Synergistic regulation of serotonin- and opioid signaling contribute to pain-insensitivity in $Na_v1.7^{-/-}$ mice

We show for the first time that a voltage gated sodium channel such as Nav1.7 controls intracellular pain signaling pathways and that loss of Nav1.7 shifts the homeostatic balance from pro- to anti-nociceptive signaling.

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Abstract

Loss of the voltage-gated sodium channel $Na_v1.7$ results in lifelong insensitivity to pain in humans and mice. This seems to involve the upregulation of endogenous opioid precursors. If there are changes of intracellular pain signaling and if the opioid signaling can escape changes compensating the increased extracellular opioids in $Na_v1.7$-/- is unknown. We now analyzed the balance of pro- and anti-nociceptive GPCR signaling using a novel cell-based assay for the activity of endogenous type II protein kinase A (PKA-II) in sensory neurons. Contrary to expected upregulation of counterbalancing pro-nociceptive signaling, we show that loss of $Na_v1.7$ decreases the pro-nociceptive serotonergic 5-HT_4 receptor and its intracellular mediator, PKA-RIIβ. Simultaneously, the efficacy of anti-nociceptive opioid signaling is not reduced but strongly increased lowering efficiently TTX-r currents in nociceptive neurons. Thus $Na_v1.7$ controls the intracellular homeostatic interplay of pro- and anti-nociceptive signaling in a synergistic and long-lasting manner contributing to lifelong endogenous analgesia.

Introduction

Novel strategies to treat pain are urgently required as over 20% of patients do not respond to current analgesics (1, 2). In addition, therapy with existing analgesics requires urgent improvement. Opioids, which are considered benchmark analgesics, achieve only 10-15% pain relief on average (3-5). Beyond the problem of in part severe side-effects (6, 7), one aspect further compounding the use of opioids is that patients quickly desensitize and thereby require ever increasing

doses. Despite extensive research, at present desensitization due to the prolonged presence of opioids can only be reduced but not abolished (8).

Voltage-gated sodium channels such as $Na_v1.7$ are central for the sensitivity and activation of nociceptive neurons in response to pain-initiating stimuli. Loss of function of $Na_v1.7$ results in lifelong absence of pain in mice and humans which, apart from anosmia, are otherwise seemingly normal (9-11). The central role of $Na_v1.7$ in pain and its predominant expression in nociceptive neurons makes it an excellent potential target for novel drugs. This topic is currently under intense academic and commercial investigation (12, 13). Nevertheless, potent $Na_v1.7$ blockers fail to alleviate pain (14).

The exact mechanisms responsible for pain insensitivity due to loss of $Na_v1.7$ are incompletely understood (12). Surprisingly, it was recently reported that $Na_v1.7$-deficiency upregulates the expression of met-enkephalin in sensory neurons and that blocking opioid receptors with naloxone recovers the ability to perceive pain in $Na_v1.7$ null mice and humans (15). These data indicate that, in addition to its role in electrical signaling, $Na_v1.7$ is likely to have other functions which contribute to pain sensitivity substantiating its potential as an exciting analgesic drug target. If $Na_v1.7$ controls only the expression of the extracellular opioid, if an increase in extracellular opioids is necessary for a reduced nociceptor activity, or if $Na_v1.7$ also changes long-term intracellular analgesic signaling is currently unknown.

Pain sensitivity is strongly regulated by balancing the interplay of pro- and anti-nociceptive signaling (16, 17). Gene expression profiling in dorsal root ganglia of $Na_v1.7$-deficient mice indicated the downregulation of the metabotropic serotonin

receptor 5-HT_4 (15). This suggests that $Na_v1.7$ may control an even more profound change than merely increased opioid ligand expression by changing both, the pro- and anti-nociceptive intracellular signaling. Whether $Na_v1.7$ thereby controls 5-HT_4 receptor-mediated intracellular pro-nociceptive signaling, and whether this acts synergistically with the increased opioid expression in the very same cell remains to be investigated with single cell resolution.

Using a novel assay for the activation of type II protein kinase A (PKA-II) and quantifying more than one million single primary neurons demonstrates that $Na_v1.7$ controls both pro- and anti-nociceptive cellular inputs resulting in increased opioid-receptor signaling and, in the same neurons, in a synergistic and sustained decrease in 5-HT activity resulting in strongly increased opioid efficacy on TTXr currents. Thus, Nav1.7 appears to control the homeostatic set point of the counterbalancing pro- and anti-nociceptive intracellular signaling cascades.

Results

We set out to characterize if loss of $Na_v1.7$ and the concomitant long-term upregulation of opioid-precursors (15) alters intracellular signaling. Specifically, we tested for changes in the pro-nociceptive and PKA-activating serotonin signaling as well as its counterbalancing PKA-inhibiting antagonistic opioid signaling. To do so, first, it needed to be identified, which 5HT receptor has the strongest influence on pro-nociceptive PKA signaling as well as to develop a system to monitor the interplay of 5HT-signaling and opioid signaling in adult primary neurons on a single cell level.

