

1 **Selective Activation of TASK-3-containing K⁺ Channels Reveals Their**
2 **Therapeutic Potentials in Analgesia**

3 Ping Liao^{1*}, Yunguang Qiu^{3,8*}, Yiqing Mo^{2*}, Jie Fu^{2*}, Zhenpeng Song^{1*}, Lu Huang^{1*},
4 Suwen Bai⁴, Yang Wang⁴, Jia-Jie Zhu⁵, Fuyun Tian³, Zhuo Chen¹, Nanfang Pan¹, Er-Yi
5 Sun¹, Linghui Yang¹, Xi Lan³, Yinbin Chen², Dongping Huang², Peihua Sun⁶, Lifen
6 Zhao³, Dehua Yang³, Weiqiang Lu², Tingting Yang⁷, Junjie Xiao⁷, Wei-Guang Li⁵,
7 Zhaobing Gao³, Bing Shen⁴, Qiansen Zhang², Jin Liu¹, Hualiang Jiang^{3,8}, Ruotian Jiang^{1†},
8 Huaiyu Yang^{2†}

9

10 ¹Laboratory of Anesthesia and Critical Care Medicine, Department of Anesthesiology,
11 Translational Neuroscience Center, West China Hospital of Sichuan University, Chengdu
12 610000, Sichuan, China.

13 ²Shanghai Key Laboratory of Regulatory Biology, Institute of Biomedical Sciences,
14 School of Life Sciences, East China Normal University, Shanghai 200241, China.

15 ³State Key Laboratory of Drug Research and CAS Key Laboratory of Receptor Research,
16 Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203,
17 China.

18 ⁴Department of Physiology, Anhui Medical University, Hefei 230032, Anhui, China.

19 ⁵Collaborative Innovation Center for Brain Science, Department of Anatomy and
20 Physiology, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China.

21 ⁶Jiangsu Key Laboratory of Neuropsychiatric Diseases and College of Pharmaceutical
22 Sciences, Soochow University, Suzhou, Jiangsu 215123, China

23 ⁷Cardiac Regeneration and Ageing Lab, School of Life Science, Shanghai University,
24 Shanghai 200444, China.

25 ⁸University of Chinese Academy of Sciences, Beijing 100049, China

26

27 * These authors contributed equally to this work.

28 †Corresponding authors. hyyang@bio.ecnu.edu.cn for H.Y.; ruotianjiang@scu.edu.cn for
29 R.J..

30

31 **ABSTRACT**

32 **The paucity of selective agonists for TASK-3, a member of two-pore domain K⁺**
33 **(K2P) channels, limited our understanding of its biological functions. Targeting a**
34 **novel druggable transmembrane cavity using structure-based drug design approach,**
35 **we discovered a biguanide compound CHET3 as a highly selective allosteric**
36 **activator for TASK-3-containing K2P channels including TASK-3 homomer and**
37 **TASK-3/TASK-1 heteromer. CHET3 displayed unexpectedly potent analgesia *in vivo***
38 **in a variety of acute and chronic pain models in rodents that could be abolished by**
39 **pharmacology or genetic ablation of TASK-3. We further found that**
40 **TASK-3-containing channels anatomically define a unique subset population of**
41 **small-sized, TRPM8, TRPV1 or Tyrosine Hydroxylase positive nociceptive sensory**
42 **neurons and functionally regulate their membrane excitability, supporting the**
43 **CHET3 analgesia in thermal hyperalgesia and mechanical allodynia under chronic**

44 **pain. Overall, our proof-of-concept study reveals TASK-3-containing K2P channels**
45 **as a novel druggable target for treating pain.**

46

47 **One Sentence Summary:** Identification of a novel drug target and its new hit
48 compounds for developing new-generation non-opioid analgesics.

49

50 **INTRODUCTION**

51 Currently available analgesics do not treat pain completely and some of them, particularly
52 opioids, provoke social problems (1). It is an urgent need to discover new therapeutic
53 targets for developing new-generation analgesics. In particular, targets that treat a variety
54 of pain with similar potency but less side effects compared to μ -opioid receptor are
55 keenly awaited. In this regard, two-pore domain K^+ (K2P) channels hold great promise
56 (2), since they produce background leak K^+ currents (3) and the activation of which in
57 nociceptors theoretically inhibits pain signaling (4-6). The expression of TASK-3 (*Kcnk9*),
58 a K2P channel, has been detected in peripheral and central nervous system (7, 8)
59 including in human dorsal root ganglia (9). Recent evidence suggested that TASK-3 is
60 involved in perception of cold (10), and the variations in *Kcnk9* gene are associated with
61 breast pain in breast cancer patients (11). However, its functional and anatomical
62 involvement in chronic pain remain largely unknown. More importantly, the paucity of
63 selective agonists limits the drug target validation of TASK-3, leaving the notion that

64 selective activation of TASK-3 alleviates pain hypothetical. Here we sought to discover
65 selective activators for TASK-3 and to use the activators as tool compounds to reveal and
66 the translational potentials and the underlying mechanisms of TASK-3 in treating pain.

67

68 **RESULTS**

69 **Discovery of selective activator CHET3 for TASK-3-containing channels**

70 We set out to discover selective activators for TASK-3 via structure-based virtual
71 screening. Since no crystal structure of TASK-3 has been determined yet, we sought to
72 build a structural model using homology modeling. Firstly, a crystal structure was chosen
73 as the template. To this end, Fpocket 2.0 server (12) was applied to detect pockets in the
74 reported crystal structures of K2P channels. In this computation, druggability score more
75 than 0.5 (the threshold) means the pocket might be druggable. We found that the cavity
76 under the intracellular side of the filter and the nearby crevice between TM2 and TM4 in
77 four crystal structures (with PDB codes 4RUE, 3UKM, 4XDK and 4XDL) (13-15) have
78 druggability scores more than 0.5 (fig. S1). Thus, this cavity may be a drug binding
79 pocket. Among the four crystal structures, the structure of TREK-2 channel (PDB code
80 4XDL) is a suitable template for building the structural model of TASK-3, because this
81 structure has good sequence identity (31%) and expectation value (3E-32) in the
82 sequence alignment generated using BLAST program (blastp algorithm) and Clustal
83 Omega server (16, 17). Moreover, this TREK-2 structure stood out from the template

84 searching results (with the best QSQE value: 0.66) in SWISS-MODEL server (18, 19).
85 Thus, the structural model of TASK-3 was built based on this crystal structure with
86 Modeller (20). Then, based on this model, we performed virtual screening targeting the
87 pocket (Fig. 1, A and B) with SPECS and ChemBridge databases. A few hits were
88 selected for the whole-cell patch-clamp electrophysiological tests in HEK-293 cells
89 overexpressing recombinant human TASK-3, which led to the discovery of a biguanide
90 compound CHET3, a novel TASK-3 activator (half-maximum effective concentration
91 (EC_{50}) $1.4 \pm 0.2 \mu\text{M}$, Fig. 1, C to F). CHET3 enhanced TASK-3-mediated K^+ currents
92 maximally by ~4-fold which can be reversed by washout (Fig. 1D) or with
93 pharmacological blockade by PK-THPP ($86 \pm 3\%$ inhibition at $0.5 \mu\text{M}$, $n = 6$ cells,
94 representative traces shown in Fig. 1E), a TASK-3 specific inhibitor (21). In
95 single-channel recordings by inside-out patches, CHET3 enhanced the channel openings
96 mediated by TASK-3 (Fig. 1, G to K), further supporting that CHET3 directly activated
97 TASK-3. Increases in open probability and conductance were observed in both the inward
98 and outward directions in response to $3 \mu\text{M}$ CHET3 (Fig. 1, I to K).

99 In addition to forming homomer channels, TASK-3 subunit can efficiently form
100 heteromer channels with TASK-1 subunit (22). Electrophysiological assays showed that
101 CHET3 could activate TASK-3/TASK-1 (23, 24) with an EC_{50} value of $2.5 \pm 0.2 \mu\text{M}$
102 with a reduced maximal efficacy of ~2.4 fold (Fig. 1F), which were also blocked by
103 PK-THPP ($90 \pm 2\%$ inhibition at $0.5 \mu\text{M}$, $n = 5$ cells, representative traces shown in fig.

104 S2A). However, CHET3 did not activate TASK-1 channels up to 10 μ M (Fig. 1F and fig.
105 S2B). Thus CHET3 is an activator specific for TASK-3 homomer and TASK-3/TASK-1
106 heteromer, two TASK-3-containing channels, with high selectivity against the structurally
107 most related K⁺ channel TASK-1. In the subsequent sections, we use TASK-3-containing
108 channels to represent TASK-3 homomer and TASK-3/TASK-1 heteromer.

109 Next, we further examined the selectivity of CHET3. Electrophysiological assays on
110 several other K2P channels, including TREK-1, TREK-2, TRAAK, TRESK and THIK-1,
111 further supported that CHET3 has high subtype selectivity among the K2P family (Fig.
112 1L and fig. S2B). Further, we found that 10 μ M CHET3 has high selectivity against
113 human ether-à-go-go-related gene (hERG) channel, voltage-gated K⁺ channel subfamily
114 B member 1 (Kv2.1), large conductance Ca²⁺-activated K⁺ channel (BK), three K⁺
115 channels sharing similarity with K2P in filter structure and dynamics (25), and transient
116 receptor potential cation channel subfamily M member 8 (TRPM8) and transient receptor
117 potential cation channel subfamily V member 1 (TRPV1) (Fig. 1M, fig. S2, C to G).

118 We also excluded agonizing and antagonizing functions of CHET3 on pain-related G
119 protein-coupled receptors (GPCRs) by testing the cellular function of μ -opioid receptor
120 (μ OR), 5-hydroxytryptamine receptor 1B (5-HT_{1B}R) and cannabinoid receptor type 1
121 (CB₁R) upon 10 μ M CHET3 (fig. S3). Collectively, these results indicate that CHET3 is a
122 selective activator of TASK-3-containing channels.

123 **Activation mechanism of CHET3**

124 Binding models derived from docking simulation were optimized by molecular
125 dynamics (MD) simulations (fig. S4), which revealed the predominant binding mode of
126 CHET3 within the pocket (Fig. 2, A and B). We next examine the ligand-channel
127 interactions in this binding mode using mutagenesis experiments and RosettaLigand (26,
128 27). Residues T93 and T199 indirectly interacts with CHET3 by water bridges (Fig. 2, A
129 and B). The two residues belong to the filter region, and the mutations T93A and T199A
130 led to non-functional channels (fig. S5, A and B). F125 may form a π - π interaction with
131 CHET3, and other surrounding residues, including I118, F125, T198, L232, I235, F238
132 and L239, likely contribute hydrophobic interactions with the ligand. RosettaLigand
133 computations based on this MD mode predicted that the I118A, F125A, L232A, I235A,
134 F238A and L239A mutants decrease CHET3 binding, while the T198A mutant should not
135 (Fig. 2C). Indeed, a saturating concentration of CHET3 (10 μ M) showed no activation on
136 the mutants of F125A, I235A, F238A and reduced activation on mutant L239A, whereas
137 CHET3 did activate T198A similarly to the WT (Fig. 2D). Mutant L232A is
138 non-functional (fig. S5C). Although CHET3 did not show reduced activation on I118A
139 (Fig. 2D), the experimental results are generally consistent with the computational
140 predictions. To gain further insights into action mechanism of CHET3, MD simulations
141 were carried out on the *apo* TASK-3 to compare with the CHET3-bound TASK-3 (Fig. 2,
142 E and F). In two out of three independent simulations for the *apo* system, the channel
143 selectivity filter tended to stay in a nonconductive-like conformational state (Fig. 2, E and

144 F). By contrast, in all the three simulations for the CHET3-bound system, the channel
145 filter adopted a conductive-like state (Fig. 2, E and F). Further, our simulations supported
146 the previous report by González *et al.* that the residue H98 plays roles in modulating the
147 extracellular ion pathway in TASK-3 (28). In the simulations of the CHET3-bound
148 TASK-3, H98 adopted a conformation to open the extracellular ion pathway (Fig. 2E and
149 fig. S6A). By contrast, in ligand-free mode, H98 has a high probability to adopt a
150 conformation to close this pathway (Fig. 2E and fig. S6B).

