstrates regulation of Na_vs *in vivo* by Nedd4-2 [13]. In this study Nedd4-2 was shown to regulate cell surface levels of Na_vs in the adult dorsal root ganglia (DRG) neurons [13]. In the same study, Nedd4-2 was found to be down-regulated in an animal model of neuropathic pain, thus suggesting a crucial role for Nedd4-2 in regulating neuronal excitability. To date, however, there is no evidence for the regulation of Na_vs in the central nervous system (CNS) by Nedd4-2. Furthermore, the expression profile of Na_vs in DRG neurons undergoes dynamic alterations with development, and thus, it is possible that Nedd4-2 exhibits different roles in the adult and embryonic DRG. Using Nedd4-2 knockout (Nedd4-$2^{-/-}$) mice here we determine whether Na_vs in both foetal cortical and DRG neurons are regulated by Nedd4-2. Given that these knockout mice die at birth [9], we have used DRG and cortical neurons (CNS) isolated from E18.5 foetuses for our analysis.

Materials and Methods

Nedd4-2 knockout mice and animals husbandry

Animals were maintained at the SA Pathology animal care facility. All animal procedures were approved by the SA Pathology Animal Ethics committee. Nedd4-2 knockout pups were generated from Nedd4-2 heterozgyote timed matings [9], and harvested at foetal day 18.5.

Isolation of primary neurons

For cortical neurons both hemispheres of the cortex were dissected, minced and placed in 0.05% trypsin for 20 min. The digested tissue was allowed to settle at the bottom of the tube and resuspended in plating medium (Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 1% penicillin/streptomycin). After trituration ~30 times with a P1000 pipette, cells were plated onto poly-L-lysine-coated 13 mm glass coverslips at a density of 2000 cells/coverslip. The next day, the medium was changed to Neurobasal medium supplemented with 1/50 B-27 and 1% penicillin/streptomycin. Cells were used after 7 days in culture. The DRG neurons were prepared as previously described [14].

Patch clamp experiments

Whole cell voltage-gated Na^+ currents were recorded in cultured cortical or DRG neurons using the whole-cell patch clamp recording technique. Patch pipettes had a resistance of 2.3-3.5 MΩ (cortical neurons) and 0.8-1.5 MΩ (DRG neurons). Transient depolarization-activated Na^+ currents were recorded using a HEKA EPC9 computer-controlled patch clamp amplifier and HEKA Pulse software. Series resistance was routinely compensated by 70–80% and capacitive and leakage currents were digitally

subtracted. Patch pipettes were filled with an internal solution containing (in mM): 10 NaCl, 90 CsF, 40 CsCl, 10 EGTA, 2 $MgCl_2$, 10 HEPES-CsOH, pH 7.2. The experiments with veratridine were conducted with the bath solution containing (in mM): 70 NaCl, 70 N-methyl-D-glucamine Cl, 2 $MgCl_2$, 1 $CaCl_2$, 4 CsCl, 10 TEA-Cl, 10 HEPES-TEA-OH, pH 7.35. The experiments with monensin and ouabain were conducted using the following bath solution (in mM): 140 NaCl, 2 $MgCl_2$, 1 $CaCl_2$, 4 CsCl, 10 TEA-Cl, 10 HEPES-TEA-OH, pH 7.35. Veratridine, monensin and ouabain were diluted in the culture medium to the final concentrations of 30, 10 and 1 µM and incubated with the cells for 1h, 2h and 3h respectively. No morphological changes or toxicity were apparent during these incubation periods.

Voltage-dependence of activation

Voltage-dependence of activation was determined by measuring the amplitude of the Na^+ current elicited by depolarization to various membrane potentials. Voltage-dependent Na^+ conductance *(G)* was determined from transformations of current-voltage relationship (*I-V*) curves using the formula: $G = [\ I\ /\ (V - V_r)]$, where I is peak current amplitude, V is the test membrane potential, and V_r is the measured or extrapolated reversal potential. Current activation curves were fitted with a sigmoidal Boltzmann function that identifies the voltage at which the VGSC is half-maximally activated: $G\ /\ G_0 = [1\ /\ (1 + \exp\ (V_{1/2}\ act - V)\ /\ K_v)]$, where G represents the conductance at various membrane potentials, G_0 is peak conductance, $V_{1/2}act$ is the voltage where the VGSCs are half-maximally activated, V is the depolarized membrane potential and K_v is the slope constant.