5-HT$_4$ receptor is critical for the activation of PKA-II

Which of the Gα_s coupled metabotropic serotonin receptors (5-HT$_4$, 5-HT$_6$, 5-HT$_7$) drive PKA activation in nociceptive neurons thus far remained controversial (18-20). Recently, we introduced a “High Content Screening (HCS)” microscopy approach to detect the phosphorylated form of PKA-II regulatory subunits RIIα and RIIβ (pRII) for the analysis of endogenous cAMP/PKA dynamics in sensory neurons (21, 22) (Fig. 1A, B). Using this assay we now observed that 5-HT as well as the 5-HT$_4$-specific agonist SC-53116 (23) increased phospho-RII (pRII) intensity with similar potency in rat sensory neurons after 3 min stimulation (Fig. 1C, EC_{50} = 30 nM and 89 nM, respectively). The 5-HT response was fully blocked by the 5-HT$_4$-specific antagonist GR113808 (24) indicating that the 5-HT$_4$ receptor is both sufficient and necessary for 5-HT-induced pRII sensitization-signaling in sensory neurons (Fig. 1D, IC_{50} = 8.6 nM). Thus, the downregulation of 5-HT$_4$ mRNA in $Na_v1.7^{-/-}$, as identified in Minett’s et al. transcriptome data (15), indicate the potential reduction of an important pro-nociceptive signaling pathway.

Opioids inhibit 5-HT-induced PKA-II activation

To examine if the upregulation of opioids could attenuate 5-HT signaling on a single cell basis, we extended our recent pRII HCS microscopy approach to monitor not only pro- but also anti-nociceptive GPCR signaling. We increased endogenous pRII signals with a 5-HT test stimulus and observed opioid activity as a reduction in the 5-HT-induced pRII signal. Indeed, the clinically relevant opioid analgesic fentanyl and also the μ-opioid-receptor (MOR)-specific agonist DAMGO dose-dependently inhibited the 5-HT (200 nM)-induced pRII signal (Fig. 1E) in rat

sensory neurons. As expected, fentanyl was more potent than DAMGO (IC_{50} = 97 nM vs. 321 nM, $F_{1,47}$ = 4.2, n = 4, P < 0.05 for pIC_{50}, extra-sum-of-squares F test). Co-application of fentanyl with 5-HT produced long-lasting inhibition of 5-HT-responses at all tested doses ($F_{3,84}$ = 77.2, n = 4, P < 2e-16, two-way ANOVA), while basal pRII intensity was not affected (Fig. 1F). The competitive opioid receptor antagonist naltrexone (NTX, 10 µM) completely reversed the fentanyl-induced inhibition of pRII intensity ($F_{1,42}$ = 136, n = 4, P < 9.5e-15, two-way ANOVA, Fig. 1G). NTX had no effect on basal or 5-HT-induced pRII intensity, which demonstrates the absence of constitutive opioid receptor activity in cultured rat sensory neurons (Fig. 1H). Also the MOR-specific antagonist CTOP (10 µM) did not affect baseline or 5-HT induced pRII intensity, but only partially reverted the fentanyl-induced inhibition of pRII ($F_{1,42}$ = 12, n = 4, P < 0.01, two-way ANOVA, Fig. 1I-J). Applying the MOR-specific agonist DAMGO resulted in a similar dose-dependent inhibition of 5-HT-induced pRII immunoreactivity ($F_{3,56}$ = 51, n = 3, P < 5e-16, two-way ANOVA, Fig. 1K), which was fully reversed by NTX (Fig. 1L) and partially by CTOP (Fig. 1M). Further experiments demonstrated that opioids also inhibit PKA-II activity induced by the IP_1 receptor agonist prostacyclin (PGI_2) or the adenylyl cyclase activator forskolin (Fsk) (Supplementary Fig. S1).

To investigate if opioids and 5-HT act on the same subgroup of nociceptive neurons, we performed co-stainings of pRII with RIIβ after stimulation with Fsk (2 µM), 5-HT (0.2 µM) or PGI_2 (1 µM) while activating opioid receptors with fentanyl (2 µM) (Fig. 2A). We recently identified RIIβ as a novel integrative marker of nociceptors that is coexpressed with opioid receptors (21, 25). After compensating for potential spill-over between fluorescence channels (Fig. 2B), we found that Fsk

increased the pRII intensity levels in all rat sensory neurons, including RIIβ(-) neurons, whereas 5-HT and PGI_2 were predominately activating RIIβ(+) neurons (Fig. 2A, C, D). Confirming that RIIβ predicts sensitivity to opioids, we found inhibitory effects of fentanyl on Fsk-induced pRII to be restricted to the RIIβ(+) subgroup (Fig. 2A, C, D).

5-HT signaling is reduced in $Na_v1.7^{-/-}$ mice

Next, we tested if loss of $Na_v1.7$ results in long-lasting changes in nociceptors in addition to the reported upregulation of the opioid ligand, pre-proenkephalin (15). More specifically, we analyzed the counterbalancing between the pro-nociceptive serotonergic signaling by $5\text{-}HT_4$ receptors and the anti-nociceptive opioid system at the level of their intracellular mediator cAMP/PKA-II in pain-insensitive $Na_v1.7^{-/-}$ mice and $Na_v1.8^{-/-}$ mice (10, 26). General features such as the pRII increase after Fsk treatment (10 µM), cell numbers, cell size and neuronal marker (UCHL1) distribution were highly similar indicating comparability among the genotypes (Fig. 3A-D).