151 **CHET3-induced analgesia in rodents**

152 Next, we evaluated CHET3 in analgesia systematically. The anti-nociceptive effect of
153 CHET3 was firstly assessed by the tail immersion test at 52 °C. CHET3 displayed
154 dose-dependent analgesia with a fast onset (30 min) after intraperitoneal (i.p.) injection
155 with a maximal effect at a dose of 10 mg/kg (Fig. 3A). Hereafter, i.p. injection with 10
156 mg/kg was used for most of the following animal studies. Interestingly, CHET3 was only
157 effective in response to a noxious cold stimulus (5 °C) or noxious heat stimuli (46 °C and
158 52 °C) but not to physiological stimuli (20 °C and 40 °C) (Fig. 3A). Importantly, the
159 CHET3 analgesia was fully blocked by the co-administration of PK-THPP, and PK-THPP
160 alone also produced nociceptive effect in tail immersion test at 46 °C (Fig. 3A). Next,
161 both the early and the late phases (29) of acute inflammatory pain induced by formalin
162 were attenuated by CHET3 (Fig. 3B), suggesting at least a peripheral effect of CHET3.
163 CHET3 reduced mechanical pain revealed by paw pressure test in mice, and the effect

164 was fully blocked by PK-THPP (Fig. 3C). Next, we evaluated the analgesic effects of
165 CHET3 on chronic pathological pain. In the spared nerve injury (SNI)-induced
166 neuropathic pain mouse model, CHET3 significantly reduced the frequency of hind paw
167 lifting (Fig. 3D), an indicator of spontaneous/ongoing pain behavior in SNI model (30).
168 In the cold plantar test, CHET3 attenuated the cold hyperalgesia in SNI at the
169 development (SNI 7 d) and maintenance (SNI 14 d and 21 d) stages of the chronic pain,
170 which could be reversed by PK-THPP. PK-THPP alone, however, had no effect in cold
171 plantar test in SNI mice (Fig. 3E). Compared to Pregabalin, a first line agent for the
172 treatment of neuropathic pain, CHET3 was more effective in relieving cold hyperalgesia
173 (Fig. 3F). In SNI mice, CHET3 has little effect on alleviating mechanical allodynia in the
174 von Frey test. However, in SNI rats, CHET3 at the dose of 10 mg/kg attenuated the
175 mechanical allodynia throughout the different stages of the chronic pain in the von Frey
176 test, which could be reversed by PK-THPP (Fig. 3G). PK-THPP alone had no effect in the
177 von Frey test in SNI rats (Fig. 3G). The analgesic effect of CHET3 at the dose of 20
178 mg/kg exhibited a faster onset (30 min post injection) with similar efficacy compared to
179 these at 10 mg/kg (fig. S7) in the von Frey test in SNI rats. In chronic inflammatory pain
180 induced by the Complete Freund's Adjuvant (CFA), CHET3 reduced heat hyperalgesia in
181 the Hargreaves test, which was blocked by PK-THPP (Fig. 3H). In addition, PK-THPP
182 injection alone aggravated heat hyperalgesia (Fig. 3H).

183 Chronic pain may induce anxiety (31, 32). Compared with the Sham group, the SNI

184 mice spent less time in open arms in the elevated plus maze test, and spent less time in
185 the light box in the light/dark box test, suggesting anxiety-like behaviors in the SNI mice.
186 Administration of CHET3 30 min before the test significantly alleviated anxiety-like
187 behaviors in both tests (Fig. 3, I and J). Together, our data suggest CHET3 potently and
188 efficaciously attenuated acute and chronic pain and pain-associated anxiety in rodents,
189 and the analgesic effects of CHET3 can be pharmacologically blocked by the TASK-3
190 blocker PK-THPP. Importantly, CHET3 was inactive in grip strength, rotarod and open
191 field tests (fig. S8, A to C), suggesting that CHET3 had no effect on the locomotion
192 activities in mice. Since TASK-3 was found to be expressed in mouse carotid body type-1
193 cells (33), we also evaluated the possible side effects of CHET3 on the cardiovascular
194 functions in mice or rats. We monitored the blood pressure as well as heart functions
195 using echocardiography, and we did not observe any significant change in blood pressure
196 (fig. S8, D to F) or heart functions including Ejection Fraction (EF) and Fractional
197 Shortening (FS) (table S1) in a post-injection time window between 45 min–90 min
198 where CHET3-induced analgesia peaked in most cases. We also monitor the body
199 temperature change following CHET3 systemic administration, and no significant
200 hyperthermia or hypothermia was observed (fig. S8G).

201 **Further on-target validation using chemical and genetic approaches**

202 Was CHET3 truly targeting TASK-3 containing K⁺ channels to be analgesic? We then
203 performed additional target validation experiments using chemical and genetic

204 approaches. Medicinal chemistry yielded CHET3-1 and CHET3-2 (Fig. 4A), two
205 derivatives of CHET3. In the CHET3-TASK-3 binding model (Fig. 2, A to B), the
206 dioxane ring may form a π - π interaction with the residue F125. CHET3-1, where the
207 dioxane ring was replaced with an aromatic ring, should keep the π - π interaction.
208 CHET3-2 should lose it since the dioxane was replaced by a steric bulk *tert*-butyl group.
209 Binding energy computations based on the binding model suggested that the binding
210 affinity of CHET3-1 to TASK-3 was similar with CHET3, while that of CHET3-2 should
211 decrease (fig. S9). In accord, CHET3-1 activated TASK-3 with an EC₅₀ value of 0.5 ± 0.1
212 μ M, while CHET3-2 was inactive (Fig. 4B), further supporting the putative binding
213 model. We reasoned that CHET3-1 should be bioactive in analgesia whereas CHET3-2
214 should not, if CHET3 truly targets TASK-3-containing channels to be analgesic. Indeed,
215 CHET3-1 attenuated cold hyperalgesia in SNI mice (Fig. 4C) and mechanical allodynia
216 in SNI rats (Fig. 4D), and heat hyperalgesia in CFA mice (Fig. 4E), all these effects could
217 be reversed by PK-THPP (fig. S10). In contrast, CHET3-2 was completely inactive in all
218 the experiments above (Fig. 4, C to E).

219 We generated the *Kcnk9* gene knockout (TASK-3 KO) mice (fig. S11). Knocking out
220 *Kcnk9* should abolish the function of TASK-3 homomer and TASK-3/TASK-1 heteromer
221 *in vivo*. In the TASK-3 KO and their WT control mice, we measured the basal sensitivity
222 to nociception, thermal hyperalgesia and mechanical allodynia, and we also evaluated the
223 analgesic effect of CHET3 in these mice. Tail immersion (Fig. 5, A and B), paw pressure

224 tests (Fig. 5C) or von Frey tests in naive animals (Pre SNI, Fig. 5D) did not reveal any
225 significant difference in baseline nociceptive sensitivity between TASK-3 KO and WT;
226 however, cold plantar (Pre SNI, Fig. 5F) and Hargreaves tests (Pre CFA, Fig. 5G)
227 revealed increased nociceptive cold and heat sensitivity in TASK-3 KO mice.
228 Furthermore, in the chronic pain models, von Frey, cold plantar and Hargreaves tests
229 revealed that TASK-3 KO mice exhibited aggravated mechanical allodynia (Fig 5D),
230 spontaneous neuropathic pain behavior (Fig. 5E), and thermal hyperalgesia (Fig. 5, F and
231 G). CHET3, as expected, in TASK-3 KO mice, was completely inactive in all the tests
232 described above (Fig. 5, A to G). Thus, using tool compounds (Fig. 4) and mouse genetics
233 (Fig. 5), we provided strong evidence to show that CHET3 targets TASK-3-containing
234 channels to be analgesic, and the loss of TASK-3 contributed to the
235 generation/maintenance of both acute and chronic pain.

236 **Distribution of TASK-3-containing channels in sensory neurons**

237 Our pharmacokinetic profile of CHET3 (table S2) showed a negligible brain
238 concentration ($C_{\max} = 79.1$ ng/mL) of CHET3 and a high concentration in the plasma (C_{\max}
239 = 1112.0 ng/mL), suggesting CHET3 mainly acted peripherally. The peripheral effect of
240 CHET3 was also supported by the fact that CHET3 attenuated the early phase of
241 formalin-induced pain (Fig. 3B). These along with the previous finding that TASK-3 in
242 dorsal root ganglion (DRG) neurons mediates cold perception (10) together strongly
243 suggest that peripheral TASK-3-containing channels contribute largely, if not all, to

244 CHET3 analgesia. Therefore, we evaluated the TASK-3 functions/distributions in
245 peripheral nervous system, particularly in DRG.

246 We used fluorescence in situ hybridization (ISH) (RNAscope technique) to map the
247 mRNA expression of TASK-3 in DRG and trigeminal ganglia (TG). The specificity of the
248 fluorescent signals was validated by positive control probe and negative control probe
249 (see methods). *Kcnk9* was identified in a subset of neurons (~7% of total neurons) in
250 DRG, predominantly in small-sized neurons (diameter $\leq 20 \mu\text{m}$) (Fig. 6, A and C),
251 indicative of its specific expression in nociceptors. Interestingly, in TG, a much higher
252 expression level of *Kcnk9* (~14% of total neurons) was found (Fig. 6, A and B). In DRG,
253 approximately 95% of *Kcnk9*⁺ neurons express TASK-1 subunit, suggesting a possible
254 formation of TASK-3/TASK-1 heteromer in DRG, and approximately 50% of *Kcnk9*⁺
255 neurons express TRPV1, a well-known noxious heat sensor predominantly expressed in
256 peptidergic nociceptive sensory neurons (34). More than 95% of *Kcnk9*⁺ neurons express
257 TRPM8, and very little *Kcnk9*⁺ neurons express TRPA1, two well-known noxious cold
258 sensors (34). Further, approximately 50% of *Kcnk9*⁺ neurons express Tyrosine
259 Hydroxylase (TH), a marker for c-low threshold mechanoreceptors (c-LTMRs)
260 predominantly found in non-peptidergic nociceptors (35), whereas *Kcnk9* rarely
261 colocalized with *P2rx3* (P2X3), which labels mainly TH⁻ negative, IB4⁺ non-peptidergic
262 nociceptors (36), nor did they colocalize with *Ntrk2* (TrkB) a marker for A δ -LTMRs (35).
263 Thus, TASK-3 marks a unique subpopulation of both peptidergic and non-peptidergic

264 nociceptive sensory neurons enriched in thermal sensors (TRPV1, TRPM8) or
265 mechanoreceptors (TH⁺ c-LTMRs) (Fig. 6, D to F), in line with its functional
266 involvement in thermal and mechanical sensation *in vivo*. In line with the previous study
267 (37), we found that *Kcnk9* expression was down regulated in SNI mice and CFA mice (fig.
268 S12), further suggesting the down-regulation of TASK-3-containing channels contributes
269 to the generation/maintenance of chronic pain.

270 **Functional roles of TASK-3-containing channels in nociceptive neurons**

271 The functional roles of TASK-3-containing channels were examined by whole-cell
272 patch-clamp recordings in dissociated DRG neurons. Recordings were focused on small
273 sized DRG neurons (diameter of ~20 μ m, cell capacitance of ~30 pF) based on the ISH
274 data. To isolate K⁺ currents, the voltage ramps from -120 mV to -30 mV were applied. In
275 total 89 cells recorded, 16 cells responded to CHET3 ($20.3 \pm 6.3\%$, 11 mice). In the
276 CHET3-sensitive cells, CHET3 enhanced the whole cell current density by approximately
277 18%, which could be further inhibited by PK-THPP by approximately 38% at -30 mV
278 (Fig. 7A). We subtracted the CHET3-sensitive current, and we found this current was
279 strongly outwardly rectifying, and was tiny between -120 mV and -60 mV, leaving the
280 reversal potential of the CHET3-sensitive current difficult to be resolved (Fig. 7B). We
281 further sought to isolate current carried by TASK-3-containing channels by subtracting
282 PK-THPP-sensitive current, and consistently, we found a similar profile for
283 PK-THPP-sensitive current (fig. S13A), further suggesting the low basal conductance at

284 the hyperpolarized membrane potentials and strong outwardly rectifying represents an
285 intrinsic property of K^+ currents mediated by TASK-3-containing channels in DRG under
286 our experimental conditions. To increase the drive force of the K^+ currents in the
287 hyperpolarized potentials, we increased the extracellular K^+ concentration to 143 mM.
288 Under this condition, the CHET3-sensitive current was reversed at around 6.7 mV, which
289 was close to the theoretical value of 1.5 mV for K^+ conductance (Fig. 7B).

290 The tiny CHET3- or PK-THPP-mediated currents around -60 mV suggests that the
291 basal activity of TASK-3-containing channels around the resting membrane potentials
292 (RMP) range was low, and thus that CHET3 or PK-THPP was unlikely to be able to
293 regulate the RMP. To systematically evaluate the regulatory role of CHET3 on the
294 excitability of nociceptive neurons, first, we applied a cocktail solution containing
295 Menthol and Capsaicin, two agonists for TRPM8 and TRPV1, respectively, to better
296 identify the nociceptive neurons that likely express TASK-3-containing channels. Only
297 neurons responding to the cocktail (fig. S13B) were furthered studied in the following
298 experiments. Consistent with low activity of TASK-3-containing channels around -60 mV,
299 application of CHET3 or PK-THPP or Vehicle (Control) did not hyperpolarize the RMP,
300 rather, they all slightly depolarized it by ~2 mV with no significant difference among the
301 three groups, suggesting CHET3 or PK-THPP had no specific roles in altering RMP (Fig.
302 7C). Next, we explored how CHET3 regulates action potentials. In 12 out of 27 neurons,
303 application of CHET3 markedly increased by ~70% the rheobase required for eliciting

304 the action potentials (APs) and decreased by ~65% the frequency of APs evoked by
305 suprathreshold current injections (Fig. 7, D and E), whereas in the other 15 cells, CHET3
306 had no effect on the rheobase and slightly increased by 10% the frequency of APs evoked
307 by suprathreshold current injections (fig. S13, C and D). In 7 out of these 12
308 CHET3-sensitive cells, we were able to further apply PK-THPP, which reversed the
309 effects of CHET3 (fig. S13, E and F). Furthermore, in another independent set of
310 experiment, we co-applied CHET3 and PK-THPP in naive cells. In 11 out of 27 cells, the
311 co-application of CHET3 and PK-THPP markedly decreased by ~40% the rheobase and
312 increased by ~50% the frequency of APs evoked by suprathreshold current injections (Fig.
313 7, F and G), whereas in the other 16 cells, co-application of CHET3 and PK-THPP
314 slightly increased the rheobase by ~20% but had no effect on the APs frequency evoked
315 by suprathreshold current injections (fig. S13, G and H). Collectively, our
316 electrophysiological data suggest the functional presence of K⁺ currents mediated by
317 TASK-3-containing channels, and the enhancement of which reduces the excitability of
318 nociceptive neurons without affecting the RMP.