Results

Nedd4-2 is not involved in regulating Na^+ steady-state current density in foetal DRG neurons

As DRG neurons display large Na^+ currents, we used primary DRGs for whole-cell patch clamp experiments. We compared the Na^+ current density of DRG neurons isolated from foetal (E18.5) WT and Nedd4-$2^{-/-}$ mice and found that there was no significant difference in Na^+ current density between the two groups of cells (Fig. 1A).

Nedd4-2 is not involved in activation-induced down-regulation of Na^+ current density in DRG neurons

We then studied the potential role of Nedd4-2 in activation-induced down-regulation of Na^+ current density in DRG neurons. Previous studies have shown that Na_v channels in brain neurons undergo activation-induced internalisation, resulting in reduced cell surface levels [15]. This was achieved using tritiated saxitoxin (^{3}H-STX) to label Na_v channels, and the Na_v activator veratridine to induce prolonged persistent channel activation. Due to the unavailability of ^{3}H-STX, we instead used whole-cell patch clamping to determine Na^+ current density as a measurement of cell surface density of Na_vs. Na_vs were activated with veratridine for 1h after which the cells were thoroughly washed to remove any traces of the toxin. Na^+ current density was measured within 15 min after veratridine wash-out. As shown previously [15,16], a prolonged incubation

with veratridine led to a significant reduction in Na^+ current density in WT neurons to 9 ± 1 % of control (n=22-26; p <0.001; Fig. 1B). Nedd4-2 did not appear to be involved in the activation-induced down-regulation of Na^+ current density in the DRG, as veratridine incubation reduced Na^+ current density in Nedd4-$2^{-/-}$ neurons to a similar level as in WT neurons (14 ± 3 % of control; n=22-26; Fig. 1B,C).

We also determined the conductance-voltage relationship in veratridine-treated and control WT and Nedd4-$2^{-/-}$ DRG neurons. We found that after incubation with veratridine, there was a significant difference (~9 mV) in the voltage-dependence of activation of Na^+ current between WT and Nedd4-$2^{-/-}$ DRG neurons (Fig. 1D). These data suggest that Nedd4-2 may be involved in the selective activation-induced down-regulation of a Na_v subtype in the DRG.

Nedd4-2 does not regulate steady-state Na^+ current density in cortical neurons

To investigate whether Nedd4-2 regulates Na_v channels in a different neuronal cell type, which expresses different populations of Na_vs, we compared the Na^+ current density of cortical neurons also isolated from E18.5 WT and Nedd4-$2^{-/-}$ mice and found that there was no significant difference in steady-state Na^+ current density between the two groups of cells (Fig. 2A).

Nedd4-2 regulates veratridine-triggered down-regulation of Na^+ current density in cortical neurons

We then studied the potential role of Nedd4-2 in activation-induced down-regulation of Na^+ current density in cortical neurons. A prolonged incubation with veratridine led to a significant reduction in Na^+ current density in WT neurons to 22±5 % of control (n=17-25; p <0.001; Fig. 2B, C). In contrast, veratridine incubation had no effect on the Na^+ current density in cortical neurons isolated from Nedd4-$2^{-/-}$ mice (n=16-21; Fig. 2B,C). These data strongly suggest that Nedd4-2 is crucial for activation-induced down-regulation of Na^+ current density in these neurons.

To investigate whether individual Na_v subtypes with distinct biophysical properties were differentially regulated by veratridine or Nedd4-2, we compared the conductance-voltage relationship between the four groups (veratridine-treated and untreated WT and Nedd4-$2^{-/-}$ neurons). We found no significant differences in voltage-dependence of activation (Fig. 2D), suggesting that neither Nedd4-2 nor veratridine specifically regulate certain Na_v subtypes in these neurons.

Nedd4-2 is required for Na_v regulation in response to increased intracellular Na^+ in cortical neurons

Veratridine induces persistent Na_v channel activation, which in turn causes an increase in intracellular Na^+ and resultant channel down-regulation. We next determined whether a non-specific elevation of intracellular Na^+ could also induce Na_v down-regulation. WT cortical neurons were preincubated with either the Na^+-H^+ ionophore monensin or a Na^+-K^+ ATPase inhibitor ouabain, treatements that rapidly increase the concentration of intracellular Na^+. The data in Fig. 3 show that pre-incubation with

monensin and ouabain both resulted in the down-regulation of Na_v current in WT cortical neurons. In Nedd4-2$^{-/-}$ neurons, however, there was no Na_v down-regulation following treatment with either monesin or ouabain. These important results clearly indicate that Nedd4-2 is essential for the down-regulation of Na_vs in response to elevated intracellular Na^+ in cortical neurons.