Using qPCR, we corroborated the downregulation of $5\text{-}HT_4$ receptors in sensory ganglia of $Na_v1.7^{-/-}$ mice identified in a microarray by Minett et al. (15) (Fig. 3E). In contrast, $5\text{-}HT_4$ receptor mRNA levels remained unchanged in $Na_v1.8^{-/-}$ mice (Fig. 3F). To test for accompanying functional cellular changes at the level of GPCRs, adenylyl cyclase, and PKA-II, we performed dose response experiments with 5-HT, PGI_2, Fsk, and the cell-permeable cAMP analog Sp-8-Br-cAMPS-AM (Fig. 3G-K). Corroborating our finding of reduced expression of $5\text{-}HT_4$ receptor mRNA, specifically pRII responses to 5-HT but not PGI_2, Fsk, or Sp-8-Br-cAMPS-

AM were reduced in sensory neurons of $Na_v1.7^{-/-}$ mice (Fig. 3G, H). Confirming decreased receptor abundance, only the maximum amplitude of the response was significantly reduced by 37% ($F_{1,64} = 53.6$, $n = 5$, $P < 0.0001$, extra-sum-of-squares F test), while the EC_{50} values remained unchanged (Fig. 3H). In addition to the reduced average signal amplitude, also the number of responding cells was reduced in $Na_v1.7^{-/-}$ mice as evaluated by thresholding the single cell data (see Fig. 3G at 1 µM 5-HT: 19.1 ± 1.2% in $Na_v1.7^{-/-}$ vs. 27.8 ± 1.3% in $Na_v1.7^{+/+}$, $n = 5$, $P < 0.001$, Student's t-test). In contrast, responses to PGI_2 were not altered, indicating specificity for 5-HT/5-HT_4 receptors (Fig. 3I). In addition, dose-responses of the downstream activators Fsk and Sp-8-Br-cAMPS-AM were similar in both genotypes (Fig. 3J, K) corroborating a primary change at the receptor level.

RIIβ is downregulated in $Na_v1.7^{-/-}$ mice

The pRII dose response data in Fig. 3H-K were normalized. But not-normalized data as presented in the kinetic experiments of Fig. 4D revealed an additional reduction of pRII baseline intensity values by 8% in $Na_v1.7^{-/-}$ mice compared to wildtype litters ($F_{1,36} = 22.9$, $n = 4$, $P < 3e\text{-}05$, two-way ANOVA). This was highly unexpected, since the baseline activity of PKA-II is commonly tightly controlled and maintained at a constant level. Thus, the newly observed reduction in baseline activity indicates a further intracellular alteration induced by the loss of $Na_v1.7$. Thereby, the reduced pro-nociceptive 5-HT_4 receptor input is further reduced on the level of intracellular signaling. As for 5-HT_4 receptors, this effect was specific for $Na_v1.7^{-/-}$ mice and absent in $Na_v1.8^{-/-}$ mice (Fig. 4K-Q).

A reduction of the pRII levels could be the result of reduced expression or reduced phosphorylation of the respective regulatory subunits. We therefore analyzed the abundance of RIIβ, the major PKA-RII regulatory subunit in rat nociceptive neurons (21). In mice we also found RIIβ to be enriched especially in small-to-medium sized sensory neurons (Fig. 4A, B). $Na_v1.7$-deficiency did not affect the number of RIIβ(+) neurons (47.1 ± 1.2% vs. 48.7 ± 0.3%), but resulted in a general shift towards lower RIIβ intensities. Indeed, mean RIIβ intensities were 11% lower in RIIβ(+) neurons of $Na_v1.7^{-/-}$ mice compared to wildtype litters (1589 ± 19 vs. 1779 ± 34, $n = 5$, $P < 0.0001$, two-way ANOVA with Bonferroni's test) (Fig. 4C).

Downregulation of 5-HT_4-mediated input and of the RIIβ-mediator could be compensated by e.g. an increased homeostatic PKA-activity. But, not only the RIIβ intensities but also the phosphorylation signals of pRII were lower in $Na_v1.7^{-/-}$ mice (Fig. 4D-J). The phosphorylation signals remained lower than in wildtype also in response to Fsk stimulation (Fig. 4J). This effect was again specific to $Na_v1.7^{-/-}$ mice as it was absent in $Na_v1.8^{-/-}$ mice.

Opioid signaling is amplified in $Na_v1.7^{-/-}$ mice

Having established a synergistic downregulation of the 5-HT_4 receptor input as well as of the intracellular mediator RIIβ, we next investigated the effect of $Na_v1.7$ and $Na_v1.8$ on the cellular activity of the common antipode of sensitization, the anti-nociceptive opioid receptor system.

We tested the effect of fentanyl (2 µM) and NTX (10 µM) on changes of pRII intensity. Treatment with neither fentanyl nor NTX altered the baseline pRII levels

in neurons from $Na_v1.7^{-/-}$ or $Na_v1.8^{-/-}$ mice (Fig. 4E, F, L, M). Thus, not only wildtype rat nociceptors (Fig. 1G, H), but also murine cells from $Na_v1.7^{-/-}$ mice did not show any constitutive opioid receptor activity in culture.

Next we tested the effect of fentanyl and NTX on changes of pRII intensity induced by 5-HT. Commonly, prolonged opioid exposure as suspected to be the case in $Na_v1.7^{-/-}$ mice induces desensitization. In contrast, we now found the inhibition of the 5-HT-signaling by fentanyl to be substantially amplified in sensory neurons of $Na_v1.7^{-/-}$ mice ($F_{1,35} = 37$, $n = 4$, $P < 6e\text{-}07$, two-way ANOVA, Fig. 4H). While there was few reduction of 5-HT-induced pRII signals in wildtype animals (Fig. 4G vs. 4H, black line), fentanyl completely abolished the 5-HT response in $Na_v1.7^{-/-}$ mice (Fig. 4G vs. 4H, red line). This fentanyl effect was indeed opioid receptor mediated as NTX effectively antagonized the fentanyl effect (Fig. 4I). Again, this phenotype was absent in $Na_v1.8^{-/-}$ mice suggesting a specific role of $Na_v1.7$ in regulating intracellular opioid-receptor signaling (Fig. 4K-Q).

