Supplementary material for

TREK-1 channel activation as a new analgesic strategy devoid of opioid adverse effects

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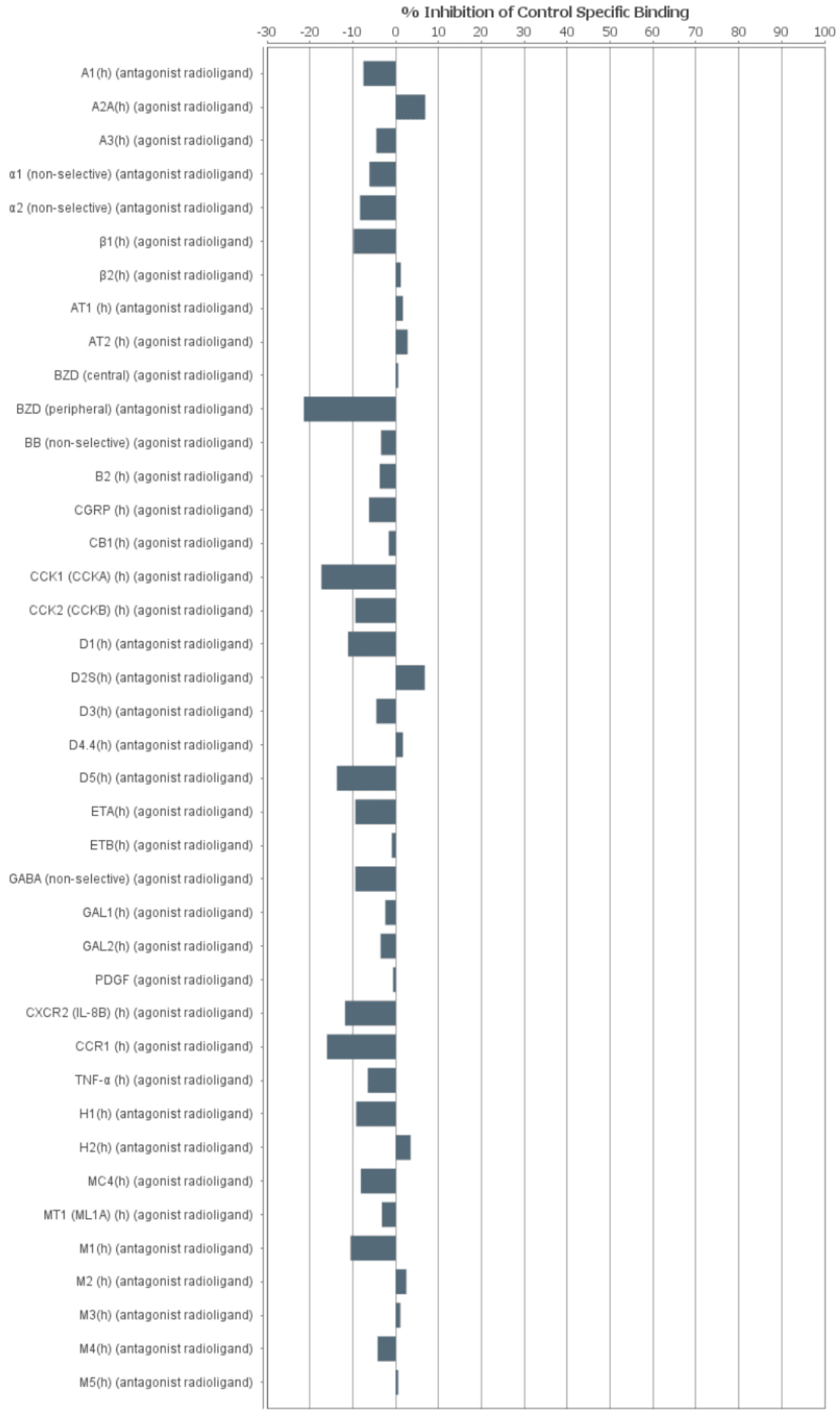
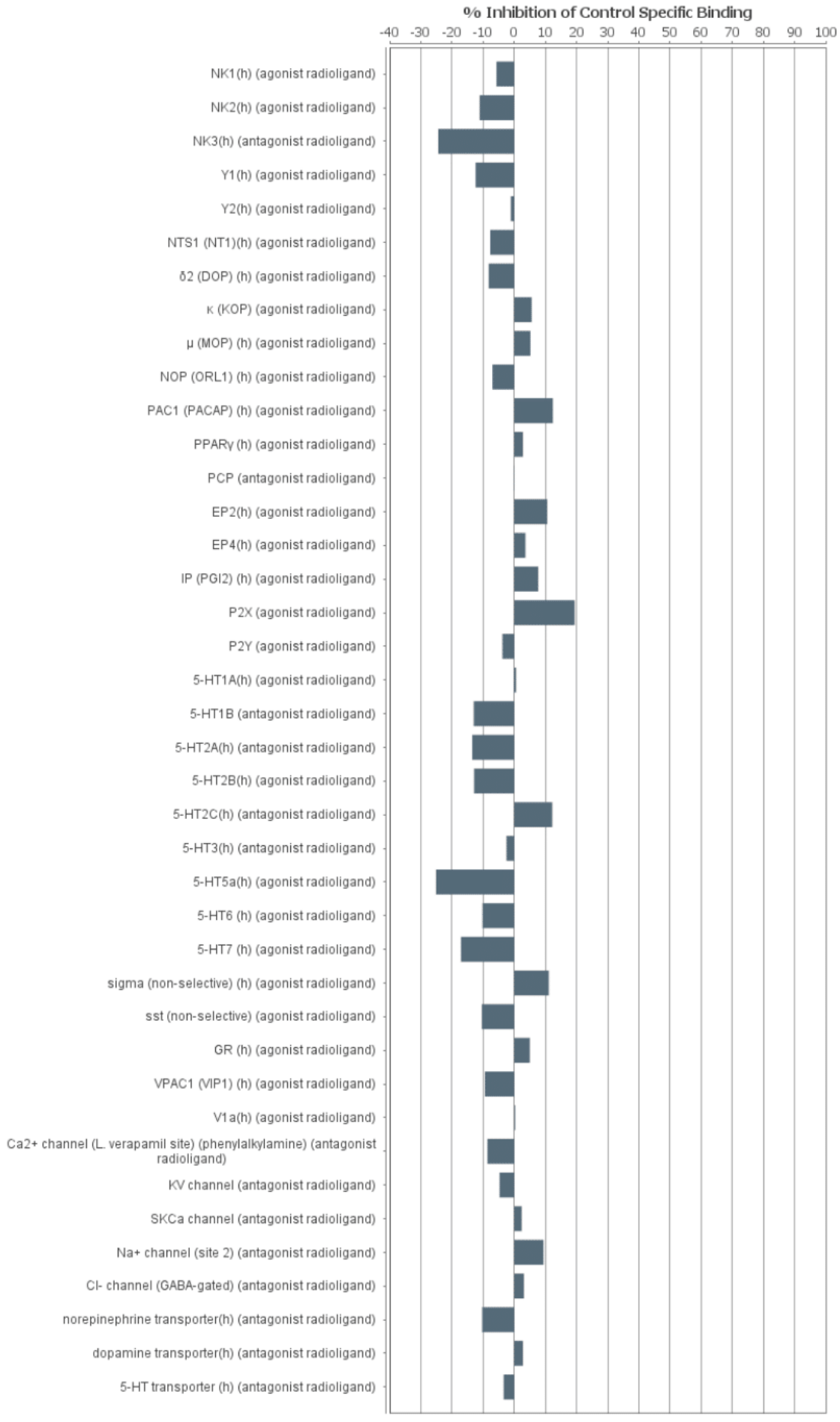
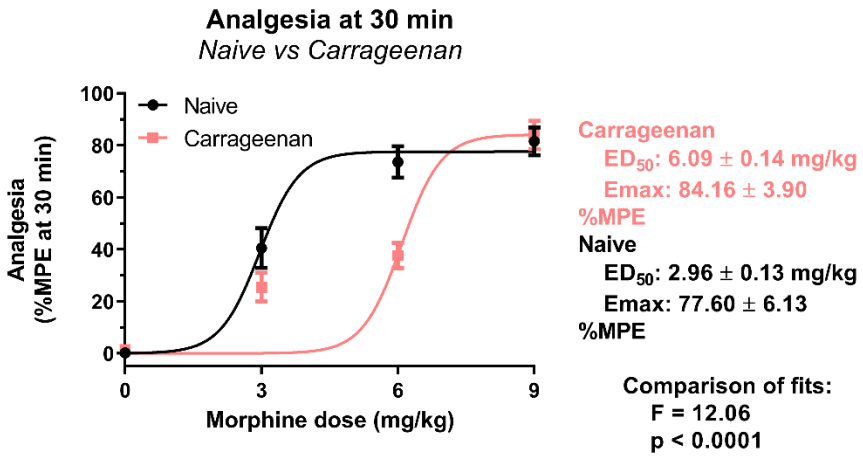
Supplementary Figures