319 Last, Ca²⁺ imaging experiments were performed in acutely dissociated DRG neurons to
320 measure how the activation of TASK-3-containing channels contributes to the thermal
321 sensitivity of DRG neurons. Thermal stimulations elicited Ca²⁺ signals in a portion of
322 small-sized DRG neurons (Fig. 7H, cells having ratio $F_{340}/F_{380} \geq 0.2$ were considered as
323 responding cells shown in black, and those having ratio $F_{340}/F_{380} < 0.2$ were considered as

324 non-responding cells shown in grey). We confirmed that these Ca²⁺ signals were
325 temperature-dependent and were mediated by TRP channels as the heat-induced
326 responses can be blocked by 5 μM AMG9810 (TRPV1 antagonist) (fig. S14, A and B),
327 and the cool-induced responses can be blocked by 10 μM BCTC (TRPM8 antagonist) and
328 20 μM HC030031 (TRPA1 blocker) (fig. S14, A and B). Bath application of 10 μM
329 CHET3 significantly and markedly inhibited the Ca²⁺ signals evoked by cool- or heat-
330 stimulations in small-sized DRG neurons (Fig. 7, H and I), suggesting the activation of
331 TASK-3-containing channels was able to lower the excitability of the nociceptive neurons
332 in response to external thermal stimulations.

333

334 **DISCUSSION**

335 The current study has three major findings: first, we discovered selective agonists for
336 TASK-3-containing channels by targeting a transmembrane cavity under the selectivity
337 filter using the structure-based approaches. Second, *in vivo* activation of peripheral
338 TASK-3-containing channels displayed potent analgesia, suggesting a TASK-3-based
339 therapeutic strategy for treating chronic inflammatory and neuropathic pain. Third, our
340 anatomical and functional data highlight the roles of peripheral TASK-3-containing
341 channels in controlling the excitability of nociceptive neurons.

342 Very recently, Schewe *et al.* reported a class of negatively charged activators (NCAs)
343 that could activate K2P channel, hERG channel and BK channel and revealed that the site

344 below the selectivity filter is the binding site of the NCAs (25). In the present work we
345 obtained CHET3 acting on this site, a non-charged compound, by virtual screening,
346 further supporting the finding that the site below the selectivity filter is a ligand binding
347 site. It is noteworthy that NCAs are non-selective activators for a variety K^+ channels
348 while CHET3 is highly selective for TASK-3-containing channels, suggesting the
349 versatility of this binding site. Plus, NCAs and CHET3 may share some common
350 activation mechanisms on K2P channels by both influencing the conformation of
351 selectivity filter. Notably, the activation mechanism we provided in this study could not
352 fully explain the selectivity of CHET3 at present. In particular, TASK-1 and TASK-3 are
353 closest relative to each other, and the residues below selectivity filter are conserved as
354 well as H98. Further studies to elucidate the differential responses of TASK-1 and
355 TASK-3 to CHET3 may be helpful for understanding the selective modulation principle
356 in K2P.

357 In most cases, the initial proof-of-concept identification of a protein as a potential
358 target is dependent on genetic methods. However, genetic deletion may produce
359 modifications on other genes. This off-target genetic side effects discredit target
360 validation work. This is particularly the case in the field of pain medicine: genetically
361 mutated mice, e.g., $Na_v1.7$ -null mice, or human exhibited remarkable insensitivity to pain,
362 whereas potent selective antagonists have weak analgesic activity (38, 39). Another
363 example more related to K2P field is that migraine-associated TRESK mutations lead to

364 the inhibition of TREK-1 and TREK-2 through frame shift mutation-induced alternative
365 translation initiation (fsATI) to increase sensory neuron excitability and is linked to
366 migraine (40). Using chemical probes to validate targets pave another way for the later
367 translational research. For in *vivo* applications of chemical probes in target identification
368 and validation, a major issue is whether the observed phenotypes are indeed relevant to
369 the on-target of the probes. In this study, we provided three independent lines of evidence
370 to show CHET3 targets TASK-3-containing channels to be analgesic. First, TASK-3
371 inhibitor PK-THPP could block the CHET-3 induced analgesia. Second, two structurally
372 highly close analogs were discovered and used in *in vivo* tests. CHET3-1, a TASK-3
373 activator structurally highly close to CHET3, is bioactive in analgesia, which could be
374 also blocked by PK-THPP. CHET3-2, another analog structurally highly close to CHET3
375 inactive on TASK-3, was completely inactive in all the analgesia tests. Last, CHET3 was
376 completely inactive in analgesia in TASK-3 KO mice in all the tests. Collectively, our
377 data suggest that the on-target activity of CHET3 links to the analgesic phenotypes.

378 Although CHET3 has a higher activation efficacy on TASK-3 over TASK-3/TASK-1,
379 we suggest that both TASK-3 homomer and TASK-3/TASK-1 heteromer channels likely
380 contribute to CHET3-induced analgesia based on the following reasons: 1) *Kcnk9* is
381 highly colocalized with *Kcnk3* in DRG; 2) TASK-3/TASK-1 heteromer has been found
382 assembled efficiently and functionally in cerebellar granule cells (41), motoneurons (42)
383 and carotid body glomus cells (43).

384 We found that CHET3 decreased the excitability with no change in RMP of the
385 nociceptive neurons. No change of RMP could be well explained by the fact that
386 CHET3-or PK-THPP-mediated currents are negligible around -60 mV. One may argue
387 that there may be a strong depolarizing “off-target” activity of CHET3 through another
388 unknown channel/receptor, thereby masking the hyperpolarizing effect mediated by
389 CHET3 on TASK-3 containing channels. However, if this were the case, one would at
390 least expect PK-THPP to depolarize the RMP, since PK-THPP, a molecule structurally
391 distinct to CHET3, is unlikely produce the hyperpolarizing “off-target” activity through
392 the same unknown channel/receptor.

393 CHET3 acted mainly on peripheral TASK-3-containing channels. Peripheral targets
394 are much less likely to produce central side effects including dependence/addiction.
395 Although the utility of CHET3 and its derivatives as pre-clinical candidate compounds
396 require to be further assessed with systematically non-clinical safety tests performed in
397 GLP (Good Laboratory Practice) in rodents and other animals, it seems that the activation
398 of peripheral TASK-3-containing channels does not produce obvious severe acute side
399 effects on cardiovascular system where TASK-3-containing channels were also expressed.
400 Interestingly, we found that TASK-3 was expressed in TG with a higher expression rate
401 than DRG. Further studies are needed to evaluate the translational potentials of activation
402 TASK-3 (TASK-3/TASK-1) in TG to treat chronic pain related to trigeminal neuralgia
403 and migraine. Last, although TASK-3 was found expressed in human DRG (9) and

404 variation of *KCNK9* was involved in breast pain in breast cancer patients (11), direct
405 evidence for functional involvement of TASK-3 in pain signaling in human remains
406 lacking. Future functional studies on human tissues or studies with genetic screening of
407 TASK-3-related mutations in human would greatly aid the assessment of the translational
408 potential of TASK-3 in treating pain in human.

409

410 **MATERIALS AND METHODS**

411 **Study design**

412 Structure-based drug design methods were used to perform initial virtual screening and
413 patch-clamp electrophysiology was mainly used to study the activity/mechanism of
414 candidate compounds on TASK-3-containing channels. Analgesic effects of TASK-3
415 activators were then studied in acute and chronic pain models in mice and rats.
416 Pharmacokinetic analysis was performed to assess how CHET3 was distributed. KO mice
417 were used to confirm the on-target activity of CHET3. Last, *in situ* hybridization with
418 RNAscope technique was used to map the distribution of TASK-3 in DRG and TG. The
419 functional roles of TASK-3 were assessed by measuring how CHET3 and PK-THPP
420 modulate the K⁺ currents, action potential firings and sensitivities to thermal stimulations
421 in nociceptive neurons.

422 Sample size and replicates: For mutations of TASK-3 led to no-functional currents, 3
423 cells per mutation were tested. For other studies of ion channels in cells including these in

424 cell line system and acutely prepared DRG cells, at least 5 cells per condition were tested.

425 For experiments in DRG neurons, at least 3 independent preparations of DRG culture

426 were performed. For *in vivo* studies with animals, 6-10 animals per condition were used.

427 No power analysis was performed to determine the sample size.

428 **Homology modeling for TASK-3 structure**

429 Sequence alignment was generated by using Clustal Omega server (16). Notably, the two

430 pore domain and selectivity filter sequence motif were highly conserved among the K2P

431 channels, which were largely used to guide the alignment. Conserved residues E30 and

432 W78 in TASK-3 helped to locate the position of non-conserved cap domain.

433 **Virtual screening**

434 Docking was performed by using Schrödinger Glide software (New York, NY, USA).

435 Compounds were screened using the high-throughput virtual screening (HVS) module

436 followed by a standard docking module SP in Glide. Glide G-score was used to rank the

437 result list. Allowed for diversities of molecule structure, binding mode and drug-like

438 properties, twelve hits were selected for bioassay.

439 **Chemicals**

440 PK-THPP was purchased from Axon Medchem. CHET3 purchased from commercial

441 sources was used in the initial electrophysiological screening. Then CHET3 was

442 synthesized in the lab for the following studies in this paper. Synthesis routes and

443 characterization of CHET3 and its derivatives CHET3-1 and CHET3-2 are outlined in the

444 Supplementary Materials.

445 For electrophysiology, stock solutions of CHET3 and derivatives (50 mM) were
446 prepared in dimethyl sulfoxide (DMSO) and diluted in the extracellular solution before
447 use.

448 For animal studies, CHET3 and PK-THPP were both dissolved in 10% DMSO, 5%
449 tween80 and 85% saline, CHET3-1 was dissolved in 10% DMSO, 5% Castor Oil
450 Ethoxylated, 35% Poly (Ethylene Glycol) and 50% corn oil, and CHET3-2 was dissolved
451 in 14% DMSO, 5% tween80 and 81% saline. The solvents were used as vehicle controls.

452 **Detailed modeling of the CHET3-TASK-3 binding poses**

453 Initially, the configuration of CHET3 was determined by Ligprep module in the
454 Schrödinger Maestro and Gaussain09 (Gaussian, Inc). Detailed descriptions are displayed
455 in the Supplementary Materials. The configuration of tautomer with lowest energy was
456 adopted to generate multiple ring conformations. CHET3 conformations were docked to
457 the TASK-3 channel model by standard Glide as described for the virtual screening. Two
458 binding modes (G-score values at -8.3 and -7.9, separately) were obtained from docking.
459 In the best pose (1st model in fig. S4A), the guanidyl group in CHET3 establishes
460 hydrogen bond with backbone NH of L232 residues in TM2, while the guanidyl group in
461 the additional mode of binding (2nd model in fig. S4B) faces towards the selectivity filter
462 and interacts with hydroxyl group of T199. To identify the accurate binding mode of
463 CHET3, two models from docking were further studied using molecular dynamics (MD)

464 simulations (see below).

465 **MD simulations**

466 The TASK-3 model obtained from the homology modeling and two binding models of
467 CHET3-bound TASK-3 were used to build the models of the *apo* TASK-3 and the
468 CHET3-bound TASK-3, respectively. Models were inserted in a POPC
469 (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) lipid bilayer to establish the
470 CHET3-bound system and the *apo* system, respectively. MD simulations were carried out
471 by using GROMACS 5.1.4 (44) with CHARMM36 parameters (45).

472 **Comparison of the binding of CHET3, CHET3-1 and CHET3-2**

473 RosettaLigand application (26, 27) was applied to dock CHET3, CHET3-1 and CHET3-2.
474 The best binding mode obtained from MD simulations were adopted as the initial docking
475 model. For each docking trial, top 1000 models were sorted by total score and the binding
476 energy between three compounds and the channel were calculated. Also, *in silico* alanine
477 scan were conducted by individually changing residue to alanine without otherwise
478 changing the conformation of protein or ligands in Rosetta. To explore the distribution of
479 binding interactions between compounds and proteins, the average energy of top 10
480 models with the lowest binding energies (interface score) were calculated. To compare
481 the binding of CHET3, CHET3-1 and CHET3-2, top 50 models of each compound with
482 the lowest binding energies were used to calculate the total score and interface score.