To analyze how these differences of averaged response signals distribute over subgroups of sensory neurons, we plotted the pRII intensities of all analyzed neurons versus their size and applied a fixed threshold to evaluate the number of responding neurons (Fig. 4R). Stimulation with 5-HT for 1 min increased pRII signals in 17% of smaller sized sensory neurons from $Na_v1.7^{-/-}$ mice and in 21% from wildtype litters, respectively. Simultaneous application of fentanyl reduced the number of 5-HT responsive neurons from 17% to 6% in $Na_v1.7^{-/-}$ and from 21% to 17% in wildtypes (Fig. 4R, red arrow). Thus, not only the signaling amplitude but

also the number of cells responding to 5-HT in the presence of fentanyl was much reduced in $Na_v1.7^{-/-}$ animals.

The pro- and anti-nociceptive input is considered to be balanced at a single cell level in a stimulus-response relationship (27). Thus, a reduced 5-HT pro-nociceptive input should result in a less effective anti-nociceptive dose-response relationship, i.e. an increased IC_{50}. We tested for the dose-response relationship of fentanyl onto one constant 5-HT (0.2 µM) response (Fig. 5A, B). Interestingly, the IC_{50} values were not significantly different between the two genotypes suggesting a similar receptor-ligand affinity (IC_{50} = 16 vs. 36 nM in $Na_v1.7^{-/-}$ mice and wildtype litters). Nevertheless, we observed a substantial reduction in the response amplitude of fentanyl over the full dose-response-curve in $Na_v1.7^{-/-}$-deficient neurons (Fig. 5A, $F_{2,56}$ = 13.6, n = 4, P < 0.0001, extra-sum-of-squares F test). This was similarly reflected in the subpopulation data (Fig. 5B). While even high doses of opioids left a substantial proportion of wildtype RIIβ(+) neurons responding to 5-HT (Fig. 5B, upper panel), fentanyl reduced pRII levels to almost baseline in $Na_v1.7$-deficient neurons indicating strongly increased effectiveness of the opioid system (Fig. 5B, lower panel). Very similar results were obtained for DAMGO ($F_{2,36}$ = 7.7, n = 3, P < 0.01, extra-sum-of-squares F test, IC_{50} = 155 vs. 250 nM in $Na_v1.7^{-/-}$ mice and wildtype litters, Supplemental Fig. S2).

Next we tested if the enhanced effectiveness of the opioid system is restricted to 5-HT or if this is a general phenomenon. To analyze this independent of the stimulatory GPCR input, we stimulated sensory neurons for 3 min with increasing doses of fentanyl in the presence of Fsk (2 µM). The effectiveness of fentanyl was

again strongly enhanced in $Na_v1.7$-deficient neurons (Fig. 5C, $F_{1,50} = 32$, $n = 4$, $P < 0.0001$ for bottom values; extra-sum-of-squares F test). Analyzing the single cell data showed that the number of Fsk-stimulated neurons were 23.1 ± 0.4% ($Na_v1.7^{-/-}$) vs. 28.4 ± 2.6% ($Na_v1.7^{+/+}$) in the absence of fentanyl ($n = 4$, $P < 0.01$, Student's t-test). Application of fentanyl (2 µM) reduced the number of Fsk-responsive neurons to 9.0 ± 0.6% in $Na_v1.7^{-/-}$ vs. 17.0 ± 0.7% in wildtype litters ($n = 4$, $P < 0.0001$, Student's t-test). Thus, the relative as well as the absolute reduction of the responding cell number was substantially increased in $Na_v1.7^{-/-}$ (Fig. 5D).

Synergistic dampening of pain signaling renders opioids more effective on sodium current reduction

Finally we tested if the general and long-lasting increased effectiveness of the opioid system can also be measured on functional downstream effectors of the PKA system. PKA shows a strong sensitization of the $Na_v1.8$ encoded TTX-r current in nociceptive neurons, which contributes to nociceptive drive. We used in vitro cultures of sensory neurons from wild type and $Na_v1.7^{-/-}$ mice to examine the level of expression of TTXr sodium currents and the effect of fentanyl. Consistent with the cellular studies of PKA activity, we found that fentanyl lowered the expression of $Na_v1.8$ TTXr currents twice as effectively in $Na_v1.7^{-/-}$ mice than in their wild type littermates. This demonstrates that the mechanistic studies carried out in cell-based assays are relevant to the control of nociceptive drive by opioids in sensory neurons, and are likely to play a significant role in the analgesic phenotype of mouse and human $Na_v1.7^{-/-}$ mutants

In conclusion, our data indicate that the loss of $Na_v1.7$ results in a strongly reduced 5-HT pro-nociceptive input, with a concomitant reduction of the intracellular mediator PKA-RIIβ, as well as a synergistic enhancement of the effectivity of the opioid receptor on general cAMP mediated anti-nociceptive input. This synergistic reduction of the counterbalancing system of pain-sensitization signaling is long-lasting and cell autonomous. Further, as this is induced specifically by the loss of $Na_v1.7$, but not of $Na_v1.8$, this suggests that the cellular homeostatic pro- versus anti-nociceptive counterbalancing is controlled specifically by $Na_v1.7$.