Discussion

In this report we have shown that at steady-state, the expression of Na_v channels on the plasma membrane of DRG and cortical neurons is not regulated by Nedd4-2. We have consolidated previous findings [15,16] showing that prolonged activation of Na_v channels in brain neurons results in their down-regulation, and extended on these findings to show that Na_v channels in the DRG are also down-regulated in response to persistent activation. We have demonstrated for the first time that Nedd4-2 is involved in activation-induced down-regulation of Na_vs in neurons isolated from the embryonic cerebral cortex, but not in embryonic DRG neurons. We have also shown that the activation-induced down-regulation involving Nedd4-2 occurs in response to increased intracellular Na^+. Combined with the prevailing understanding of ENaC and Nav regulation, the most likely mechanism of this feedback inhibition is that Nedd4-2 mediated ubiquitination of the Na_vs leads to endocytosis and subsequent degradation of the channels. Thus in the absence of Nedd4-2, Na_vs persist at the plasma membrane, leading to sustained and elevated current density and a failure of the feedback control mechanism.

It has been known for some time that regulatory feedback exists between the electrical activity of a neuron and channel density. In response to elevated intracellular Na^+, caused by Na^+ channel activators (such as veratridine) or Na^+ ionophores there is a rapid partial disappearance of surface Na^+ channels in brain neurons developing in culture [15]. Later, it was found that in cultured foetal neurons Na_v density was controlled by internalisation and degradation following Na^+ influx [16]. However, the precise molecular mechanism for this down-regulation of Na_v channels has remained elusive. In this report, we show that Nedd4-2 regulates Na_v channels and this is at least partly responsible for channel down-regulation after veratridine exposure in cortical neurons, but not DRG neurons. As Nedd4-2 was recently shown to regulate Na_v channels in the adult DRG [13], it is possible that Nedd4-2 has different roles in foetal and adult sensory neurons, in particular since the expression profile of Na_v subtypes undergoes significant and largely uncharacterised alterations with development. For example, $Na_v1.3$ is expressed at high levels during embryonic development but is completely absent in the adult DRG [17], expression of $Na_v1.8$ and $Na_v1.9$ in sensory neurons increases with age [18], and the heart Na_v subtype $Na_v1.5$ is expressed selectively in embryonic DRG neurons while an alternative splice variant, $Na_v1.5a$, is present but only at low levels in the adult DRG [19].

Similarly, differential expression of Na_v subtypes or their modulators may explain the differential roles of Nedd4-2 in embryonic cortical versus DRG neurons. Indeed,

opposing phenotypes have been observed for a single ion channel mutation in $Na_v1.7$ in different cell types in which the channel is expressed, rendering DRG neurons hyperexcitable, and sympathetic ganglion neurons hypoexcitable [20]. Most simply, in our system this could be due to a Nedd4-2 regulation of Na_v subunits with greater expression in cortical neurons than in the DRG. Seven of the nine mammalian Na_v channels contain a PY motif and have been shown to interact with the WW3 and WW4 domains of Nedd4-2 *in vitro* [4]. Subsequent ubiquitination and inhibition by Nedd4-2 has been demonstrated for all the channels assessed thus far including $Na_v1.2$, $Na_v1.3$, $Na_v1.5$, $Na_v1.7$, and $Na_v1.8$ [4,10,11]. Recently $Na_v1.6$ was also shown to be inhibited by Nedd4-2 in isolated hippocampal neurons of the CNS, via a complex regulation also involving phosphorylation of $Na_v1.6$ by p38 MAPK [12]. Determining the identity of the Na_vs regulated by Nedd4-2 in CNS neurons is the subject of ongoing investigations in our laboratory.

Another possibility to explain the differences in cortical and DRG neurons is a potential functional redundancy between Nedd4-2 and Nedd4. These proteins are highly similar in structure, and may regulate some of the same targets [21]. Indeed, Nedd4 binds to multiple Na_v channels, but channel ubiquitination and down-regulation is more robust by Nedd4-2 [4,11]. Perhaps a lack of Nedd4-2 in cortical neurons is sufficient to cause a mis-regulation of Na_vs, but redundancy with another Nedd4 family member in DRG neurons is able to compensate and allow normal Na_v channel activity and down-regulation following increased intracellular Na^+.