% Inhibition of Control Specific Binding
-30
-20
-10
0
10
20
30
40
50
60
70
80
90
100
A1(h) (antagonist radioligand)
A2A(h) (agonist radioligand)
A3(h) (agonist radioligand)
α1 (non-selective) (antagonist radioligand)
α2 (non-selective) (antagonist radioligand)
β1(h) (agonist radioligand)
β2(h) (agonist radioligand)
AT1 (h) (antagonist radioligand)
AT2 (h) (agonist radioligand)
BZD (central) (agonist radioligand)
BZD (peripheral) (antagonist radioligand)
BB (non-selective) (agonist radioligand)
B2 (h) (agonist radioligand)
CGRP (h) (agonist radioligand)
CB1(h) (agonist radioligand)
CCK1 (CCKA) (h) (agonist radioligand)
CCK2 (CCKB) (h) (agonist radioligand)
D1(h) (antagonist radioligand)
D2S(h) (antagonist radioligand)
D3(h) (antagonist radioligand)
D4.4(h) (antagonist radioligand)
D5(h) (antagonist radioligand)
ETA(h) (agonist radioligand)
ETB(h) (agonist radioligand)
GABA (non-selective) (agonist radioligand)
GAL1(h) (agonist radioligand)
GAL2(h) (agonist radioligand)
PDGF (agonist radioligand)
CXCR2 (IL-8B) (h) (agonist radioligand)
CCR1 (h) (agonist radioligand)
TNF-α (h) (agonist radioligand)
H1(h) (antagonist radioligand)
H2(h) (antagonist radioligand)
MC4(h) (agonist radioligand)
MT1 (ML1A) (h) (agonist radioligand)
M1(h) (antagonist radioligand)
M2 (h) (antagonist radioligand)
M3(h) (antagonist radioligand)
M4(h) (antagonist radioligand)
M5(h) (antagonist radioligand)