Discussion

Genetic loss of function of $Na_v1.7$ causes congenital insensitivity to pain in mice and humans (9, 11, 28), while acute block of $Na_v1.7$ with potent and selective $Na_v1.7$ antagonists does not (14). This contradiction could be resolved if $Na_v1.7$ contributes not only to electrical activity but also to other cellular processes as well. Indeed, Minett et al. found in $Na_v1.7^{-/-}$ mice that the substance P release in the spinal cord is abolished (10) and that the opioid activity is tonically increased (15). These observations may be the consequence of reduced depolarization and thus reduced secretion while other nociceptor regulatory mechanisms might be left unchanged. In contrast, our data now prove not only that indeed there are further cellular pain mechanisms controlled by voltage-gated sodium channels but present the very first evidence that $Na_V1.7$ especially controls intracellular nociceptive signaling (see overview scheme in Fig. 7).

Our results highlight the intimate interaction of pro- and anti-nociceptive inputs. 5-HT has been described as a counterplayer of opioids. Accordingly, agonists of 5-

290 HT_4 receptors are used to counteract chronic opioid induced constipation (29, 30)
291 and in opioid-induced respiratory depression (31). Also at a systemic level,
292 serotonin-reuptake inhibitors counteract spinal pain-input by increasing the activity
293 of inhibitory neurons. Our results further support this antagonistic role by showing
294 5-HT_4 receptor signaling and opioid signaling to be tightly functionally interlinked on
295 a single cell level in a subgroup of peripheral nociceptive neurons.

296 The pro- and anti-nociceptive input has been shown to scale with stimulus
297 intensity. Thus, an increased pro-nociceptive input results in a concomitant
298 increase of the counterbalancing opioid anti-nociceptive intracellular signaling
299 thereby maintaining a constant net-signaling (27). On the basis of these
300 observations, our results indicate that $Na_v1.7$ controls this scaling-ratio (Fig. 7). We
301 find the reduction of the pro-nociceptive serotonergic input in $Na_v1.7^{-/-}$ is not
302 followed by a corresponding reduction of the opioid input. On the contrary, the
303 effectiveness of opioid signaling was much increased resulting in stronger inhibition
304 of pRII. Furthermore, $Na_v1.7$ seems not only to control the ratio but also the
305 maximal endpoint of the opioid effect. While in wildtype animals' significant pro-
306 nociceptive signaling remains after opioid treatment, this was completely abolished
307 in $Na_v1.7^{-/-}$ neurons (Fig. 5A). This is extremely interesting in the light of the
308 problem of therapy induced opioid desensitization (8) as well as the low level of
309 average opioid-induced pain reduction (3-5). Our cellular data now suggest that
310 $Na_v1.7$ controls synergistically both intracellular changes leading to complete loss
311 of pain in $Na_v1.7^{-/-}$ mice and humans.

Pre-proenkephalin is upregulated in complete absence of $Na_v1.7$ activity but not after partial blockade (15). However, GPCRs are known to be modulated quickly by non-transcriptional changes initiated for example by sodium. Indeed, beyond transcription sodium regulates a range of cellular processes such as GPCR ligand binding and allosteric regulation of biased signaling such as the uncoupling of opioid receptors from its α_i signaling while increasing the constitutive signaling activity through the β-arrestin pathway (32-35). A reduction of intracellular sodium should therefore result indeed in the observed increased α_i signaling activity. But the specificity of our observations to $Na_v1.7^{-/-}$ but not to $Na_v1.8^{-/-}$ mice, argues against large scale sodium changes. Instead, one could speculate about the importance of differential subcellular localization and of differential $Na_v1.7$/opioid receptor signaling hubs. But as the knowledge about the role of sodium in signal transduction still gains momentum, future work needs to detail the mechanism leading to the described synergistic dampening of pain signaling.

The loss of function of $Na_v1.7$ appears to cause long-term changes to intracellular nociceptive signaling. We find them to be independent of the continuous presence of extracellular opioids. These changes result synergistically in reduced pro-nociceptive input but increased long-lasting effectiveness of anti-nociceptive opioids. This gives proof that sustained dampening of the intracellular pain signaling in nociceptive neurons can be achieved. Potentially, $Na_v1.7$ therefore regulates the homeostasis set point of pain signaling. This interpretation would be in agreement with previous *in vivo* reports showing the close link of $Na_v1.7$ expression and pain (15, 27, 36, 37). Therefore it is now important to use our novel cellular endpoints (reduced RIIβ expression, decreased pro-nociceptive input,

increased relative effectivity of opioid signaling as well as increase of the maximal effectivity for longtime) together with our novel assay of cellular opioid signaling to investigate the drugability of this regulator of intracellular pain-signaling. Current inhibitors of $Na_v1.7$ activity are lacking convincing direct analgesic activity on their own. Therefore, it will now be of special interest to investigate, in which clinically relevant pain states the pharmaceutical modulation of $Na_v1.7$ might in combination with opioid therapeutic translate into prolonging as well as enhancing opioid effectiveness in mice and humans thereby offering indirectly better pain treatment.

Materials & Methods

Antibodies

The following antibodies were used in this study: chicken polyclonal anti-UCHL1 (1:2000, Novus, Cambridge, UK, #NB110-58872), rabbit monoclonal anti phospho RIIα (S96) (1:1000, clone 151, Abcam, Cambridge, UK, #ab32390), mouse monoclonal anti-PKA RIIβ (1:2000, BD Transduction Laboratories, #610625), highly cross-adsorbed Alexa 647, 594, and 488 conjugated secondary antibodies (Invitrogen, Carlsbad, CA).