483 **Electrophysiology**

484 Electrophysiology tests of hTASK-3, hTASK-1, hTREK-1, mTREK-2, hTRAAK,
485 hTHIK-1, hTRESK, hTASK-3/hTASK-1, hTRPM8 and hTRPV1 were done with
486 transiently transfected HEK-293T cells. The cDNA of hTASK-3, hTASK-1, hTHIK-1,
487 hTRESK and hTASK-3/hTASK-1 were subcloned into the pCDNA3 vector (Invitrogen).
488 The cDNA of hTREK-1, mTREK-2, hTRAAK, hTRPM8 and hTRPV1 were subcloned
489 into the pEGFPN1 expression vector (Invitrogen). For the hTASK-3/hTASK-1
490 concatemer products were designed for the 3' and 5' ends of TASK-3 and TASK-1
491 ensuring that the stop codon of TASK-3 was removed.

492 Electrophysiology tests of hERG, Kv2.1 and BK were done with stable cell lines. The
493 CHO-hERG stable cell line was generated in-house and was based on a standard
494 CHO-K1 cell line. The HEK293-human Kv2.1 stable cell line and the CHO-human BK
495 stable cell line were generated by Ion Channel Explore (Beijing, China).

496 Whole-cell recordings of ion channels were performed with patch-clamp amplifiers
497 (EPC10, HEKA or Axon 700B, Molecular Devices) at 23-25 °C. The current signals were
498 filtered at 2 kHz and digitized at 10 kHz. The pipettes for whole cell recordings were
499 pulled from borosilicate glass capillaries (World Precision Instruments) and had a
500 resistance of 3-7 MΩ. For recordings of K⁺ channels, the standard pipette solution
501 contained (in mM): 140 KCl, 2 MgCl₂, 10 EGTA, 1 CaCl₂, 10 HEPES (pH 7.3, adjusted
502 with KOH), and the external solution contained (in mM): 150 NaCl, 5 KCl, 0.5 CaCl₂,
503 1.2 MgCl₂, 10 HEPES (pH 7.3, adjusted with NaOH). For recordings of the TRPV1 and

504 TRPM8 currents, the internal solution contained (in mM): 140 CsCl, 0.1 CaCl₂, 1 MgCl₂,
505 10 HEPES, 5 EGTA (pH 7.2, adjusted with CsOH), and the external solution contained
506 (in mM): 140 NaCl, 5 KCl, 1 MgCl₂, 0.5 EGTA, 10 HEPES (pH 7.4, adjusted with
507 NaOH). For recordings of hERG, the outward current of hERG channels was elicited by a
508 2.5-second depolarization step to +30 mV from a holding potential of -80 mV followed
509 by a 4-second repolarization step to -50 mV to measure the tail current. For recordings of
510 Kv2.1, currents were evoked by a 200-millisecond depolarization step to +60 mV from a
511 holding potential of -80 mV. For recordings of BK, currents were evoked by a 1-second
512 depolarization step to +70 mV from a holding potential of -80 mV. For recordings of
513 TRPV1 and TRPM8, currents were recorded using a ramp protocol from -100 mV to
514 +100 mV over a period of 400-millisecond at a holding potential of 0 mV.

515 Inside-out patches were done by using EPC10 (HEKA) at 23-25 °C. The pipettes for
516 single-channel recordings had resistances of 7-15 MΩ. The standard pipette and bath
517 solutions contained (in mM): 140 KCl, 1 CaCl₂, 2 MgCl₂, 10 HEPES, 10 EGTA (pH 7.4,
518 adjusted with KOH). Currents were recorded at a pipette potential of -60 mV and +60
519 mV respectively.

520 **Ethics statement**

521 All experiments with animals were approved by the Animal Research Committee of East
522 China Normal University (PROTOCOL No.: m20171020 and m20180112) and the
523 Animal Research Committee of West China Hospital of Sichuan University

524 (PROTOCOL No.: 2018175A). For tissue collection, mice were given a lethal dose of
525 pentobarbital intraperitoneally.

526 **Animals**

527 BALB/c mice and Sprague-Dawley rats were used in most animal studies, and TASK-3
528 KO mice and WT control littermates were on a C57BL/6 background. Male mice or rats
529 aged 8-10 weeks were used for behavior tests, unless stated otherwise. Animals were
530 housed in a conventional facility at 21 °C on a 12 h light-dark cycle with unrestricted
531 access to food and water.

532 **TASK-3 KO mice generation**

533 To generate a *Kcnk9* knockout C57BL/6 mouse line by CRISPR-Cas9 genome editing
534 system, two single-guide RNAs (sgRNA-1, 5'-CCGCTTCATGGCCGCGAAGAAGG-3',
535 and sgRNA-2, 5'-AGGAACCGGCGAATTTCCACTTGG-3') flanking exon1 were
536 designed (Bioray Laboratories). A 241-bp deletion was bound to the exon 1 of the *Kcnk9*
537 gene locus, resulting *Kcnk9*^{ΔΔ} mice with a frameshift mutation. Additional information is
538 provided upon request.

539 **Spared nerve injury model**

540 Unilateral spared nerve injury (SNI) surgery was performed. The experiment animal was
541 laid on prone position. After disinfection with povidone iodide and 75% ethanol, a
542 minimal skin incision is made at the mid-thigh level in order to exposing the sciatic nerve
543 and its three branches via separating the muscle layers. The tibial and common peroneal

544 nerves were tightly ligated with 5.0 silk threads and a 1-2 mm length section was
545 removed between the proximal and distal parts of nerves. The sural nerve was
546 restrictively preserved to avoiding any harmful injury. The muscle layer and skin were
547 closed after surgery and animals were transferred to a warm pad to recover from
548 anesthesia.

549 **Chronic inflammatory pain model**

550 A volume of 20 μ L Complete Freund's Adjuvant (CFA) (Sigma-Aldrich) was
551 subcutaneously injected into the left hindpaw of mouse to induce chronic inflammatory
552 pain in mouse. After injection the syringe was maintained for at least 30 s to avoid
553 overflow.

554 **Tail immersion**

555 Mice were restrained in the test tube with the tails stretching out and moving freely 15
556 min twice daily for 3 days. The distal third tails were immersed into a water bath at 5 °C,
557 20 °C, 40 °C, 46 °C, or 52 °C. The three measurements of tail flick latency (in second) to
558 stimulation as indicated by rapid tail flexion were averaged. A cutoff value of 15 seconds
559 was adopted to prevent unexpected damage.

560 **Formalin test**

561 Mice were housed individually in Plexiglas chambers, after habituation to the testing
562 environment for at least 30 min, the left hindpaw of the mice were injected
563 subcutaneously with formalin (20 μ L of 2.5% formalin, diluted in saline) and the mice

564 were put into the chamber of the automated formalin apparatus where movement of the
565 formalin-injected paw was recorded by an action camera (SONY, HDR-AS50). The
566 number of paw flinches was counted at 5 min intervals for 60 min by a blind
567 experimenter. Time spent exhibiting these pain behaviors was recorded for the first (0-10
568 min) and second phases (10-60 min).

569 **Paw pressure**

570 The effects of the mechanical nociception were evaluated with an Analgesimeter (model
571 37215; Ugo-Basile, Varese, Italy). Mice were placed in the testing room for 3 continuous
572 days to acclimate environment. The hindpaw of mice was pressed with a constant
573 pressure of 450 g using a cone-shaped paw-presser with a rounded tip and immediately
574 stopped as soon as the animal showed a struggle response, and the reaction latency was
575 recorded in second. The analgesic effects for TASK-3 agonists were evaluated 30 min
576 after i.p. injection.

577 **Spontaneous pain test**

578 After 3 days acclimation, the SNI mice were placed in an elevated transparent cage (20 ×
579 20 × 14 cm) with a wire mesh floor (0.5 × 0.5 cm). A 5 min duration was videoed by an
580 action camera (SONY, HDR-AS50) for each mouse, and the number of left hindpaw
581 flinching was calculated by a blind experimenter.

582 **Cold plantar test**

583 Mice were allowed to acclimate to the testing environment 2-3 h daily for 3 continuous

584 days. The cold probe produced freshly with fine dry ice powder into a 5 mL syringe was
585 held against a 6 mm depth of flat glass. The center of hindpaw was targeted and the
586 withdrawal latency manifesting as quick flick or paw licking was recorded. A cutoff time
587 of 30 s was used to prevent potential tissue damage.

588 **von Frey test**

589 The SNI rats and mice were individually placed in the chamber as described in the
590 spontaneous pain test. Mechanical sensitivity was assessed by two methods:

591 Method 1 (for all the von Frey tests described except Fig. 5D): The mechanical paw
592 withdrawal threshold was assessed using von Frey filaments with an ascending order. The
593 tip of filament was perpendicularly targeted to the region comprising of the sural nerve
594 territory and sufficient stimulation was held for 1 s. Rapid paw withdrawal or flinching
595 was considered as a positive response and the bending force for which at least 60% of the
596 application elicited positive response was recorded as the mechanical paw withdrawal
597 threshold.

598 Method 2 (up-down method): Mechanical responses were tested by stimulating the
599 region comprising of the sural nerve territory with von Frey monofilaments by using the
600 up-and-down method, starting with 0.04 g. Biting, licking, and withdrawal during or
601 immediately following the 3 s stimulus were considered as a positive response.

602 **Hargreaves test**

603 The hindpaw sensitivity to thermal noxious stimulus was assessed using a radiant heat

604 source (model 37370; Ugo-Basile, Varese, Italy), stimulus intensity was set to produce an
605 approximate latency of 10 s at baseline, and a cut-off value was set at 20 s to avoid
606 unexpected damage. Mice were allowed to acclimate in Plexiglas chambers with a glass
607 floor for 3 days, and the time to paw withdrawal was measured per mouse with a 5 min
608 inter-stimulation period. Three trials were averaged to yield the withdrawal latency.

609 **RNAscope *in situ* hybridization**

610 Sequences of target probes, preamplifier, amplifier and label probes are proprietary and
611 commercially available (Advanced Cell Diagnostics). *In situ* hybridization was
612 performed on frozen DRG sections (10 μ m) using RNAscope Multiplex Fluorescent
613 Reagent Kit v2 (ACDbio, Cat#323100) and RNAscope 4-Plex Ancillary Kit for
614 Multiplex Fluorescent Kit v2 (ACDbio, Cat#323120). The hybridization assay as
615 described by vendor's protocol. *In situ* probes include: *Kcnk3* (Cat#534881), *Kcnk9*
616 (Cat#475681), *Trpa1* (Cat#400211), *Trpv1* (Cat#313331), *Trpm8* (Cat#420451), *Rbfox3*
617 (Cat#313311), *Th* (Cat#317621), *Ntrk2* (Cat#423611), *P2rx3* (Cat#521611). The
618 specificity of the fluorescent signals was validated by RNAscope 3-plex Positive Control
619 Probe (Cat#320881) and RNAscope 3-plex Negative Control Probe (Cat#320871).
620 Fluorescent images were taken using a NIKON A1R⁺MP Two-photon confocal scanning
621 microscope and were analyzed using ImageJ software.

622 **Acutely dissociated DRG neuron preparation and electrophysiology**

623 3-6 week-old male Sprague-Dawley rats were sacrificed. The DRGs were collected in a

624 35-mm tissue culture dish and digested in 3% collagenase for 20 min, followed by 1%
625 trypsin for another 30 min. After titrating by sucking up and down, the DRG neurons
626 were cultured in Neurobasal containing 2% B27 medium for 2-4 h. The bath solution
627 contained (in mM): 140 NaCl, 3 KCl, 1.3 MgCl₂, 10 HEPES, 2.4 CaCl₂, 10 glucose, pH
628 7.3. The pipette solution contained (in mM): 40 KCl, 10 HEPES, 5 EGTA, 10 NaCl, 95
629 K-gluconate, 4 Mg-ATP, pH 7.4. To minimize voltage-gated currents, the voltage ramps
630 from -120 mV to -30 mV were applied, and 1mM CsCl was added extracellularly to block
631 hyperpolarization-activated currents. To determine the reversal potential of the
632 CHET3-sensitive currents, NaCl was replaced with equimolar KCl. The whole-cell
633 recordings were performed with similar hardware settings as described in
634 electrophysiology in HEK-293 cells.

635 **Acutely dissociated DRG neuron preparation and intracellular Ca²⁺ imaging**

636 2 week-old male Sprague-Dawley rats were sacrificed. The DRGs were collected in a
637 35-mm tissue culture dish and digested in 2.5 mg/mL papain (Sigma-Aldrich) for 30 min
638 at 37 °C, followed by 2.5 mg/mL collagenase (Sigma-Aldrich) for another 30 min.
639 Digested ganglia were gently washed with neurobasal medium and mechanically
640 dissociated by passage through pipet. Neurons were seeded on laminin-coated wells
641 (Corning) and cultured overnight at 37 °C in 5% CO₂ in 2% B27 (Sigma-Aldrich),
642 supplemented neuronbasal medium, containing 50 ng/mL GDNF (PeproTech), 50 ng/mL
643 BDNF (PeproTech).