% Inhibition of Control Specific Binding
-40 -30 -20 -10 0 10 20 30 40 50 60 70 80 90 100
NK1(h) (agonist radioligand)
NK2(h) (agonist radioligand)
NK3(h) (antagonist radioligand)
Y1(h) (agonist radioligand)
Y2(h) (agonist radioligand)
NTS1 (NT1)(h) (agonist radioligand)
δ2 (DOP) (h) (agonist radioligand)
κ (KOP) (agonist radioligand)
μ (MOP) (h) (agonist radioligand)
NOP (ORL1) (h) (agonist radioligand)
PAC1 (PACAP) (h) (agonist radioligand)
PPARγ (h) (agonist radioligand)
PCP (antagonist radioligand)
EP2(h) (agonist radioligand)
EP4(h) (agonist radioligand)
IP (PGI2) (h) (agonist radioligand)
P2X (agonist radioligand)
P2Y (agonist radioligand)
5-HT1A(h) (agonist radioligand)
5-HT1B (antagonist radioligand)
5-HT2A(h) (antagonist radioligand)
5-HT2B(h) (agonist radioligand)
5-HT2C(h) (antagonist radioligand)
5-HT3(h) (antagonist radioligand)
5-HT5a(h) (agonist radioligand)
5-HT6 (h) (agonist radioligand)
5-HT7 (h) (agonist radioligand)
sigma (non-selective) (h) (agonist radioligand)
sst (non-selective) (agonist radioligand)
GR (h) (agonist radioligand)
VPAC1 (VIP1) (h) (agonist radioligand)
V1a(h) (agonist radioligand)
Ca2+ channel (L. verapamil site) (phenylalkylamine) (antagonist radioligand)
KV channel (antagonist radioligand)
SKCa channel (antagonist radioligand)
Na+ channel (site 2) (antagonist radioligand)
Cl- channel (GABA-gated) (antagonist radioligand)
norepinephrine transporter(h) (antagonist radioligand)
dopamine transporter(h) (antagonist radioligand)
5-HT transporter (h) (antagonist radioligand)

Supplementary Figure 1 RNE28 binding assay.

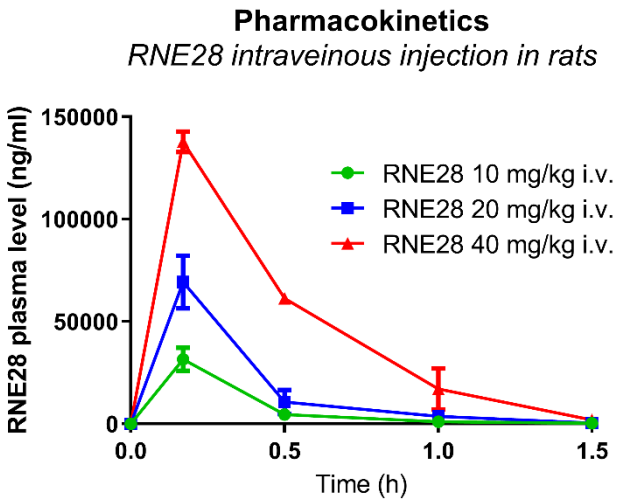
Percentage of inhibition of specific binding of radiolabeled ligands for various receptors, channels and transporters, by 10 µM RNE28 (scintillation counting). Assay performed by Eurofins-Cerep (Celle l'Evescault, France).



Supplementary Figure 2 Analgesic activity of morphine in naive and inflamed animals.

Dose-effect curves of the antinociceptive activity of morphine 30 min following oral gavage in naive (a) and carrageenan-injected (b) mice. Datasets were fitted with the least-square method and fits were compared with the extra sum-of-square F test. ED_{50}, E_{max}, and p and F values are shown on the right.

n = 10 and represent mice.

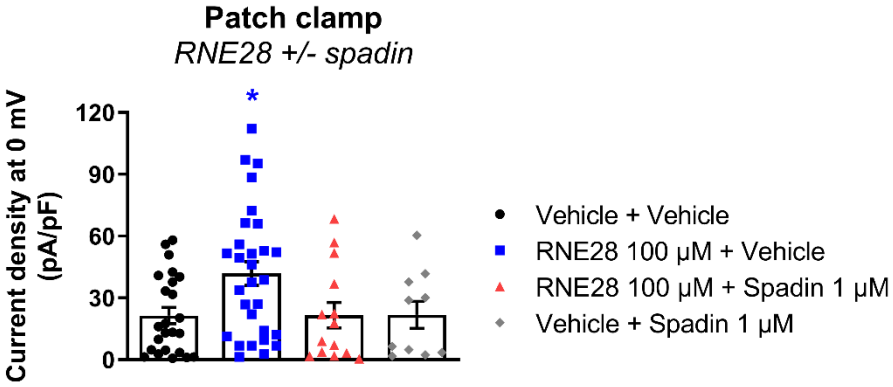


Treatment	C_{max} (ng/ml)	T_{max} (h)	AUC (ng/ml*h)	$t_{1/2}$ (h)
RNE28 10 mg/kg i.v.	31539	0.17	10421	0.3
RNE28 20 mg/kg i.v.	69336	0.17	23751	0.2
RNE28 40 mg/kg i.v.	137792	0.17	68907	0.2

Supplementary Figure 3 RNE28 pharmacokinetics.

Rats were injected with 10, 20 or 40 mg/kg RNE28 via the intravenous route. Blood samples were taken before and 10, 30, 60 and 90 min after injection, and RNE28 plasmatic concentration was determined by HPLC. Assay performed by CERB (Baugy, France).