This regulation of Na_vs parallels the Nedd4-2 regulation of ENaC. It has long been known that Nedd4 and Nedd4-2 mediate the ubiquitin-dependent down-regulation of Na^+ channel activity in response to increased intracellular Na^+ [7,8]. Our observations here raise some interesting questions, such as whether Nedd4-2$^{-/-}$ mice present any phenotypes that can be attributed to misregulation of Na_vs as well as ENaC. Additionally, it is worth considering that Nedd4-2 polymorphisms, which have been linked to hypertension in human patients, may affect Na_v regulation, in addition to ENaC regulation.

Author Contribution

SK and PP conceived and supervised the project; JAK, GR, SK and PP designed the study; JAK, NAB, GR and JM performed experiments; JAK, GR, NAB, SK and PP analysed data; and JAK, NAB, JM and SK wrote the paper. Authors declare no conflict of interest.

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Figure legends

Figure 1. Nedd4-2 does not mediate veratridine-induced down-regulation of Na^+ current amplitude in foetal DRG neurons. (**A**) Voltage-gated Na^+ currents recorded from DRG neurons by depolarizing cells to voltages ranging from -60 to +50 mV from a holding potential of -70 mV. To calculate Na^+ current density, the current amplitude was normalised to cell capacitance (pA/pF). Error bars show mean ± SEM of 22-26 cells from 3 WT and 3 Nedd4-$2^{-/-}$ (KO) mice. (**B**) Voltage-gated Na^+ currents recorded from DRG neurons by depolarizing cells to voltages ranging from -60 to +50 mV from a holding potential of -70 mV in the presence of veratridine (30 µM). To calculate Na^+ current density, the current amplitude was normalised to cell capacitance (pA/pF). Error bars show mean ± SEM of 22-26 cells from 3 WT and 3 Nedd4-$2^{-/-}$ (KO) mice. (**C**) Na^+ current density in veratridine-treated and untreated DRG neurons from embryonic WT and Nedd4-$2^{-/-}$ (KO) mice. Cells were incubated with 30 µM veratridine or vehicle control for 1h after which Na^+ current density (in pA/pF) was recorded by depolarizing the cells to the voltage corresponding to peak Na^+ current amplitude. (*) significantly different from untreated WT and Nedd4-$2^{-/-}$ neurons ($p < 0.001$, One-way Anova with Tukey's post test). Error bars show mean ± SEM of 22-26 cells from 3-4 WT and 3-4 Nedd4-$2^{-/-}$ mice. (**D**) Conductance (G)-voltage relationship for Na^+ currents in DRG neurons from WT and Nedd4-$2^{-/-}$ (KO) mice incubated with or without veratridine (30 µM). Error bars show mean ± SEM of 22-26 cells. (*) Nedd4-$2^{-/-}$ veratridine treated neurons significantly different from all other groups ($p < 0.001$, One-way Anova, Tukey's post test).

Figure 2. Nedd4-2 is crucial for the veratridine-induced down-regulation of Na^+ current density in foetal cortical neurons. (**A**) Voltage-gated Na^+ currents recorded from cortical neurons by depolarizing cells to voltages ranging from -60 to +50 mV from a holding potential of -70 mV. To calculate Na^+ current density, the current amplitude was normalised to cell capacitance (pA/pF). Error bars show mean ± SEM of 21-25 cells from 3 WT and 3 Nedd4-$2^{-/-}$ (KO) mice. (**B**) Voltage-gated Na^+ currents recorded from cortical neurons by depolarizing the cells to voltages ranging from -60 to +50 mV from a holding potential of -70 mV in the presence of veratridine. To calculate Na^+ current density, the current amplitude was normalised to cell capacitance (pA/pF). Error bars show mean ± SEM of 16-17 cells from 3 WT and 3 Nedd4-$2^{-/-}$ (KO) mice (**C**) Na^+ current density in veratridine-treated and untreated WT and Nedd4-$2^{-/-}$ (KO) cortical neurons. Cells were incubated with 30 µM veratridine or vehicle control for 1h after which Na^+ current density (in pA/pF) was recorded by depolarizing the cells to the voltage that generated peak Na^+ current amplitude. Error bars show mean ± SEM of 16-25 cells from 3 WT and 3 Nedd4-$2^{-/-}$ mice. (*) significantly different from all other groups ($p < 0.001$, One-way Anova with Tukey's post test). (**D**) Conductance (G)-voltage relationship for Na^+ currents in WT and Nedd4-$2^{-/-}$ (KO) cortical neurons incubated with or without veratridine (30 µM). Error bars show mean ± SEM of 16-25 cells.