644 Changes in intracellular Ca^{2+} concentration were monitored using ratiometric
645 Fura-2-based fluorimetry. Neurons were loaded with 2 μM Fura-2-acetoxymethyl ester
646 (Yeasen) dissolved in bath solution and 0.02% Pluronic F127 (Sigma-Aldrich) for 30 min
647 at 37°C. Fluorescence was measured during alternating illumination at 340 and 380 nm
648 using Olympus IX73 inverted fluorescence microscopy system. The bath solution
649 contained (in mM): 138 NaCl, 5.4 KCl, 2 CaCl_2 , 2 MgCl_2 , 10 glucose, and 10 HEPES,
650 pH 7.4, adjusted with NaOH. At the end of each experiment, cells were subjected to a
651 depolarizing solution containing 50 mM KCl, and non-responsive cells to 50 mM KCl
652 were excluded from analysis. Bar graphs in Fig. 7I and fig. S14B were pooled data from
653 both responding cells and non-responding cells in different conditions.

654 **Thermal stimulation**

655 Coverslip pieces with culture cells were placed in a recording chamber and continuously
656 perfused (about 1 mL/min).

657 Cool stimulation: the temperature was adjusted with ice bag cool perfuse solution, and
658 controlled by a feedback device. Cold sensitivity was investigated with a ~2 min duration
659 ramp-like temperature drop from 37 °C to ~15 °C.

660 Heat stimulation: the temperature was adjusted with a water-heated device (model
661 TC-324B/344B, America), with the temperature of the perfuse solution raise, and
662 controlled by a feedback device. Heat sensitivity was investigated with a ~5 min duration
663 ramp-like temperature rise from 25 °C to ~43 °C.

664 **Statistical analysis**

665 Statistical analyses were carried out using the Origin 9.0 software (Origin Lab
666 Corporation, Northampton, USA). Data were analyzed as described in the figure legends.
667 Normality of the data distribution was determined before appropriate statistical methods
668 were chosen. The drug was assessed as significantly active by using statistical tests
669 performed between values of the baseline and those of given time points unless specified.
670 No statistical methods were used to predetermine sample sizes.

671

672 **SUPPLEMENTARY MATERIALS**

673 Materials and Methods

674 Fig. S1. A potential druggable pocket identified in several structures of K2P channels.

675 Fig. S2. Representative current traces for whole-cell recordings on several K2P channels
676 and other ion channels.

677 Fig. S3. 10 μ M CHET3 does not show effect on three pain-related GPCRs.

678 Fig. S4. Binding modes of CHET3 suggested by docking and MD simulations.

679 Fig. S5. Whole-cell path-clamp current recording for three TASK-3 mutants.

680 Fig. S6. Conformations of the extracellular ion pathway in MD simulations.

681 Fig. S7. Dose-dependent analgesia by CHET3 in mechanical allodynia.

682 Fig. S8. Effects of CHET3 on the locomotion activities, blood pressure and body
683 temperature in rodents.

684 Fig. S9. Comparison of the binding of CHET3, CHET3-1 and CHET3-2.
685 Fig. S10. Blockade of CHET3-1 analgesia by PK-THPP.
686 Fig. S11. Generation and characterization of TASK-3 gene (*Kcnk9*) knockout mice.
687 Fig. S12. Down-regulation of peripheral TASK-3 under chronic pain.
688 Fig. S13. Effects of CHET3 and PK-THPP on nociceptive neurons.
689 Fig. S14. Thermal stimulation induced Ca^{2+} signals were mediated by TRP channels.
690 Table S1. Echocardiographic evaluation of CHET3 on mice.
691 Table S2. CHET3 pharmacokinetics in plasma and brain following a single
692 intraperitoneal administration at 10 mg/kg to male C57BL/6 mice.

693

694 REFERENCES AND NOTES

- 695 1. N. D. Volkow, A. T. McLellan, Opioid abuse in chronic pain—misconceptions and
696 mitigation strategies. *Eur. J. Neurosci.* **374**, 1253-1263 (2016).
- 697 2. A. S. Yekkirala, D. P. Roberson, B. P. Bean, C. J. Woolf, Breaking barriers to novel
698 analgesic drug development. *Nat. Rev. Drug Discov.* **16**, 544-563 (2017).
- 699 3. S. A. N. Goldstein, D. Bockenhauer, I. O'Kelly, N. Zilberberg, Potassium leak
700 channels and the KCNK family of two-P-domain subunits. *Nat. Rev. Neurosci.* **2**,
701 175-184 (2001).
- 702 4. J. Busserolles, C. Tsantoulas, A. Eschalier, J. A. Lopez Garcia, Potassium channels in
703 neuropathic pain: advances, challenges, and emerging ideas. *Pain* **157**, S7-S14

- 704 (2016).
- 705 5. S. G. Waxman, G. W. Zamponi, Regulating excitability of peripheral afferents:
706 emerging ion channel targets. *Nat. Neurosci.* **17**, 153-163 (2014).
- 707 6. A. Mathie, E. L. Veale, Two-pore domain potassium channels: potential therapeutic
708 targets for the treatment of pain. *Pflugers Arch.* **467**, 931-943 (2015).
- 709 7. Y. Kim, H. Bang, D. Kim, TASK-3, a new member of the tandem pore K⁺ channel
710 family. *J. Biol. Chem.* **275**, 9340-9347 (2000).
- 711 8. S. Rajan, E. Wischmeyer, G. X. Liu, R. P. Muller, J. Daut, A. Karschin, C. Derst,
712 TASK-3, a novel tandem pore domain acid-sensitive K⁺ channel - An extracellular
713 histidine as pH sensor. *J. Biol. Chem.* **275**, 16650-16657 (2000).
- 714 9. T. K. Baumann, P. Chaudhary, M. E. Martenson, Background potassium channel
715 block and TRPV1 activation contribute to proton depolarization of sensory neurons
716 from humans with neuropathic pain. *Eur. J. Neurosci.* **19**, 1343-1351 (2004).
- 717 10. C. Morenilla-Palao, E. Luis, C. Fernández-Peña, E. Quintero, J. L. Weaver, D. A.
718 Bayliss, F. Viana, Ion channel profile of TRPM8 cold receptors reveals a role of
719 TASK-3 potassium channels in thermosensation. *Cell Rep.* **8**, 1571-1582 (2014).
- 720 11. D. J. Langford, C. West, C. Elboim, B. A. Cooper, G. Abrams, S. M. Paul, B. L.
721 Schmidt, J. D. Levine, J. D. Merriman, A. Dhruva, J. Neuhaus, H. Leutwyler, C.
722 Baggott, C. W. Sullivan, B. E. Aouizerat, C. Miaskowski, Variations in potassium
723 channel genes are associated with breast pain in women prior to breast cancer surgery.

- 724 *J. Neurogenet.* **28**, 122-135 (2014).
- 725 12. V. Le Guilloux, P. Schmidtke, P. Tuffery, Fpocket: an open source platform for ligand
726 pocket detection. *BMC bioinformatics* **10**, 168 (2009).
- 727 13. A. N. Miller, S. B. Long, Crystal structure of the human two-pore domain potassium
728 channel K2P1. *Science* **335**, 432-436 (2012).
- 729 14. M. Lolicato, P. M. Riegelhaupt, C. Arrigoni, K. A. Clark, D. L. Minor, Jr.,
730 Transmembrane helix straightening and buckling underlies activation of
731 mechanosensitive and thermosensitive K2P channels. *Neuron* **84**, 1198-1212 (2014).
- 732 15. Y. Y. Dong, A. C. Pike, A. Mackenzie, C. McClenaghan, P. Aryal, L. Dong, A.
733 Quigley, M. Grieben, S. Goubin, S. Mukhopadhyay, G. F. Ruda, M. V. Clausen, L.
734 Cao, P. E. Brennan, N. A. Burgess-Brown, M. S. Sansom, S. J. Tucker, E. P.
735 Carpenter, K2P channel gating mechanisms revealed by structures of TREK-2 and a
736 complex with Prozac. *Science* **347**, 1256-1259 (2015).
- 737 16. F. Sievers, A. Wilm, D. Dineen, T. J. Gibson, K. Karplus, W. Li, R. Lopez, H.
738 McWilliam, M. Remmert, J. Soding, J. D. Thompson, D. G. Higgins, Fast, scalable
739 generation of high-quality protein multiple sequence alignments using Clustal
740 Omega. *Mol. Syst. Biol.* **7**, 539 (2011).
- 741 17. S. F. Altschul, T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, D. J.
742 Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database
743 search programs. *Nucleic Acids Res.* **25**, 3389-3402 (1997).

- 744 18. M. Bertoni, F. Kiefer, M. Biasini, L. Bordoli, T. Schwede, Modeling protein
745 quaternary structure of homo- and hetero-oligomers beyond binary interactions by
746 homology. *Sci Rep.* **7**, 10480 (2017).
- 747 19. A. Waterhouse, M. Bertoni, S. Bienert, G. Studer, G. Tauriello, R. Gumienny, F. T.
748 Heer, T. A. P. de Beer, C. Rempfer, L. Bordoli, R. Lepore, T. Schwede,
749 SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic*
750 *Acids Res.* **46**, W296-W303 (2018).
- 751 20. A. Šali, T. L. Blundell, Comparative protein modelling by satisfaction of spatial
752 restraints. *J. Mol. Biol.* **234**, 779-815 (1993).
- 753 21. C. A. Coburn, Y. F. Luo, M. X. Cui, J. B. Wang, R. Soll, J. C. Dong, B. Hu, M. A.
754 Lyon, V. P. Santarelli, R. L. Kraus, Y. Gregan, Y. Wang, S. V. Fox, J. Binns, S. M.
755 Doran, D. R. Reiss, P. L. Tannenbaum, A. L. Gotter, P. T. Meinke, J. J. Renger,
756 Discovery of a pharmacologically active antagonist of the two-pore-domain
757 potassium channel K_{2P9.1} (TASK-3). *ChemMedChem* **7**, 123-133 (2012).
- 758 22. G. Czirják, P. Enyedi, Formation of functional heterodimers between the TASK-1
759 and TASK-3 two-pore domain potassium channel subunits. *J. Biol. Chem.* **277**,
760 5426-5432 (2002).
- 761 23. C. E. Clarke, E. L. Veale, P. J. Green, H. J. Meadows, A. Mathie, Selective block of
762 the human 2-P domain potassium channel, TASK-3, and the native leak potassium
763 current, I_{KSO}, by zinc. *J. Physiol.* **560**, 51-62 (2004).

- 764 24. S. Rinné, A. K. Kiper, G. Schlichthörl, S. Dittmann, M. F. Netter, S. H. Limberg, N.
765 Silbernagel, M. Zuzarte, R. Moosdorf, H. Wulf, E. Schulze-Bahr, C. Rolfes, N. Decher,
766 TASK-1 and TASK-3 may form heterodimers in human atrial cardiomyocytes. *J. Mol.*
767 *Cell. Cardiol.* **81**, 71-80 (2015).
- 768 25. M. Schewe, H. Sun, Ü. Mert, A. Mackenzie, A. C. W. Pike, F. Schulz, C. Constantin,
769 K. S. Vowinkel, L. J. Conrad, A. K. Kiper, W. Gonzalez, M. Musinszki, M. Tegtmeyer,
770 D. C. Pryde, H. Belabed, M. Nazare, B. L. de Groot, N. Decher, B. Fakler, E. P.
771 Carpenter, S. J. Tucker, T. Baukrowitz, A pharmacological master key mechanism
772 that unlocks the selectivity filter gate in K⁺ channels. *Science* **363**, 875-880 (2019).
- 773 26. I. W. Davis, D. Baker, RosettaLigand docking with full ligand and receptor flexibility.
774 *J. Mol. Biol.* **385**, 381-392 (2009).
- 775 27. G. Lemmon, J. Meiler, Rosetta Ligand docking with flexible XML protocols.
776 *Methods Mol. Biol.* **819**, 143-155 (2012).
- 777 28. W. González, L. Zúñiga, L. P. Cid, B. Arévalo, M. I. Niemeyer, F. V. Sepúlveda, An
778 extracellular ion pathway plays a central role in the cooperative gating of a K_{2P} K⁺
779 channel by extracellular pH. *J. Biol. Chem.* **288**, 5984-5991 (2013).
- 780 29. F. V. Abbott, K. B. Franklin, R. F. Westbrook, The formalin test: scoring properties of
781 the first and second phases of the pain response in rats. *Pain* **60**, 91-102 (1995).
- 782 30. A. Tappe-Theodor, R. Kuner, Studying ongoing and spontaneous pain in
783 rodents-challenges and opportunities. *Eur. J. Neurosci.* **39**, 1881-1890 (2014).