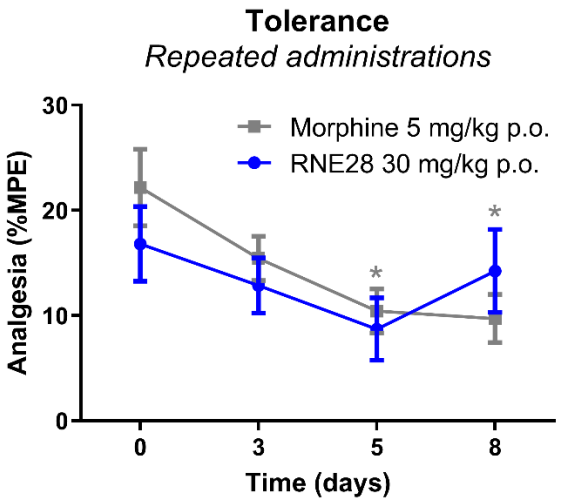
n = 5 and represent rats.



Supplementary Figure 4 Blockade of the effect of RNE28 on TREK-1 currents by spadin.

Current densities recorded in HEK-293 cells transfected with the human TREK-1 channel in response to 100 µM RNE28 (n = 29), 1 µM spadin (n = 10), both drugs (n = 14) or their respective vehicles (n = 24).

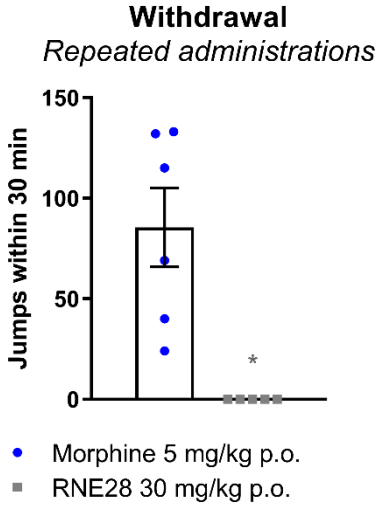
* $p < 0.05$ vs Vehicle + Vehicle. One-way ANOVA followed by Dunnett's post-hoc test. n numbers represent cells.



Supplementary Figure 5 Tolerance to the antinociceptive effect of RNE28.

Mice were given RNE28 at 30 mg/kg or morphine at 5 mg/kg per os, twice a day for 9 days. Pain thresholds were assessed at days 0, 3, 5 and 8 by paw immersion in 46°C water and plotted as %MPE.

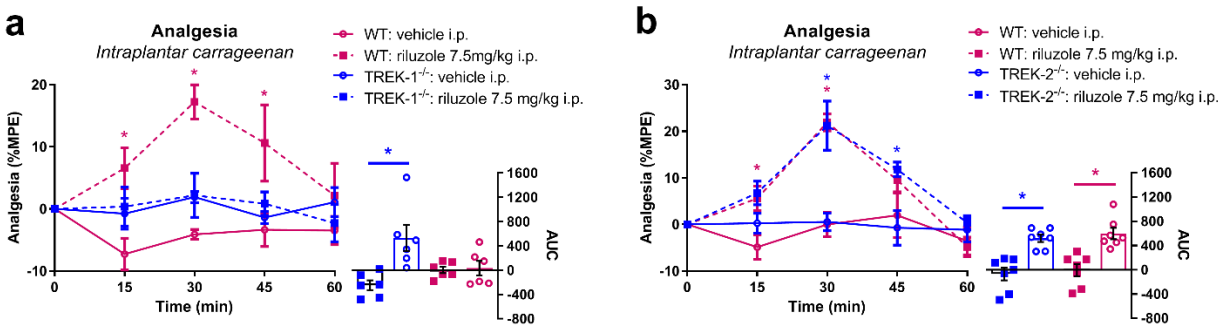
* $p < 0.05$ vs Day 0 of the same treatment. Two-way ANOVA followed by Dunnett's post-hoc test. n = 12 for both groups and represent mice.



Supplementary Figure 6 Naloxone-precipitated withdrawal in RNE28-treated mice.

Mice were given RNE28 at 30 mg/kg (n = 5) or morphine at 5 mg/kg (n = 6) per os, twice a day for 9 days. 2 h after the last administration, animals received an intraperitoneal injection of 2 mg/kg naloxone. Jumping behavior, which is characteristic of a precipitated withdrawal syndrome, was monitored for 30 min.

* $p < 0.05$ vs morphine. Mann-Whitney test. n represent mice.



Supplementary Figure 7 Analgesic activity of riluzole in TREK-1$^{-/-}$ and TREK-2$^{-/-}$ mice.

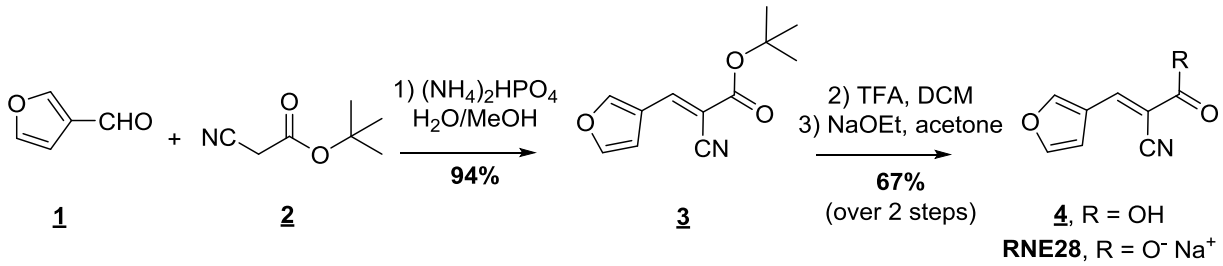
a *Left*, time-course of the analgesic effect induced by 0 or 7.5 mg/kg riluzole administered intraplantarly in WT and TREK-1$^{-/-}$ mice 2 h following paw inflammation induction by intraplantar carrageenan injection (20 µl, 2%). Pain thresholds were evaluated by paw immersion in 46°C water and plotted as %MPE. *Right*, areas under curves calculated from 0 to 60 min post-administration.