Reagents

5-HT (10 mM in dH_2O), naltrexone (100 mM in dH2O), SC-53116 (100 mM in DMSO), GR113808 (100 mM in DMSO) were purchased from Sigma-Aldrich (Munich, Germany) and dissolved as indicated. Fentanyl (10 mM in dH_2O), DAMGO (10 mM in PBS), CTOP (5 mM in DMSO), and forskolin (10 mM in DMSO)

were from Tocris (Bristol, UK). Prostacyclin (10 mM in PBS at pH9.5) was from Cayman (Ann Arbor, MI). 8-bromoadenosine 3',5'-cyclic monophosphorothioate, Sp-isomer and acetoxymethyl ester (Sp-8-Br-cAMPS-AM, 10 mM in DMSO) was from BIOLOG LSI (Bremen, DE).

Animals

Male Sprague Dawley rats (200-225 g, aged 8-10 weeks) were obtained from Harlan (Rossdorf, DE) and used for results shown in Fig. 1, 2, and S1. Conditional $Na_v1.7$ knockout mice were generated by crossing floxed (SCN9A) $Na_v1.7$ mice with Advillin-Cre mice (10, 28) and global Nav1.8 knockout mice were used (26). Female and male mice were aged between 6–20 weeks. Mice and rats were kept on a 12-h light/dark cycle and provided with food and water *ad libitum*. All animal experiments were performed in accordance with the German animal welfare law and approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen or approved by the United Kingdom Home Office according to guidelines set by personal and project licenses, as well as guidelines of the Committee for Research and Ethical Issues of IASP. Animals were sacrificed between 9-12 a.m. by CO_2 intoxication. DRGs of rats (L1-L6) or mice (lumbar and thoracic) were removed within 30 min per animal.

DRG neuron cultures

DRGs were de-sheathed, pooled and incubated in Neurobasal A/B27 medium (Invitrogen, Carlsbad, CA) containing collagenase P (Roche, Penzberg, DE) (0.2 U/ml, 1 h, 37 C, 5% CO_2). The DRGs were dissociated by trituration with fire-

polished Pasteur pipettes. Axon stumps and disrupted cells were removed by BSA gradient centrifugation (15% BSA, 120 g, 8 min). Viable cells were resuspended in Neurobasal A/B27 medium, plated in poly-L-ornithine (0.1 mg/ml)/laminin (5 µg/ml)-precoated 96-well imaging plates (Greiner, Kremsmünster, AU) and incubated overnight (37 °C, 5% CO_2). Neuron density was 1500 neurons/cm^2.

Stimulation of DRG neurons

DRG neurons were stimulated 24 hours after isolation in 96-well imaging plates. Compounds were dissolved in 12.5 µl PBS in 96-well V-bottom plates, mixed with 50 µl medium from the culture wells, and added back to the same wells. Stimulations were performed with automated 8 channel pipettes (Eppendorf, Hamburg, DE) at low dispense speed on heated blocks, stimulated cells were placed back in the incubator. The cells were fixed for 10 minutes at room temperature (RT) by adding 100 µl 8% paraformaldehyde resulting in a final concentration of 4%.

Immunofluorescence staining

Fixed cells were treated with goat serum blocking (2% goat serum, 1% BSA, 0.1% Triton X-100, 0.05% Tween 20, 1h, RT) and incubated with respective primary antibodies diluted in 1% BSA in PBS at 4 °C overnight. Subsequent to three washes with PBS (30 min, RT) cells were incubated with secondary Alexa dye-coupled antibodies (1:1000, 1h, RT). After three final washes (30 min, RT), wells of 96-well plates were filled with PBS, sealed, and stored at 4 °C until scanning.

Quantitative microscopy

Stained DRG cultures in 96-well plates were scanned using a Cellomics ArrayScan XTI with LED light source. Images of 1024 x 1024 pixels were acquired with a 10x objective and analyzed using the Cellomics software package. Briefly, images of UCHL1 stainings were background corrected (low pass filtration), converted to binary image masks (fixed threshold), segmented (geometric method), and neurons were identified by the object selection parameters size: 80-7500 μm^2, circularity (perimeter^2 / 4π area): 1-3, length-to-width ratio: 1-2, average intensity: 800-12000, and total intensity: $2x10^5$-$5x10^7$. These image masks were then overlaid on images obtained at other fluorescence wavelengths to quantify signal intensities. To calculate spill-over between fluorescence channels, three respective controls were prepared for each triple staining: (1) UCHL1 alone, (2) UCHL1 + antibody 1, and (3) UCHL1 + antibody 2. Raw fluorescence data of the controls were used to calculate the slope of best fit straight lines by linear regression, which was then used to compensate spill-over as described previously(38). Compensated data were scaled to a mean value of 1 (or 1000) for the unstimulated cells to adjust for variability between experimental days. One and two-dimensional probability density plots were generated using R packages(39). Gating of subpopulations was performed by setting thresholds at local minima of probability density plots. The mean number of analyzed neurons was 31,288 ± 3231 (L1-L6 only) per rat and 30965 ± 1438 per mouse (lumbar and thoracic DRGs).