- 784 31. E. Navratilova, F. Porreca, Reward and motivation in pain and pain relief. *Nat. Rev.*
785 *Neurosci.* **17**, 1304-1312 (2014).
- 786 32. L. A. McWilliams, B. J. Cox, M. W. Enns, Mood and anxiety disorders associated
787 with chronic pain: an examination in a nationally representative sample. *Pain* **106**,
788 127-133 (2003).
- 789 33. P. J. Turner, K. J. Buckler, Oxygen and mitochondrial inhibitors modulate both
790 monomeric and heteromeric TASK-1 and TASK-3 channels in mouse carotid body
791 type-1 cells. *J. Physiol.* **591**, 5977-5998 (2013).
- 792 34. J. Vriens, B. Nilius, T. Voets, Peripheral thermosensation in mammals. *Nat. Rev.*
793 *Neurosci.* **15**, 573-589 (2014).
- 794 35. L. Li, M. Rutlin, V. E. Abraira, C. Cassidy, L. Kus, S. Gong, M. P. Jankowski, W.
795 Luo, N. Heintz, H. R. Koerber, C. J. Woodbury, D. D. Ginty, The functional
796 organization of cutaneous low-threshold mechanosensory neurons. *Cell* **147**,
797 1615-1627 (2011).
- 798 36. L. Vulchanova, M. S. Riedl, S. J. Shuster, L. S. Stone, K. M. Hargreaves, G. Buell, A.
799 Surprenant, R. A. North, R. Elde, P2X3 is expressed by DRG neurons that terminate
800 in inner lamina II. *Eur. J. Neurosci.* **10**, 3470-3478 (1998).
- 801 37. S. L. Pollema-Mays, M. V. Centeno, C. J. Ashford, A. V. Apkarian, M. Martina,
802 Expression of background potassium channels in rat DRG is cell-specific and
803 down-regulated in a neuropathic pain model. *Mol. Cell. Neurosci.* **57**, 1-9 (2013).

- 804 38. J. R. Deus, Z. Dekan, J. S. Wingerd, J. J. Smith, N. R. Munasinghe, R. F. Bhola, W.
805 L. Imlach, V. Herzig, D. A. Armstrong, K. J. Rosengren, F. Bosmans, S. G. Waxman,
806 S. D. Dib-Hajj, P. Escoubas, M. S. Minett, M. J. Christie, G. F. King, P. F. Alewood,
807 R. J. Lewis, J. N. Wood, I. Vetter, Pharmacological characterisation of the highly
808 Nav1.7 selective spider venom peptide Pn3a. *Sci Rep.* **7**, 40883 (2017).
- 809 39. E. C. Emery, A. P. Luiz, J. N. Wood, Nav1.7 and other voltage-gated sodium channels
810 as drug targets for pain relief. *Expert Opin. Ther. Targets* **20**, 975-983 (2016).
- 811 40. P. Royal, A. Andres-Bilbe, P. Á. Prado, C. Verkest, B. Wdziekonski, S. Schaub, A.
812 Baron, F. Lesage, X. Gasull, J. Levitz, G. Sandoz, Migraine-associated TRESK
813 mutations increase neuronal excitability through alternative translation initiation and
814 inhibition of TREK. *Neuron* **101**, 232-245 (2019).
- 815 41. D. W. Kang, J. H. Han, E. M. Talley, D. A. Bayliss, D. H. Kim, Functional expression
816 of TASK-1/TASK-3 heteromers in cerebellar granule cells. *J. Physiol.* **554**, 64-77
817 (2004).
- 818 42. A. P. Berg, E. M. Talley, J. P. Manger, D. A. Bayliss, Motoneurons express
819 heteromeric TWIK-related acid-sensitive K⁺ (TASK) channels containing TASK-1
820 (KCNK3) and TASK-3 (KCNK9) subunits. *J. Neurosci.* **24**, 6693-6702 (2004).
- 821 43. D. Kim, E. J. Cavanaugh, I. Kim, J. L. Carroll, Heteromeric TASK-1/TASK-3 is the
822 major oxygen-sensitive background K⁺ channel in rat carotid body glomus cells. *J.*
823 *Physiol.* **587**, 2963-2975 (2009).

- 824 44. S. Pronk, S. Páll, R. Schulz, P. Larsson, P. Bjelkmar, R. Apostolov, M. R. Shirts, J. C.
825 Smith, P. M. Kasson, D. van der Spoel, B. Hess, E. Lindahl, GROMACS 4.5: a
826 high-throughput and highly parallel open source molecular simulation toolkit.
827 *Bioinformatics* **29**, 845-854 (2013).
- 828 45. J. B. Klauda, R. M. Venable, J. A. Freites, J. W. O'Connor, D. J. Tobias, C.
829 Mondragon-Ramirez, I. Vorobyov, A. D. MacKerell, Jr., R. W. Pastor, Update of the
830 CHARMM all-atom additive force field for lipids: validation on six lipid types. *J.*
831 *Phys. Chem. B* **114**, 7830-7843 (2010).
- 832 46. P. V. Bharatam, D. S. Patel, P. Iqbal, Pharmacophoric features of biguanide
833 derivatives: an electronic and structural analysis. *J. Med. Chem.* **48**, 7615-7622
834 (2005).
- 835 47. K. M. Tye, R. Prakash, S. Y. Kim, L. E. Fenno, L. Grosenick, H. Zarabi, K. R.
836 Thompson, V. Gradinaru, C. Ramakrishnan, K. Deisseroth, Amygdala circuitry
837 mediating reversible and bidirectional control of anxiety. *Nature* **471**, 358-362
838 (2011).

839

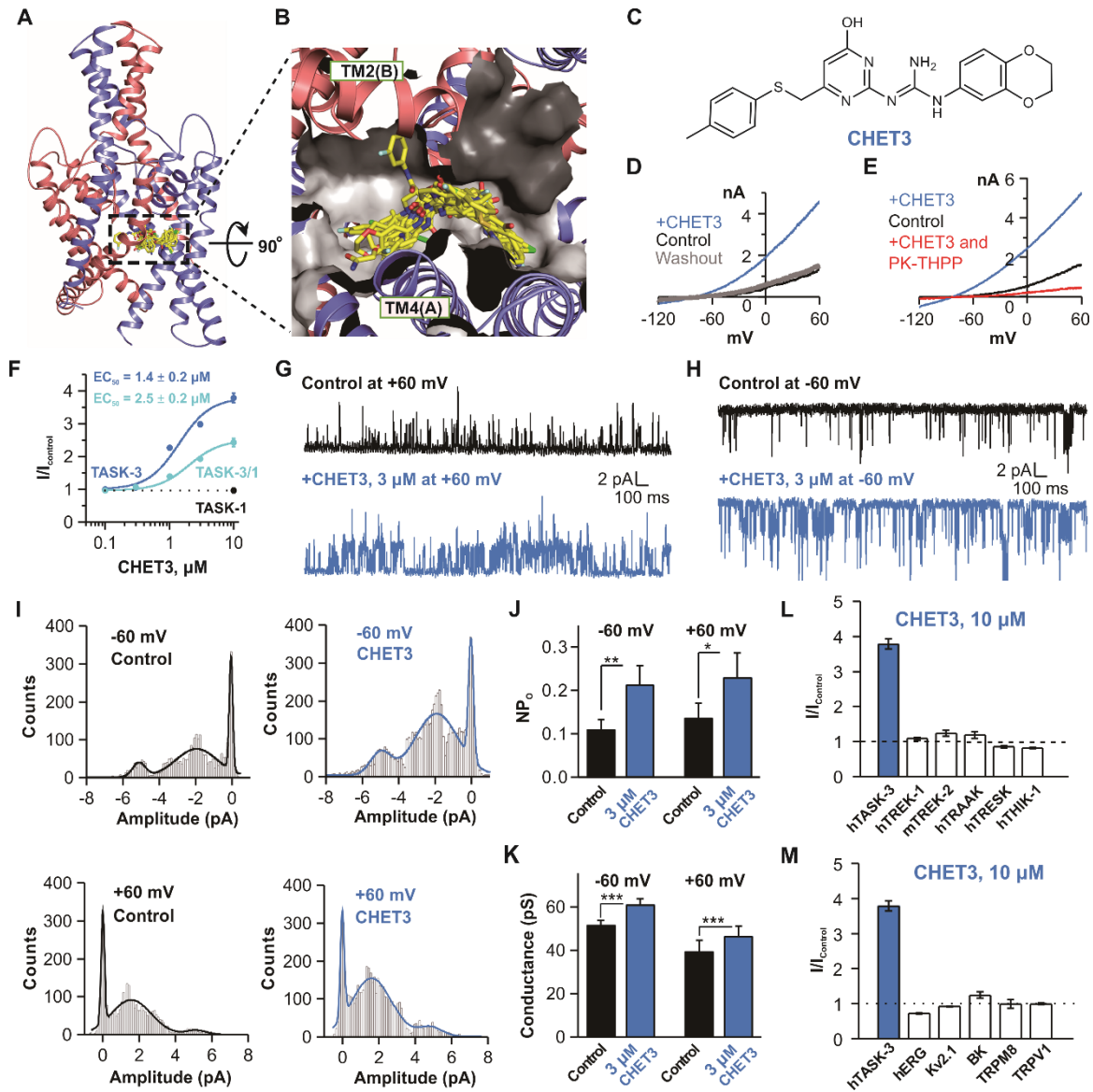
840 **Acknowledgements:** We thank Bioray Laboratories for technical support in preparing
841 *Kcnk9* knockout mice. We thank Dr. Tao Li (West China Hospital) for technical assistance
842 with blood pressure test and Miss Rongrong Cui (SIMM) for assistance on PK test. We
843 thank the supports of ECNU Multifunctional Platform for Innovation (001 and 011).

844 **Funding:** This work was funded by National Science & Technology Major Project “Key
845 New Drug Creation and Manufacturing Program” of China (2018ZX09711002 to H.J.,
846 Q.Z., and H.Y.), the National Natural Science Foundation of China (21422208 to H.Y;
847 31600832 to R.J.), Thousand Talents Plan in Sichuan Province (to R.J.), 1.3.5 Project for
848 Disciplines of Excellence (ZY2016101), West China Hospital, Sichuan University (to
849 J.L.), the "XingFuZhiHua" funding of ECNU (44300-19311-542500/006), the
850 Fundamental Research Funds for the Central Universities (to H.Y., and 2018SCUH0086
851 to R.J.) and the Special Program for Applied Research on Super Computation of the
852 NSFC-Guangdong Joint Fund (the second phase) under Grant No.U1501501, and the
853 State Key Laboratory of Bioorganic and Natural Products Chemistry. **Author**
854 **contributions:** P.L. performed behavior tests, ISH, Ca²⁺ imaging; Y.Q. performed drug
855 design and computations; Y.M. performed mouse genetics on TASK-3 KO mice and
856 behavior tests; J.F. performed electrophysiology; Z.S. performed electrophysiology,
857 behavior tests, ISH; L.H. performed electrophysiology; S.B., Y.W. and B.S. performed
858 Ca²⁺ imaging; J.Z. and W.G.L. performed elevated plus maze tests; Z.C. and N.P. assisted
859 with behavior tests and cell culture; E.S. performed dark/light box tests; L.Y. assisted
860 with behavior tests; F.T., X.L. and Z.G. performed electrophysiology for some of the
861 initial compound screenings; P.S., Y.C. and Y.M. performed pharmacokinetics study; D.H.
862 performed the qPCR experiments for TASK-3 KO mice; L.Z. performed experiments of
863 μ OR; D.Y. performed experiments of 5-HT_{1B}R; W.L. performed experiments of CB₁R;

864 T.Y., J.X. and Y.M. performed experiments of echocardiography. Q.Z. prepared the
865 derivatives of CHET3. J.L. oversaw the animal behavior tests. H.J. oversaw the
866 computations. R.J. and H.Y. initiated, supervised the project, analyzed the experiments,
867 and wrote the manuscript with input from all co-authors. **Competing interests:** The
868 authors declare no competing interests. **Data and materials availability:** All data is
869 available in the main text or the supplementary materials.