b *Left*, time-course of the analgesic effect induced by 0 or 7.5 mg/kg riluzole administered intraplantarly in WT and TREK-2$^{-/-}$ mice 2 h following paw inflammation induction by intraplantar carrageenan injection (20 µl, 2%). Pain thresholds were assessed by paw immersion in 46°C water and plotted as %MPE. *Right*, areas under curves calculated from 0 to 60 min post-administration.

* $p < 0.05$ vs vehicle of the same genotype. Two-way ANOVA followed by Sidak's post-hoc test (a-b left) and Kruskal-Wallis followed by Dunn's post-hoc test (a-b right). n = 6 (a, in all groups) and n = 7 (b, in all groups) and represent mice.

Supplementary Methods

RNE28 synthesis



To a solution of cyanoacetic acid t-butylester **2** (1.05 equiv.) in methanol (10 vol) and water (9 vol) was added ammonium phosphate dibasic (2 mol%) followed by 3-furaldehyde **1** (1 equiv.). The reaction mixture was warmed to 30 °C and stirred for 8 hours. The suspension was cooled to 15 °C and stirred for 30 minutes before being filtered. The wet cake was dried at 45 °C to allow the obtention of E)-tert-butyl 2-cyano-3-(furan-3-yl)acrylate **3** as white powder in 94% yield. ^{1}H NMR (400 MHz, DMSO-d^6): δ 8.56 (s, 1H), 8.27 (s, 1H), 7.98 (s, 1H), 7.19 (s, 1H), 1.53 (s, 9H).

A solution of (E)-tert-butyl 2-cyano-3-(furan-3-yl)acrylate **3** (1 eq) in dichloromethane (10 vol) and trifluoroacetic acid (2.5 equiv.) was stirred at room temperature (20 °C) for 24 hours before filtration. The residue was triturated with ether (5 V) and the solvent was evaporated under reduced pressure. This work up was repeated twice to give (E)-2-cyano-3-(furan-3-yl)acrylic acid **4** as a brown powder in 99% yield. ^{1}H NMR (400 MHz, DMSO-d^6): δ 13.83 (s, 1H), 8.54 (s, 1H), 8.29 (s, 1H), 7.96 (s, 1H), 7.19 (s, 1H).

Acid **4** was dissolved in acetone (9 vol) and water (1 vol) and stirred at 20 °C for 90 min until a clear solution was obtained. A sodium ethoxide solution (1 equiv.) was added and the mixture was stirred until a suspension was obtained (60 min). The pH was checked (pH 5) before the mixture was cooled to 2 °C and stirred at this temperature for 1 hour before filtration. The product RNE28Na was obtained as a white powder in 67% yield over two steps. HPLC purity 99.9% a/a, Na content 12.5% w/w (calculated 12.42%); mp. 224 °C; ^{1}H NMR (400 MHz, methanol-d^4): δ 8.11 (s, 1H), 7.95 (s, 1H), 7.65 (s, 1H), 7.21 (s, 1H).

Animals and models: TREK-2$^{-/-}$ mice

The TREK-2 gene inactivation strategy has been previously described (Guyon et al., 2009). It consists in a duplication of exon 2 introducing a frameshift which results in a premature stop codon in knock-out mice.

Binding assays

Binding assays were performed by Eurofins Cerep (Celle l'Evescault, France). RNE28 binding was calculated as the percentage of inhibition of radioactively labeled ligands specific for different target receptors/channels/transporters (scintillation counting). The table below details for each target the cells and radiolabeled ligand used, its concentration and Kd, the control used to measure non-specific binding and the incubation time and temperature used. RNE28 was used at 10 µM in every assay.
Results showing an inhibition or stimulation higher than 50% are considered to represent significant effects of the test compounds. Results showing an inhibition (or stimulation) between 25% and 50% are indicative of weak to moderate effects and results showing an inhibition (or stimulation) lower than 25% are not considered significant and mostly attributable to variability of the signal around the control level.