Electrophysiology

All electrophysiological recordings were performed using an AxoPatch 200B amplifier and a Digidata 1440A digitiser (Axon Instruments), controlled by Clampex software (version 10, Molecular Devices). Filamented borosilicate microelectrodes (GC150TF-7.5, Harvard Apparatus) were coated with beeswax and fire polished using a microforge (Narishige) to give resistances of 2-3 MΩ. For voltage-clamp experiments, the following solutions were used. Extracellular solution (values are in mM): 70 NaCl, 70 Choline.Cl, 3 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 20 tetraethylammonium (TEA).Cl, 0.1 $CdCl_2$, 0.3 tetrodotoxin (TTX), 10 HEPES, 10 glucose, pH 7.3 with NaOH. Intracellular solution (values are in mM): 140 CsF, 1 EGTA, 10 NaCl, 10 HEPES, pH 7.3 with CsOH. Unless otherwise stated standard whole-cell currents were acquired at 25 kHz and filtered at 10 kHz (low-pass Bessel filter). After achieving whole-cell configuration the cell was left for five minutes to allow for dialysis of the intracellular solution. A holding potential of -100 mV was applied and series resistance was compensated by ≥ 70%. All currents were leak subtracted using a p/4 protocol. To record TTX-resistant sodium currents, a depolarising voltage-pulse protocol was applied to cell; the cell was held at -100 mV and then stepped to -15 mV for 50 ms before returning back to -100 mV. This step was applied every 5 seconds for the duration of the experiment. The cells were continuously perfused using a gravity-fed perfusion system. All electrophysiological data were extracted using Clampfit (version 10, Molecular Devices) and analyzed using GraphPad Prism software (version 6, GraphPad Software).

Statistical Analysis

Statistical analyses were performed with Students t-tests, one-, or two-way ANOVA with respective post hoc tests as indicated in the figure legends. $P < 0.05$ was considered as statistically significant. HCS dose-response curves were generated using non-linear regression curve-fitting (three parameter, standard Hill slope) with Prism (GraphPad, La Jolla, CA). The parameters of the model (top, bottom, or pEC_{50}/pIC_{50} values) were compared using the extra-sum-of-squares F test. HCS kinetic experiments were analyzed with R(39) using ordinary two-way ANOVA. Bonferroni's post hoc analysis was applied to determine *P* values of selected pairs defined in a contrast matrix using the R library multcomp. Error bars represent the standard error of the mean (SEM) of 3-5 independent replicate experiments using cells of different animals.

Supplementary Materials

Fig. S1. Opioids inhibit PGI_2 and forskolin induced pRII increase in rat sensory neurons.

Fig. S2. Downward-shift of the DAMGO dose-response in the presence of 5-HT.

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Figure Legends

Fig. 1. 5-HT induced increase of pRII is mediated by 5-HT$_4$ receptors and inhibited by opioids in rat sensory neurons. (A) Experimental outline for analyzing G_s-and G_i-coupled GPCR signaling in sensory neurons by HCS microscopy. **(B)** Representative images of control (Ctrl) and 5-HT stimulated rat sensory neurons. Green/red encircled neurons indicate automatically selected/rejected objects. Scale bar, 100 µm. **(C)** Dose response curve showing the increase of pRII intensities by 5-HT and the 5-HT$_4$-specific agonist SC-53116. **(D)** Induction of pRII by 5-HT was dose-dependently inhibited by the 5-HT$_4$-specific antagonist GR113808. **(E)** Fentanyl (Fent) and the MOR-specific agonist DAMGO (DA) dose-dependently inhibited 5-HT-induced pRII increases. **(F)** Time-course experiment indicating long-lasting inhibition of 5-HT (0.2 µM) induced pRII increase by fentanyl (0.1-10 µM). **(G)** The opioid receptor antagonist naltrexone (NTX, 10 µM) reversed the fentanyl-induced inhibition of pRII induction. **(H)** NTX did not affect baseline or 5-HT induced pRII intensity. **(I)** The µ opioid receptor antagonist CTOP (10 µM) partially reverted the inhibition of the 5-HT response by fentanyl. **(J)** CTOP did not alter baseline or 5-HT induced pRII intensity. **(K)** The MOR-specific agonist DAMGO (0.1-10µM) inhibited the pRII increase induced by 5-HT. **(L)** The inhibitory effect of DAMGO was fully reverted by NTX and **(M)** partially by CTOP. Values in (C-M) are means ± SEM; *n* = 3-4 independent experiments; >2000 neurons/condition; two-way ANOVA with Bonferroni's test; **P*<0.05; ***P*<0.01; ****P*<0.001 indicate significance levels between baseline and stimulated

conditions; $^{\S}P<0.05$; $^{\S\S}P<0.01$; $^{\S\S\S}P<0.001$ indicate significance levels between stimulated and inhibited conditions.

Fig. 2. Opioids inhibit pRII-increases selectively in RIIβ(+) sensory neurons of rats. (A) Cell density plots showing single cell data of pRII/RIIβ-labeled rat sensory neurons stimulated with Fsk (2 µM), 5-HT (0.2 µM), or PGI_2 (1 µM) in the absence (upper panel) or presence (lower panel) of fentanyl (2 µM). Dashed lines indicate gating thresholds to discriminate between RIIβ(-) and RIIβ(+) neurons with the numbers indicating the relative percentage of cells in the respective quadrant. Combined data of *n* = 4 experiments with a total of >8000 neurons/condition. **(B)** Compensation controls showing proper removal of spill-over between fluorescence channels. **(C, D)** Mean pRII intensities in RIIβ(-) and RIIβ(+) neurons. Values are means ± SEM; *n* = 4 independent experiments; >8000 neurons/condition; two-way ANOVA with Bonferroni's test; $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$.