870

871 **Figures**



872

873 **Fig. 1. Structure-based ligand discovery of CHET3.**

874 (A) Pocket in a TASK-3 homology model used for virtual screening. Representative
 875 docking poses were shown. (B) Cytoplasmic expanded view of the pocket and the
 876 docking poses. (C) Chemical structure of CHET3. (D and E) Exemplar whole-cell
 877 path-clamp recordings showing the activation of TASK-3 by 10 μ M CHET3 and
 878 blockade of 0.5 μ M PK-THPP. (F) CHET3 dose-response curves for TASK-3 (n = 7) and

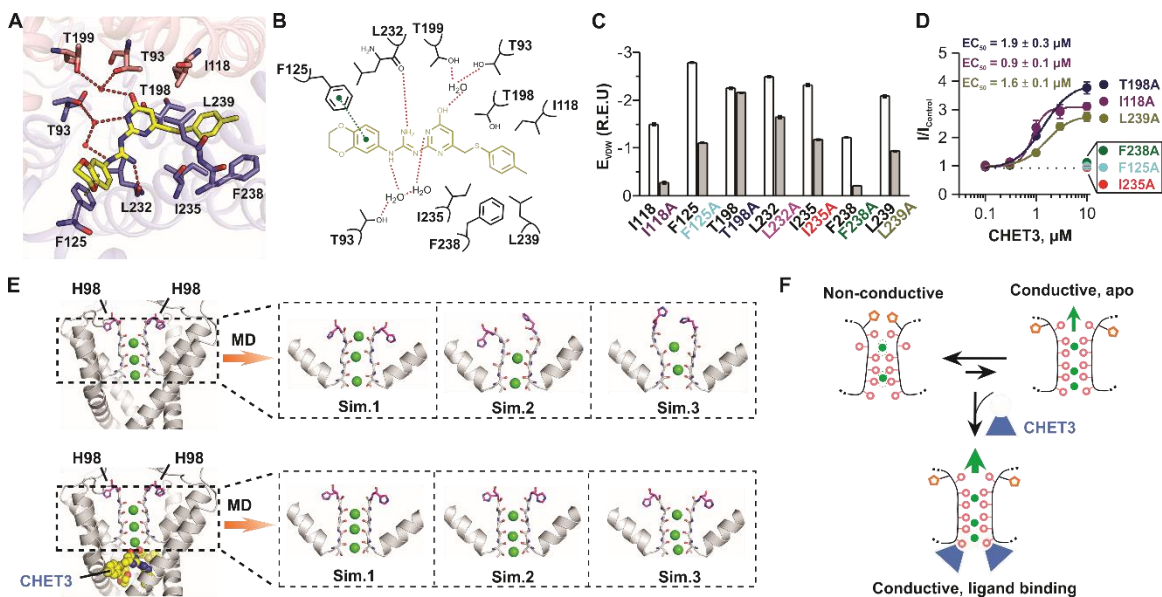
879 TASK-3/TASK-1 (n = 5). (**G** and **H**) Representative single-channel current traces from
880 inside-out patches showing the activation of TASK-3 by CHET3 at +60 mV (G) and -60
881 mV (H). (**I**) Histograms of the single-channel currents which were fitted by Gaussian
882 distributions. (**J** and **K**) Analysis of NP_o (channel number times open probability)
883 changes and conductance changes from the single-channel currents (n = 6; paired *t* test).
884 (**L**) Summary for the effects of CHET3 on several other K2P channels (n = 7-10). (**M**)
885 Summary for the effects of CHET3 on hERG, Kv2.1, BK, TRPM8 and TRPV1 channels
886 (n = 5-7). Data in (F, J to M) are shown as mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* <
887 0.001.

888

889

890

891

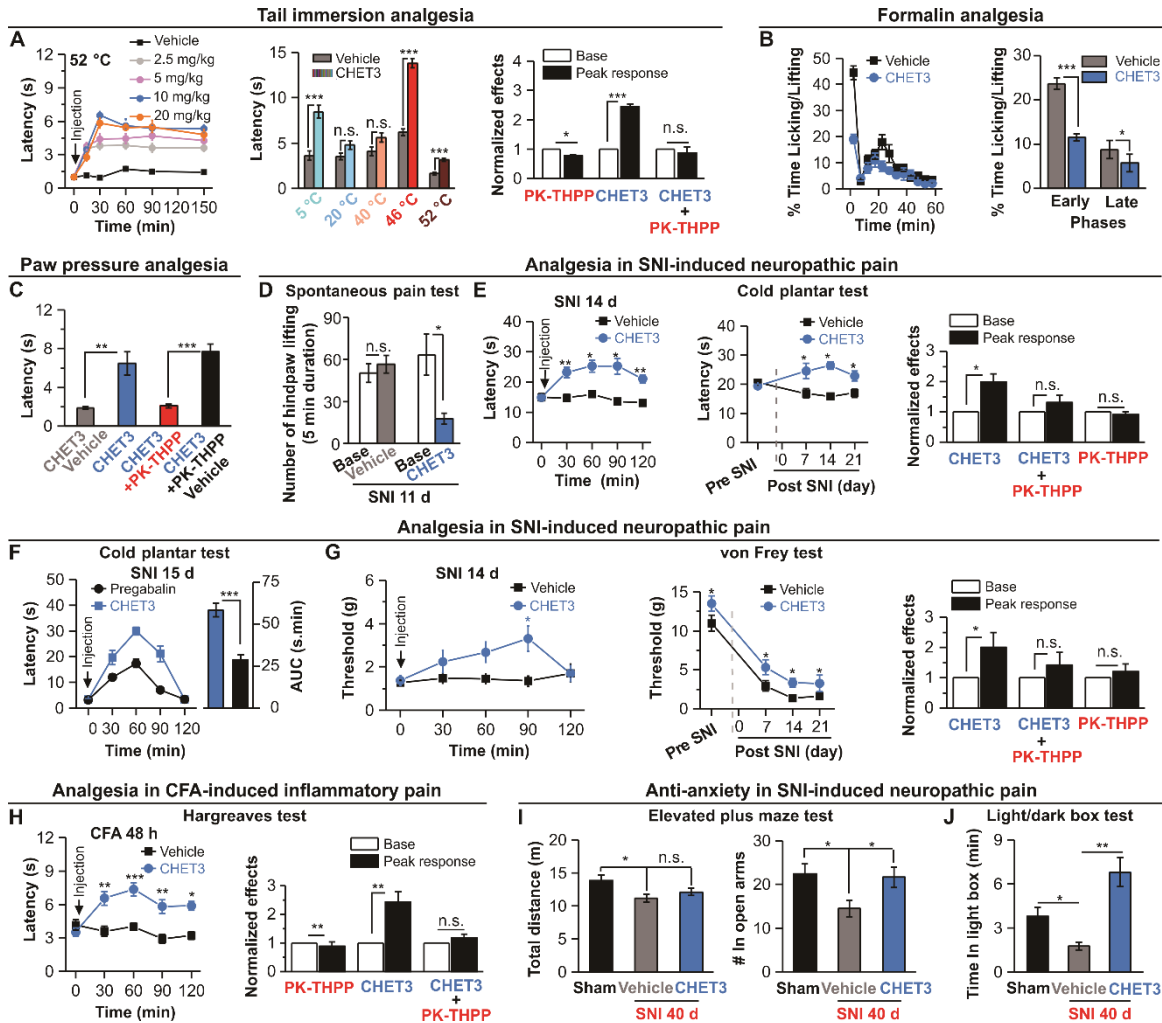


892

893 **Fig. 2. Activation mechanism of CHET3 on TASK-3.**

894 (A and B) 3-dimensional and 2-dimensional diagrams showing interactions between
895 CHET3 and TASK-3. Hydrogen bond (red dash) and π - π interaction (green dash) were
896 shown. (C) Computations showing the contributions of seven residues and their
897 mutations to CHET3 binding. Energy unit is Rosetta Energy Unit (R.E.U.). (D)
898 Dose-response curves of six mutations on CHET3 activity (n = 6). Data are shown as
899 mean \pm SEM. (E) Selectivity filter conformations of the *apo* TASK-3 and the
900 CHET3-bound TASK-3 revealed by MD simulations, including bound potassium ions
901 (green spheres), carbonyl oxygen (red sphere) rotation of filter residues, and movements
902 of residue H98 (purple sticks). (F) Schematic representation of the action mechanism of
903 CHET3 on TASK-3.

904



905

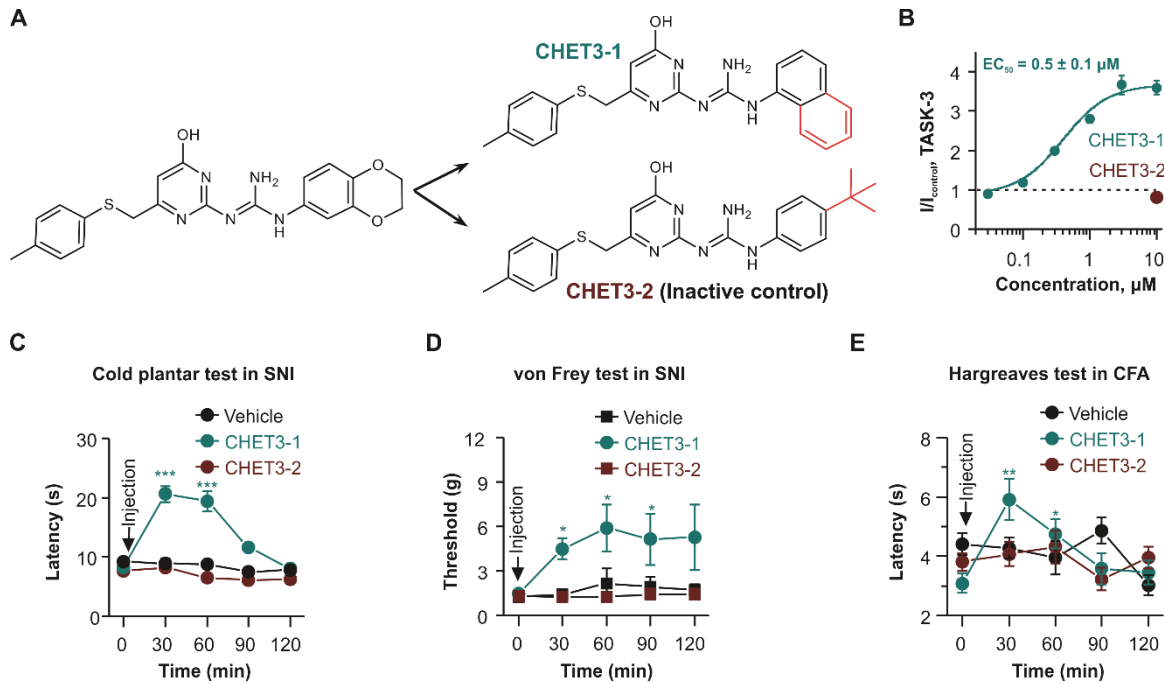
906 **Fig. 3. Analgesic effects of CHET3 in rodents.**

907 (A) *left*, Time profile for dose-dependent analgesia by CHET3 in tail immersion test at
 908 52 °C (n = 6-10); *middle*, CHET3 analgesia in tail immersion tests at different
 909 temperatures (n = 9-11; unpaired *t* test); *right*, Summary for PK-TTHPP effects (n = 8-10;
 910 unpaired *t* test). (B) Summary for CHET3 analgesia in formalin (2.5%, 20 μ l) test (n = 8;
 911 unpaired *t* test). (C) CHET3 and PK-TTHPP on the paw withdrawal latency in paw
 912 pressure test (measured at 45 min post injection, n = 8-10; paired *t* test). (D) CHET3 on
 913 spontaneous pain within a 10 min duration in mice (measured at 35 min post injection, n

914 = 6-10; unpaired *t* test). **(E)** *left*, Time profile for CHET3 on cold hyperalgesia in mice (n
915 = 10-11; paired *t* test); *middle*, Summary for CHET3 on cold hyperalgesia at different
916 stages in SNI (n = 9-11; unpaired *t* test); *right*, Summary for PK-THPP (n = 10; paired *t*
917 test). **(F)** Comparison of CHET3 and Pregabalin (30 mg/kg, i.p.) in cold plantar test in
918 mice (n = 11-12; unpaired *t* test). **(G)** *left*, Time profile for CHET3 in mechanical
919 allodynia in rats (n = 8-13; paired *t* test); *middle*, Summary for CHET3 on mechanical
920 allodynia at different stages in SNI rats (n = 8-13; paired *t* test); *right*, Summary for
921 PK-THPP effects (n = 8; paired *t* test). **(H)** *left*, Time profile for CHET3 on heat
922 hyperalgesia (n = 10-11; paired *t* test); *right*, Summary for PK-THPP effects (n = 11;
923 paired *t* test). **(I and J)** CHET3 on anxiety-like behaviors in elevated plus maze test (I) (n
924 = 8-9; unpaired *t* test) and in light/dark box tested (J) (n = 6-8; unpaired *t* test). CHET3
925 (10 mg/kg) and PK-THPP (15 mg/kg) were administrated via i.p. injections unless
926 specified. Data are shown as mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. n.s.,
927 not significant.