Assay	*Source*	*Ligand*	*Conc.*	*Kd*	*Non Specific*	*Incubation*
A1(h) (antagonist radioligand)	human recombinant (CHO cells)	[3H]DPCPX	1 nM	1.7 nM	DPCPX (1 µM)	60 min RT
A2A(h) (agonist radioligand)	human recombinant (HEK-293 cells)	[3H]CGS 21680	6 nM	27 nM	NECA (10 µM)	120 min RT
A3(h) (agonist radioligand)	human recombinant (HEK-293 cells)	[125I]AB-MECA	0.15 nM	0.22 nM	IB-MECA (1 µM)	120 min RT
α1 (non-selective) (antagonist radioligand)	rat cerebral cortex	[3H]prazosin	0.25 nM	0.09 nM	prazosin (0.5 µM)	60 min RT
α2 (non-selective) (antagonist radioligand)	rat cerebral cortex	[3H]RX 821002	0.5 nM	0.38 nM	(-)epinephrine (100 µM)	60 min RT
β1 (h) (agonist radioligand)	human recombinant (HEK-293 cells)	[3H](-)CGP 12177	0.3 nM	0.39 nM	alprenolol (50 µM)	60 min RT
β2 (h) (agonist radioligand)	human recombinant (CHO cells)	[3H](-)CGP 12177	0.3 nM	0.15 nM	alprenolol (50 µM)	120 min RT
AT1 (h) (antagonist radioligand)	human recombinant (HEK-293 cells)	[125I][Sar1,Ile8]-AT-II	0.05 nM	0.05 nM	angiotensin-II (10 µM)	120 min 37°C
AT2 (h) (agonist radioligand)	human recombinant (HEK-293 cells)	[125I]CGP 42112A	0.01 nM	0.01 nM	angiotensin-II (1 µM)	4 hr 37°C
BZD (peripheral) (antagonist radioligand)	rat heart	[3H]PK 11195	0.2 nM	1.8 nM	PK 11195 (10 µM)	15 min RT
BB (non-selective) (agonist radioligand)	rat cerebral cortex	[125I][Tyr4]bombesin	0.01 nM	0.71 nM	bombesin (1 µM)	60 min RT
B2 (h) (agonist radioligand)	human recombinant (CHO cells)	[3H]bradykinin	0.3 nM	0.32 nM	bradykinin (1 µM)	60 min RT
CGRP (h) (agonist radioligand)	human recombinant (CHO cells)	[125I]hCGRPα	0.03 nM	0.06 nM	hCGRPα (1 µM)	90 min RT
CB1(h) (agonist radioligand)	human recombinant (CHO cells)	[3H]CP 55940	0.5 nM	3.5 nM	WIN 55212-2 (10 µM)	120 min 37°C
CCK1 (CCKA) (h) (agonist radioligand)	human recombinant (CHO cells)	[125I]CCK-8s	0.08 nM	0.24 nM	CCK-8s (1 µM)	60 min RT
CCK2 (CCKB) (h) (agonist radioligand)	human recombinant (CHO cells)	[125I]CCK-8s	0.08 nM	0.054 nM	CCK-8s (1 µM)	60 min RT
D1(h) (antagonist radioligand)	human recombinant (CHO cells)	[3H]SCH 23390	0.3 nM	0.2 nM	SCH 23390 (1 µM)	60 min RT
D2S(h) (antagonist radioligand)	human recombinant (HEK-293 cells)	[3H]methyl-spiperone	0.3 nM	0.15 nM	(+)butaclamol (10 µM)	60 min RT
D3(h) (antagonist radioligand)	human recombinant (CHO cells)	[3H]methyl-spiperone	0.3 nM	0.085 nM	(+)butaclamol (10 µM)	60 min RT
D4.4(h) (antagonist radioligand)	human recombinant (CHO cells)	[3H]methyl-spiperone	0.3 nM	0.19 nM	(+)butaclamol (10 µM)	60 min RT

Assay	*Source*	*Ligand*	*Conc.*	*Kd*	*Non Specific*	*Incubation*
D5(h) (antagonist radioligand)	human recombinant (GH4 cells)	[3H]SCH 23390	0.3 nM	0.25 nM	SCH 23390 (10 µM)	60 min RT
ETA(h) (agonist radioligand)	human recombinant (CHO cells)	[125I]endothelin-1	0.03 nM	0.03 nM	endothelin-1 (100 nM)	120 min 37°C
ETB (h) (agonist radioligand)	human recombinant (CHO cells)	[125I]endothelin-1	0.03 nM	0.04 nM	endothelin-1 (0.1 µM)	120 min 37°C
GABA (non-selective) (agonist radioligand)	rat cerebral cortex	[3H]GABA	10 nM	15 nM	GABA (100 µM)	60 min RT
GAL1(h) (agonist radioligand)	human recombinant (HEK-293 cells)	[125I]galanin	0.1 nM	0.1 nM	galanin (1 µM)	60 min RT
GAL2(h) (agonist radioligand)	human recombinant (CHO cells)	[125I]galanin	0.05 nM	0.63 nM	galanin (1 µM)	120 min RT
PDGF (agonist radioligand)	Balb/c 3T3 cells	[125I]PDGF BB	0.03 nM	0.15 nM	PDGF BB (10 nM)	180 min 4°C
CXCR2 (IL-8B) (h) (agonist radioligand)	human recombinant (HEK-293 cells)	[125I]IL-8	0.025 nM	0.022 nM	IL-8 (30 nM)	60 min RT
CCR1 (h) (agonist radioligand)	human recombinant (HEK-293 cells)	[125I]MIP-1α	0.01 nM	0.02 nM	MIP-1α (100 nM)	120 min RT
TNF-α (h) (agonist radioligand)	U-937 cells	[125I]TNF-α	0.1 nM	0.05 nM	TNF-α (10 nM)	120 min 4°C
H1(h) (antagonist radioligand)	human recombinant (HEK-293 cells)	[3H]pyrilamine	1 nM	1.7 nM	pyrilamine (1 µM)	60 min RT
H2(h) (antagonist radioligand)	human recombinant (CHO cells)	[125I]APT	0.075 nM	2.9 nM	tiotidine (100 µM)	120 min RT
MC4(h) (agonist radioligand)	human recombinant (CHO cells)	[125I]NDP-α-MSH	0.05 nM	0.54 nM	NDP-α-MSH (1 µM)	120 min 37°C
MT1 (ML1A) (h) (agonist radioligand)	human recombinant (CHO cells)	[125I]2-iodomelatonin	0.01 nM	0.04 nM	melatonin (1 µM)	60 min RT
M1(h) (antagonist radioligand)	human recombinant (CHO cells)	[3H]pirenzepine	2 nM	13 nM	atropine (1 µM)	60 min RT
M2 (h) (antagonist radioligand)	human recombinant (CHO cells)	[3H]AF-DX 384	2 nM	4.6 nM	atropine (1 µM)	60 min RT
M3 (h) (antagonist radioligand)	human recombinant (CHO cells)	[3H]4-DAMP	0.2 nM	0.5 nM	atropine (1 µM)	60 min RT
M4(h) (antagonist radioligand)	human recombinant (CHO cells)	[3H]4-DAMP	0.2 nM	0.32 nM	atropine (1 µM)	60 min RT
M5(h) (antagonist radioligand)	human recombinant (CHO cells)	[3H]4-DAMP	0.3 nM	0.3 nM	atropine (1 µM)	60 min RT
NK1(h) (agonist radioligand)	U373MG uppsala	[125I]-Substance P LYS3	0.05 nM	0.04 nM	[Sar9,Met(O2)11]-SP (1 µM)	30 min RT
NK2(h) (agonist radioligand)	human recombinant (CHO cells)	[125I]NKA	0.1 nM	0.12 nM	[Nleu10]-NKA (4-10) (300 nM)	60 min RT
NK3 (h) (antagonist radioligand)	human recombinant (CHO cells)	[3H]SR 142801	0.4 nM	0.47 nM	SB 222200 (10 µM)	120 min RT