Fig. 3. Loss of $Na_v1.7$ results in downregulation of 5-HT_4 receptor and reduced 5-HT induced pRII increase. (A, B) UCHL1 and pRII intensities in sensory neurons of $Na_v1.7$ and $Na_v1.8$ knockout mice analyzed by HCS microscopy. Representative images of control (Ctrl, **A**) and forskolin (Fsk, **B**) stimulated neurons are shown. Green/red encircled neurons indicate inclusion/rejection of objects for quantification. Scale bar, 100 µm. **(C, D)** Size and UCHL1 intensity distributions of sensory neurons did not differ between genotypes. Probability density estimates (PDE) were derived from $>10^5$ neurons analyzed in *n* = 4 independent experiments (2 males and 2 females per genotype). **(E, F)** Real-time PCR quantification of *Htr4* mRNA encoding 5-HT_4 receptors in $Na_v1.7^{-/-}$ and

664 $Na_v1.8^{-/-}$ mice versus wildtype controls. Data are means ± SEM; *n* = 6 (3 males and
665 3 females per genotype), Student's unpaired t-test. **(G)** Single cell data of
666 pRII/RIIβ-labeled sensory neurons after 3 min stimulation with increasing doses of
667 5-HT. Data represent >3000 neurons/condition from *n* = 5 females per genotype.
668 **(H)** pRII dose-response curve indicating a 37% reduction of the 5-HT response
669 amplitude in $Na_v1.7$-deficient sensory neurons, but pEC_{50} values were unchanged.
670 **(I-K)** Dose-responses of PGI_2 **(I)**, Fsk **(J)**, and Sp-8-Br-cAMPS-AM **(K)** were similar
671 in both genotypes. Data in (H-K) are means ± SEM; *n* = 5 females per genotype;
672 *P*<0.0001 for 5-HT top values; extra-sum-of-squares F test.

673 **Fig. 4. Sensory neurons of $Na_v1.7^{-/-}$, but not $Na_v1.8^{-/-}$ mice, have a lower basal**
674 **pRII level and respond stronger to the opioid receptor agonist fentanyl. (A)**
675 Distribution of RIIβ expression in sensory neurons of $Na_v1.7$-deficient mice (red
676 line) and wildtype litters (black line). **(B)** $Na_v1.7$-deficiency resulted in
677 downregulation of RIIβ in small-to-medium sized sensory neurons, but did not
678 reduce relative numbers of RIIβ(+) neurons. Data represent>10^5 neurons/plot; *n* =
679 5 females per genotype. **(C)** Mean RIIβ intensities were 11% lower in RIIβ(+)
680 neurons of $Na_v1.7^{-/-}$ mice compared to wildtype litters. Data are means ± SEM; *n* =
681 5 females per genotype, two-way ANOVA with Bonferroni's test, ****p<0.0001. **(D-**
682 **J)** Time-course of pRII intensity after stimulation with fentanyl (Fen, E, 2 µM),
683 naltrexone (NTX, F, 10 µM), serotonin (5-HT, G, 0.2 µM), combinations thereof (H,
684 I), or forskolin (Fsk, J, 10 µM). **(K-Q)** The phenotype observed in $Na_v1.7^{-/-}$ mice
685 was absent in $Na_v1.8^{-/-}$ mice. **(R)** Density plots of pRII intensity vs. cell size
686 showing single cell data of all neurons shown in (D-J). Data in (D-Q) are means ±

SEM; *n* = 4 (2 males and 2 females per genotype); >3000 neurons/condition; two-way ANOVA with Bonferroni's test; **P*<0.05; ***P*<0.01; ****P*<0.001.

Fig. 5. The inhibition of pRII increase by opioids is amplified in $Na_v1.7^{-/-}$ mice. **(A)** Dose-response of fentanyl in the presence of 5-HT (0.2 µM) indicating a downward shift in $Na_v1.7$-deficient sensory neurons versus wildtype litters. Data are means ± SEM; *n* = 4 females per genotype; *P*<0.0001 for whole curve; extra-sum-of-squares F test. **(B)** Combined single cell data of pRII/RIIβ-labeled sensory neurons shown in (A) representing >2500 neurons/condition. **(C)** Dose-response relationship of pRII intensities in sensory neurons of $Na_v1.7^{-/-}$ and wildtype (WT) litters stimulated with increasing doses of fentanyl in the presence of Fsk (2 µM). Data are means ± SEM; *n* = 4 females per genotype; *P*<0.0001 for bottom values; extra-sum-of-squares F test. **(D)** Combined single cell data of pRII/RIIβ-labeled sensory neurons shown in (C) representing >2500 neurons/condition.

Fig. 6. Effect of fentanyl on TTX-resistant Na+ currents from WT and $Na_v1.7^{-/-}$ sensory neurons. **(A)** Electrophysiological current recording showing TTX-resistant Na+ current before (black) and after (red) the application of fentanyl (100 nM) in wildtype (WT) and $Na_v1.7$-deficient DRG neurons. **(B)** Change in peak current (%) of TTX-resistant Na^+ current after the application of fentanyl in WT and $Na_v1.7^{-/-}$ DRG neurons. The inhibition of pRII increase by opioids is amplified in $Na_v1.7^{-/-}$ mice. **(C)** Average change in TTX-resistant Na+ peak current (%) in WT and $Na_v1.7^{-/-}$ DRG neurons after addition of 100 nM fentanyl. Data in (B, C) represent mean ± SEM; *n* = 9 neurons per genotype; Student's unpaired t-test; ****P*<0.001.

Fig. 7. Synergistic regulation of pro- and anti-nociceptive signaling in $Na_V1.7$-deficient mice. Nav1.7-deficiency results in downregulation of 5-HT_4 receptors and their downstream kinase PKA-RIIβ. Thereby, the pro-nociceptive input in sensory neurons (red) is strongly reduced. Simultaneously, the anti-nociceptive input (green) is increased due to enhanced opioid receptor activity and upregulation of endogenous opioid peptides (enkephalins). This synergistic regulation shifts the balance toward anti-nociceptive mechanisms and thus contributes to the pain-free phenotype in $Na_V1.7$-deficient mice.