928

929



930

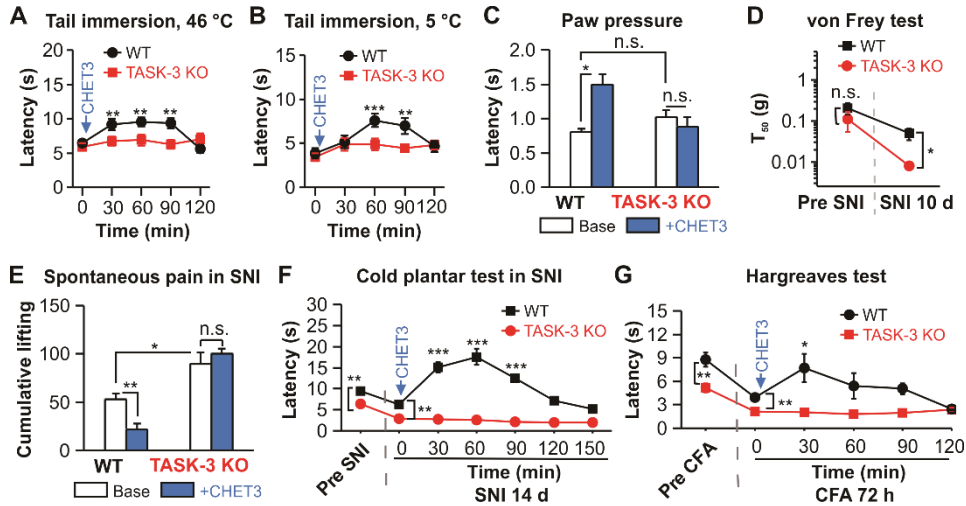
931 **Fig. 4. Validation of TASK-3 as analgesic target by using CHET3 derivatives.**

932 (A) Chemical structures of CHET3 derivatives. (B) Dose-response relationships for
933 CHET3-1 (n = 6) and CHET3-2 (n = 7) on TASK-3. (C to E) CHET3-1 and CHET3-2 in
934 cold hyperalgesia (C) (n = 9-11; paired *t* test), mechanical allodynia (D) (n = 7-8; paired *t*
935 test), and heat hyperalgesia (E) (n = 9-10; paired *t* test). Data are shown as mean \pm SEM.

936 **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

937

938



939

940 **Fig. 5. Effects of systemic administration of CHET3 on TASK-3 KO mice.**

941 (A and B) CHET3 had no analgesic effects in tail immersion tests in TASK-3 KO mice (n

942 = 9-12; paired *t* test). (C) CHET3 had no analgesic effect in paw pressure tests in TASK-3

943 KO mice (n = 8 for KO, n = 7 for WT; paired *t* test). Note that no change of baseline

944 sensitivity in nociception for TASK-3 KO mice in A to C. (D) TASK-3 KO mice in SNI

945 model exhibited enhanced mechanical allodynia (up-down method, n = 8 for KO, n = 10

946 for WT; unpaired *t* test). (E) TASK-3 KO mice in SNI model exhibited enhanced

947 spontaneous pain activities which was insensitive to CHET3 (n = 6-7; paired *t* test). (F

948 and G) CHET3 had no analgesic effect in cold plantar test (n = 6-7; paired *t* test) in SNI

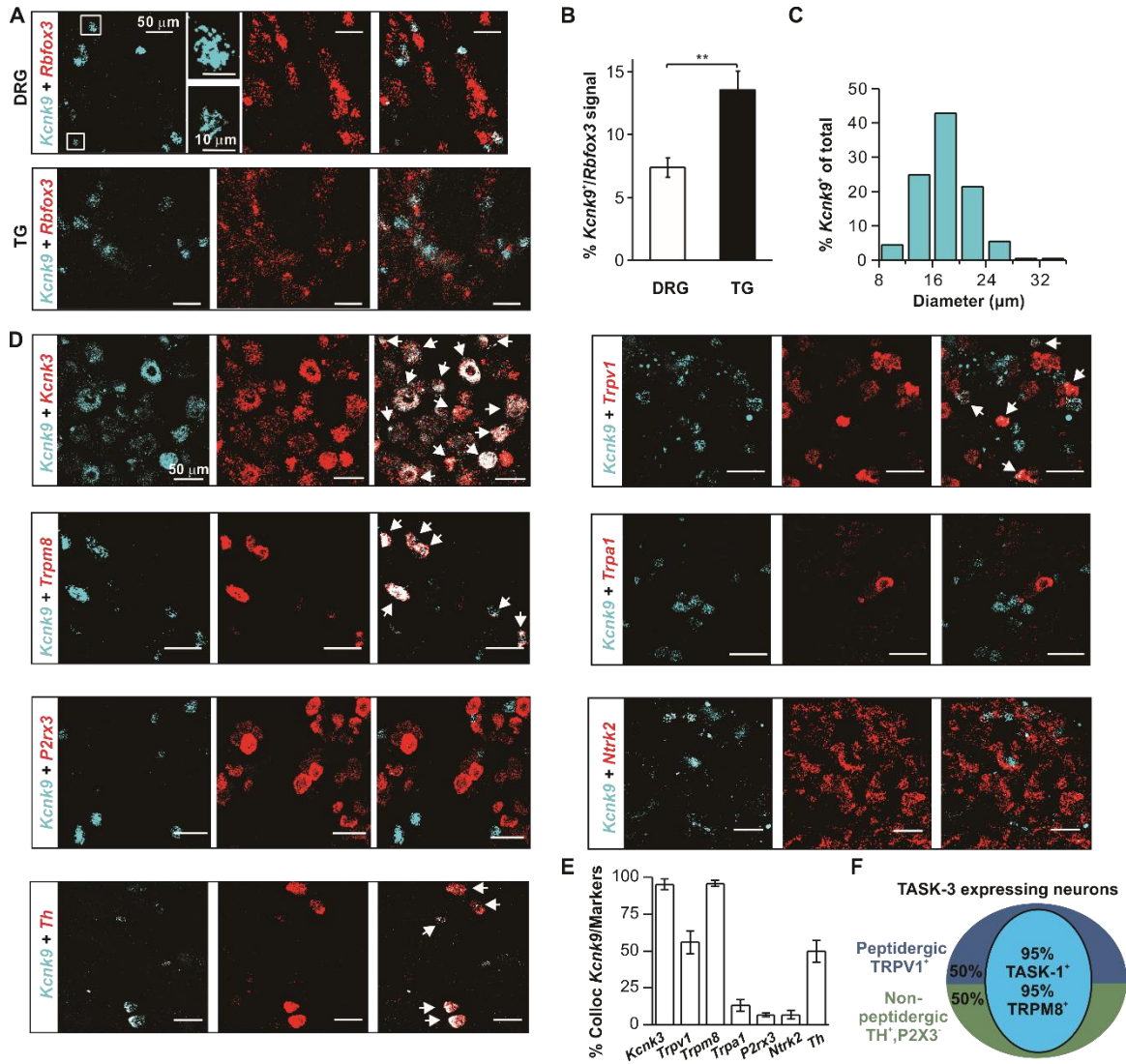
949 model and Hargreaves test (n = 8-12; paired *t* test) in CFA model in TASK-3 KO mice.

950 Note that TASK-3 KO mice had shorter paw withdraw latencies in both tests (unpaired *t*

951 test) in base conditions. Data are shown as mean \pm SEM. **P* < 0.05, ***P* < 0.01, ****P* <

952 0.001. n.s., not significant.

953



954

955 **Fig 6. Distribution of TASK-3 in DRG neurons.**

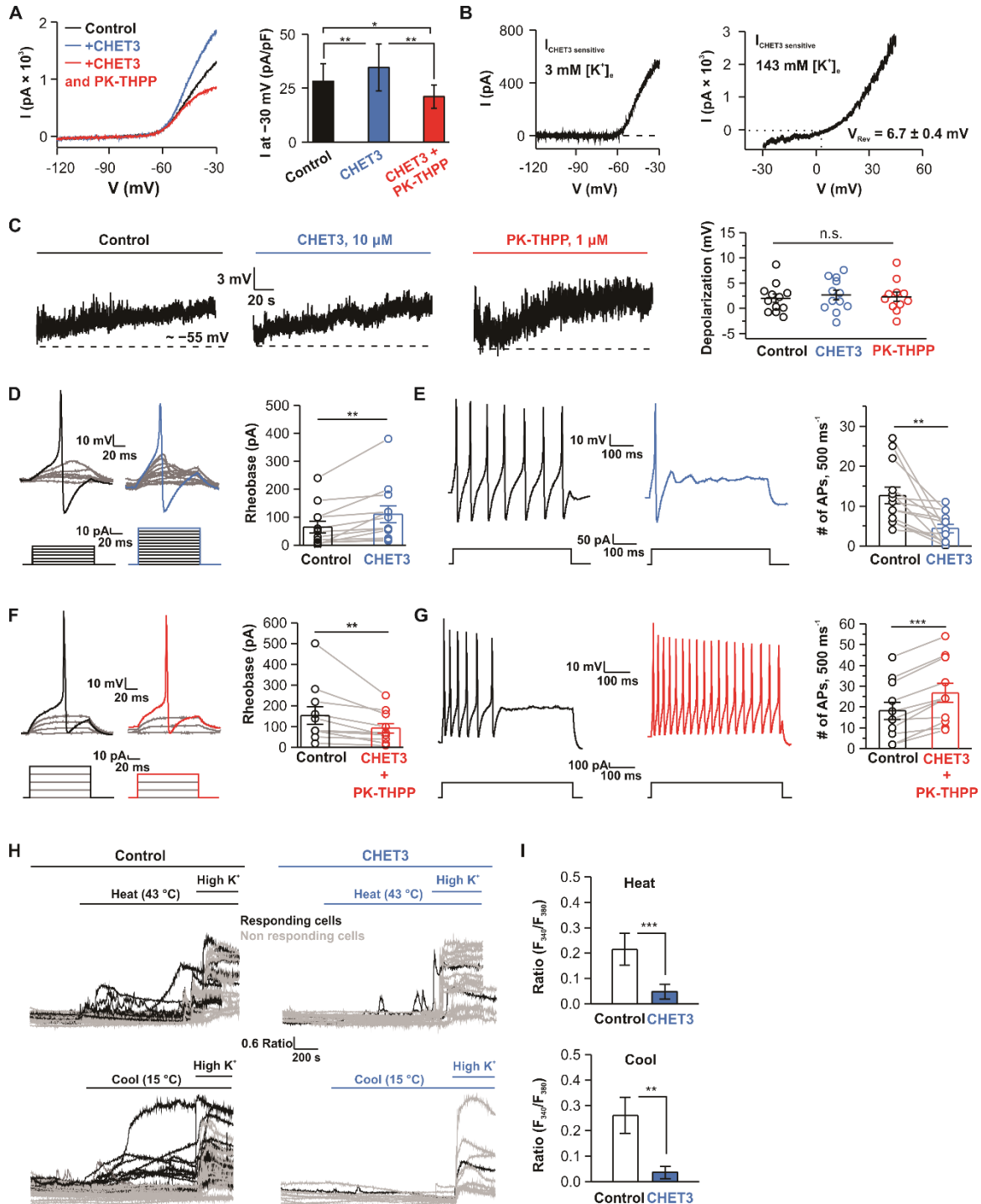
956 (A and B) Images and quantifications showing *Kcnk9* expression in sensory neurons
 957 using RNAscope (n = 7 sections from 3 mice) and TG (n = 8 sections from 3 mice)
 958 (Mann-Whitney test). Data in (B) are shown as mean ± SEM. (C) Quantification of the
 959 cell sizes of *Kcnk9*⁺ neurons (n = 6 sections from 3 mice). (D) Representative images
 960 showing *Kcnk9*⁺ neurons expression in different subtype of DRG neurons using
 961 RNAscope. (E) Bar graph summary for experiments in (D) (n = 4-9 DRG sections from

962 3-8 mice for each condition). Data are shown as mean \pm SEM. (F) Schematic summary

963 for the distribution of *Kcnk9*⁺ neurons in DRG. ***P* < 0.01.

964

965



966

967 **Fig 7. Functional roles of TASK-3 in nociceptive neurons.**

968 (A) *left*, Representative electrophysiological traces showing CHET3 ($10 \mu\text{M}$) and

969 PK-THPP ($1 \mu\text{M}$) effects on K^+ currents in DRG neurons; *right*, Bar graph summary for

970 experiments in *left* ($n = 9$ cells in 6 mice; paired t test). **(B)** Representative traces showing
971 CHET3-sensitive currents at different extracellular K^+ concentrations (V_{Rev} was
972 determined from $n = 5$ cells in 3 mice). **(C)** Representative traces and scatter plots
973 showing resting membrane potential (RMP) changes in response to Vehicle, CHET3 or
974 PK-THPP ($n = 12$ cells for Control and CHET3, $n = 11$ cells for PK-THPP in 5 mice for
975 each condition; one-way ANOVA test). **(D and E)** Traces and bar graph showing CHET3
976 effect on rheobase and firing frequency in nociceptive neurons ($n = 12$ cells in 5 mice;
977 paired sample Wilcoxon signed rank test in (D), paired t test in (E)). **(F and G)** Traces and
978 bar graph showing co-application of CHET3 and PK-THPP on rheobase and firing
979 frequency in nociceptive neurons ($n = 11$ cells in 3 mice; paired sample Wilcoxon signed
980 rank test in (F), paired t test in (G)). **(H)** Individual Ca^{2+} imaging traces from small-sized
981 DRG neurons in representative field of views in response to heat (25 °C-43 °C) , cooling
982 (37 °C-15 °C). **(I)** Bar graphs summary for experiments in (H) (Heat: $n = 143$ cells in 11
983 coverslips for control, $n = 99$ cells in 9 coverslips for CHET3; Mann-Whitney test. Cool:
984 $n = 87$ cells in 9 coverslips for control, $n = 46$ cells in 9 coverslips for CHET3;
985 Mann-Whitney test. Both experiments were from 3 independent preparations from 6
986 mice). Data are shown as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. n.s., not
987 significant.

988

989