Assay	*Source*	*Ligand*	*Conc.*	*Kd*	*Non Specific*	*Incubation*
Y1(h) (agonist radioligand)	SK-N-MC cells (endogenous)	[125I]peptide YY	0.025 nM	0.06 nM	NPY (1 µM)	120 min 37°C
Y2(h) (agonist radioligand)	KAN-TS cells	[125I]peptide YY	0.015 nM	0.01 nM	NPY (1 µM)	60 min 37°C
NTS1 (NT1) (h) (agonist radioligand)	human recombinant (CHO cells)	[125I]Tyr3-neurotensin	0.05 nM	0.22 nM	neurotensin (1 µM)	60 min 4°C
δ2 (DOP) (h) (agonist radioligand)	human recombinant (CHO cells)	[3H]DADLE	0.5 nM	0.73 nM	naltrexone (10 µM)	120 min RT
κ (KOP) (agonist radioligand)	rat recombinant (CHO cells)	[3H]U 69593	1 nM	2 nM	naloxone (10 µM)	60 min RT
µ (MOP) (h) (agonist radioligand)	human recombinant (HEK-293 cells)	[3H]DAMGO	0.5 nM	0.35 nM	naloxone (10 µM)	120 min RT
NOP (ORL1) (h) (agonist radioligand)	human recombinant (HEK-293 cells)	[3H]nociceptin	0.2 nM	0.4 nM	nociceptin (1 µM)	60 min RT
PAC1 (PACAP) (h) (agonist radioligand)	human recombinant (CHO cells)	[125I]PACAP1-27	0.015 nM	0.092 nM	PACAP1-27 (100 nM)	120 min RT
PPARγ (h) (agonist radioligand)	human recombinant (E. coli)	[3H]rosiglitazone	5 nM	5.7 nM	rosiglitazone (10 µM)	120 min 4°C
EP2(h) (agonist radioligand)	human recombinant (HEK-293 cells)	[3H]PGE2	3 nM	3 nM	PGE2 (10 µM)	120 min RT
EP4(h) (agonist radioligand)	human recombinant (HEK-293 cells)	[3H]PGE2	0.5 nM	0.3 nM	PGE2 (10 µM)	120 min RT
IP (PGI2) (h) (agonist radioligand)	human recombinant (HEK-293 cells)	[3H]iloprost	6 nM	8 nM	iloprost (10 µM)	60 min RT
P2Y (agonist radioligand)	rat cerebral cortex	[35S]dATPαS	10 nM	10 nM	dATPαS	60 min RT
5-HT1A(h) (agonist radioligand)	human recombinant (HEK-293 cells)	[3H]8-OH-DPAT	0.3 nM	0.5 nM	8-OH-DPAT (10 µM)	60 min RT
5-HT1B (antagonist radioligand)	rat cerebral cortex	[125I]CYP (+ 30 µM isoproterenol)	0.1 nM	0.16 nM	serotonin (10 µM)	120 min 37°C
5-HT2A(h) (antagonist radioligand)	human recombinant (HEK-293 cells)	[3H]ketanserin	0.5 nM	0.6 nM	ketanserin (1 µM)	60 min RT
5-HT2B(h) (agonist radioligand)	human recombinant (CHO cells)	[125I](±)DOI	0.2 nM	0.2 nM	(±)DOI (1 µM)	60 min RT
5-HT2C(h) (antagonist radioligand)	human recombinant (HEK-293 cells)	[3H]mesulergine	1 nM	0.5 nM	RS 102221 (10 µM)	120 min 37°C
5-HT5a (h) (agonist radioligand)	human recombinant (HEK-293 cells)	[3H]LSD	1.5 nM	1.5 nM	serotonin (100 µM)	120 min 37°C
5-HT6 (h) (agonist radioligand)	human recombinant (CHO cells)	[3H]LSD	2 nM	1.8 nM	serotonin (100 µM)	120 min 37°C
5-HT7 (h) (agonist radioligand)	human recombinant (CHO cells)	[3H]LSD	4 nM	2.3 nM	serotonin (10 µM)	120 min RT