Figure 3. Nedd4-2 is required for down regulation of Na_v currents in response to increased intracellular sodium in cortical neurons. Voltage gated Na^+ currents were recorded in cortical neurons incubated with either vehicle control (0.1% DMSO, 3h), monensin (10 µM, 2h) or ouabain (1 µM, 3h) using the same voltage protocol as in Fig 2A. Bars represent average peak Na^+ current density ± SEM (n= 16-21 from 3 WT and 3 Nedd4-2$^{-/-}$ (KO) mice measured under indicated conditions. (*) significantly different from untreated WT neurons (p <0.001, One-way Anova with Tukey's post test).

Fig. 1

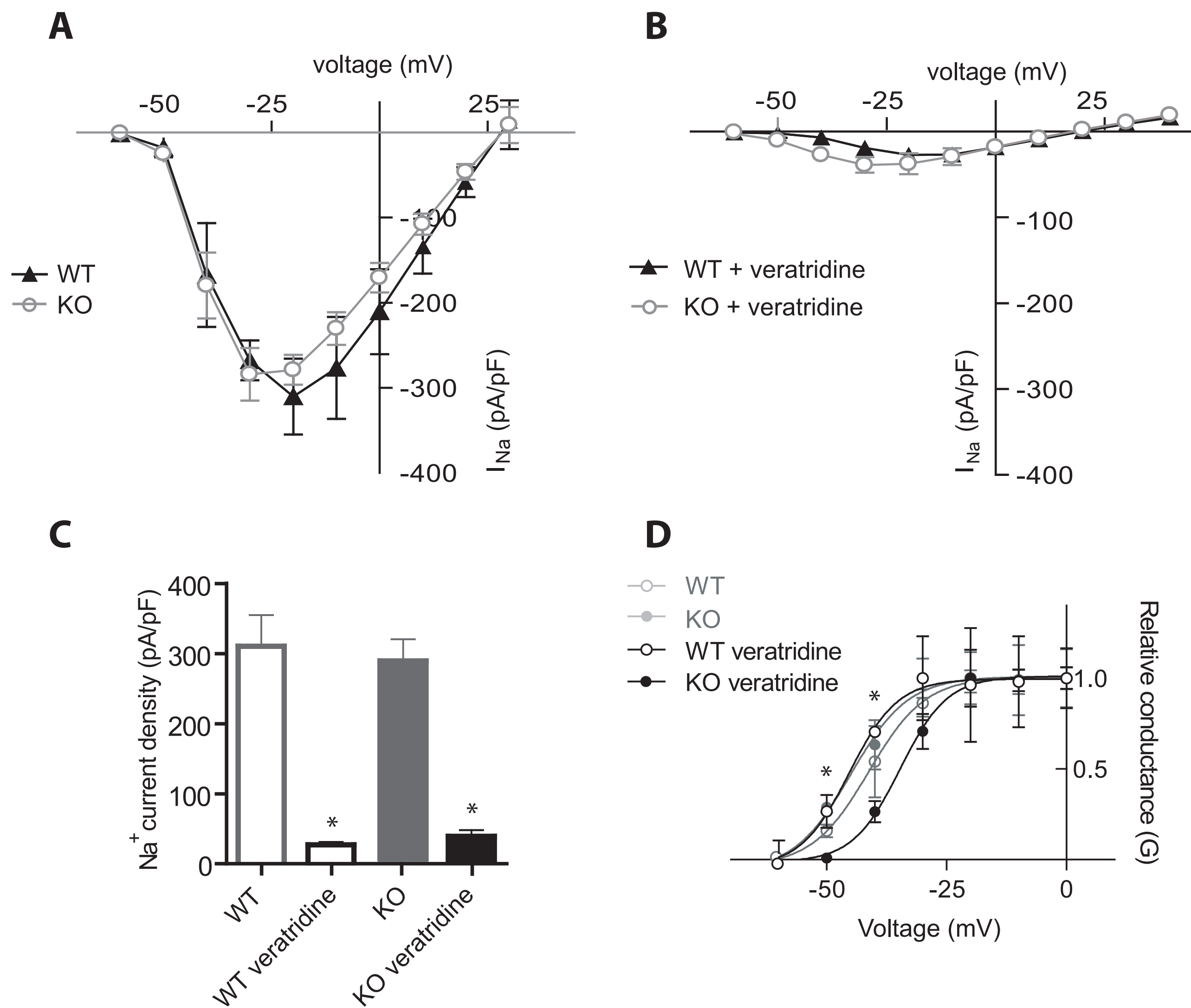

Fig. 2

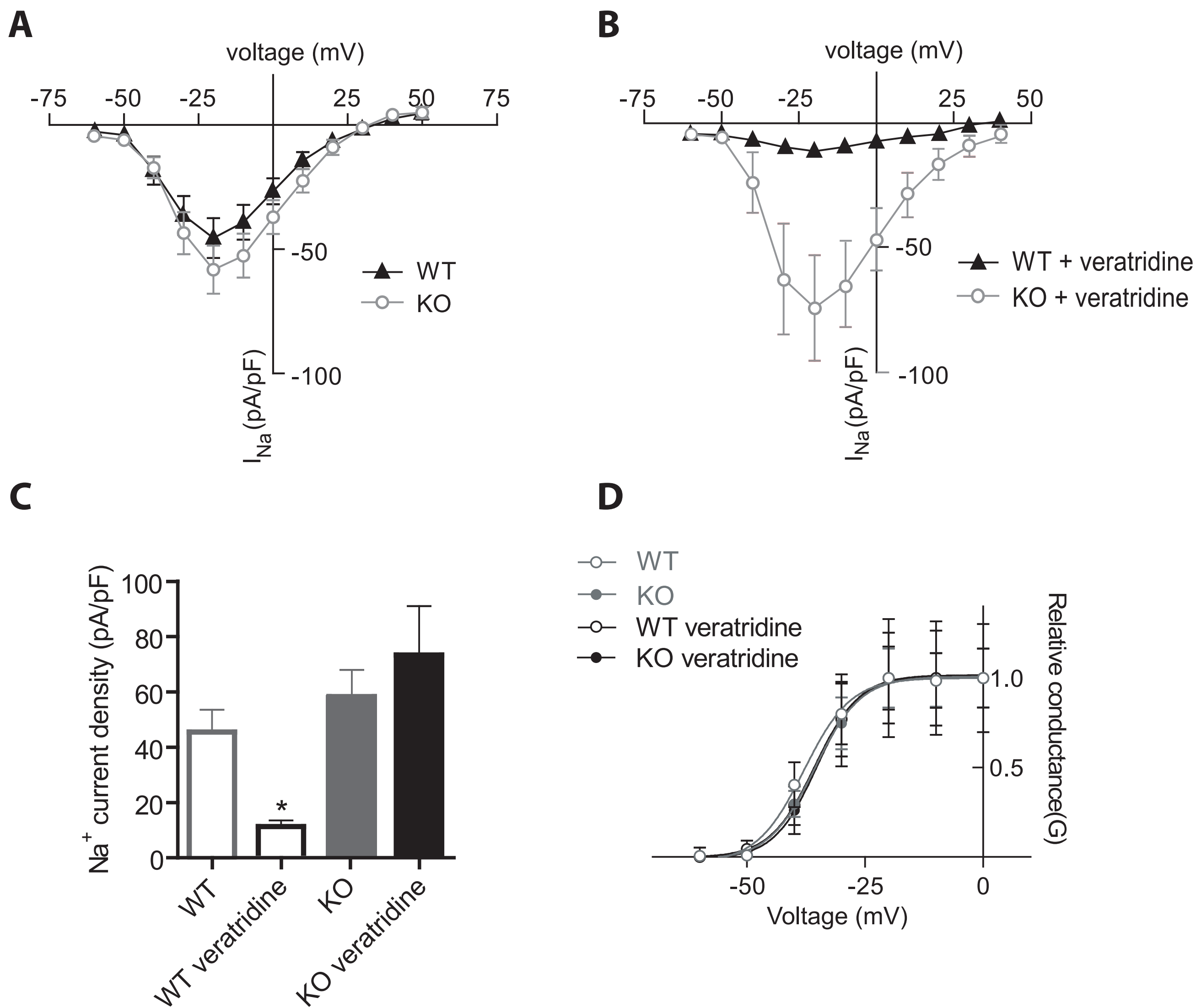

Fig. 3