Assay	*Source*	*Ligand*	*Conc.*	*Kd*	*Non Specific*	*Incubation*
sigma (non-selective) (h) (agonist radioligand)	Jurkat cells (endogenous)	[3H]DTG	10 nM	41 nM	Haloperidol (10 µM)	120 min RT
sst (non-selective) (agonist radioligand)	AtT-20 cells	[125I]Tyr11-somatostatin-14	0.05 nM	0.08 nM	somatostatin-14 (300 nM)	60 min 37°C
GR (h) (agonist radioligand)	IM-9 cells (cytosol)	[3H]dexamethasone	1.5 nM	1.5 nM	triamcinolone (10 µM)	6 hr 4°C
VPAC1 (VIP1) (h) (agonist radioligand)	human recombinant (CHO cells)	[125I]VIP	0.04 nM	0.05 nM	VIP (1 µM)	60 min RT
V1a(h) (agonist radioligand)	human recombinant (CHO cells)	[3H]AVP	0.3 nM	0.5 nM	AVP (1 µM)	60 min RT
BZD (central) (agonist radioligand)	rat cerebral cortex	[3H]flunitrazepam	0.4 nM	2.1 nM	diazepam (3 µM)	60 min 4°C
PCP (antagonist radioligand)	rat cerebral cortex	[3H]TCP	10 nM	13 nM	MK 801 (10 µM)	120 min 37°C
P2X (agonist radioligand)	rat urinary bladder	[3H]α,β-MeATP	3 nM	2.6 nM	α,β-MeATP (10 µM)	120 min 4°C
5-HT3(h) (antagonist radioligand)	human recombinant (CHO cells)	[3H]BRL 43694	0.5 nM	1.15 nM	MDL 72222 (10 µM)	120 min RT
Ca2+ channel (L, verapamil site) (phenylalkylamine) (antagonist radioligand)	rat cerebral cortex	[3H]D888	3 nM	3 nM	D 600 (10 µM)	120 min RT
KV channel (antagonist radioligand)	rat cerebral cortex	[125I]α-dendrotoxin	0.01 nM	0.04 nM	α-dendrotoxin (50 nM)	60 min RT
SKCa channel (antagonist radioligand)	rat cerebral cortex	[125I]apamin	0.007 nM	0.007 nM	apamin (100 nM)	60 min 4°C
Na+ channel (site 2) (antagonist radioligand)	rat cerebral cortex	[3H]batrachotoxinin	10 nM	91 nM	veratridine (300 µM)	60 min 37°C
Cl- channel (GABA-gated) (antagonist radioligand)	rat cerebral cortex	[35S]TBPS	3 nM	14.6 nM	picrotoxinin (20 µM)	120 min RT
norepinephrine transporter (h) (antagonist radioligand)	human recombinant (CHO cells)	[3H]nisoxetine	1 nM	2.9 nM	desipramine (1 µM)	120 min 4°C
dopamine transporter (h) (antagonist radioligand)	human recombinant (CHO cells)	[3H]BTCP	4 nM	4.5 nM	BTCP (10 µM)	120 min 4°C
5-HT transporter (h) (antagonist radioligand)	human recombinant (CHO cells)	[3H]imipramine	2 nM	1.7 nM	imipramine (10 µM)	60 min RT

RNE28 quantification in serum samples

RNE28 was assayed by CERB (Baugy, France), by reversed phase HPLC with UV detection after liquid-liquid extraction. Chromatographic separations were performed with a Varian 230 pump, Varian 410 autosampler (injection loop 200 µl, 90 µl injected) and Varian 345 detector (302 nm) coupled to a Unisphere C18 column (250 x 4.6 mm, 5 µm) and security guard C18 precolumn (4 x 3 mm). Aliquots were injected and eluted with acetonitrile-0.1% formic acid in water (30:70, v/v). Flow rate was set at 1 ml/min and pressure at 140 bars. The RNE28 peak area was measured (about 10.2 min retention time).

Patch clamp: RNE28 and spadin co-exposure

Human TREK-1 (KCNK2, NCBI RefSeq NM_001017424) was cloned into pEZ-M02 vector (GeneCopoeia). HEK-293 cells were transfected in 35 mm dishes with 2 µg plasmid using the jetPRIME kit (Polypus) according to the manufacturer's instructions, and selected by addition of 1 mg/ml G418 to the culture medium for at least two weeks. Before patch clamp, the culture medium was replaced by the patch clamp extracellular solution supplemented with 100 µM RNE28, 1 µM spadin, and/or their respective vehicles (DMSO and water, both at 0.1% final). Recordings were performed 15 to 60 min after drug exposure. Currents were recorded using a MultiClamp 200B amplifier and an Axon Digidata 1440A digitizer (Molecular Devices). Data were recorded and stored using Clampex 10 (Molecular Devices). Recordings were low-pass-filtered at 5 kHz and sampled at 20 kHz.

Naloxone-precipitated withdrawal

Mice were given RNE28 at 30 mg/kg or morphine at 5 mg/kg per os, twice a day for 9 days. 2 h after the last administration, animals received an intraperitoneal injection of 2 mg/kg naloxone. Ten minutes before naloxone treatment, mice were placed in a transparent acrylic cylinder (20 cm in diameter, 60 cm high) for habituation. The jumping behavior, characteristic of a precipitated withdrawal syndrome, was monitored during 30 min. n = 6 for both groups and represent mice.

Reference

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