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1 **Cav3.2 calcium channels: the key protagonist of the supraspinal effect of paracetamol.**

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1

2 **1. Introduction**

3 Paracetamol (acetyl-*para*-aminophenol), is a widely used analgesic and antipyretic agent.
4 Nevertheless, its analgesic mechanism remains elusive and its metabolism is complex. Several studies
5 on paracetamol metabolites have largely focused on their toxic actions, notably by hepatotoxic
6 compounds like *p*-benzoquinone (*p*-BQ) and *N*-acetyl-*p*-benzoquinoneimine (NAPQI). Recently, we
7 have proposed that a new metabolic pathway, previously described by Högestätt et al. [25], would
8 be involved in its analgesic action. Paracetamol is metabolized to *p*-aminophenol, which is itself
9 metabolized in the brain by the fatty acid amide hydrolase (FAAH) to *N*-(4-hydroxyphenyl)-
10 5Z,8Z,11Z,14Z-eicosatetraenamide (AM404), which is able to induce analgesia [34]. Interestingly, this
11 active metabolite is structurally related to lipoaminoacids, a new class of signaling molecules
12 involved in pain modulation [6,26].

13 Members of the lipoaminoacid family, such as anandamide (*N*-arachidonoyl ethanolamide)
14 and 2-AG (2-arachidonylglycerol), interact with several targets including cannabinoid [21,47] and
15 TRPV1 receptors [26,43,56], two main actors involved in pain modulation and in the analgesic action
16 of paracetamol [34,35]. But interestingly, lipoaminoacids are also able to strongly inhibit T-type
17 calcium channels, especially the Cav3.2 member, an effect which mediates their analgesic property
18 [3].

19 T-type calcium channels have the exclusive characteristic to be activated by weak
20 depolarization close to the resting membrane potential conferring them a role of cell excitability
21 modulator [42]. In the central nervous system, T-type calcium channels participate in epilepsy [5,30],
22 spontaneous firing [42], slow-wave sleep [31] and pain perception [8,15,51]. Three T-type calcium
23 channels, Cav3.1, Cav3.2, and Cav3.3, have been described [42]. Among them, Cav3.2 channels are
24 expressed in small and medium-diameter neurons of the dorsal root ganglion (DRG), in the dorsal
25 horn superficial laminæ and in several brain structures [50]. In naive and neuropathic animals, we
26 have shown that Cav3.2 inhibition by antagonists or oligonucleotide antisenses reduced nociceptive

1 reactions and hyperalgesia, respectively [8,24]. Finally, Cav3.2 knock-out mice display severe
2 impairment in pain perception in several pain tests confirming the strong role of this channel in
3 nociception [15].

4 Considering that the AM404 lipoaminoacid was the active metabolite of paracetamol [25]
5 and that lipoaminoacids were able to inhibit Cav3.2 channels, which modulate nociception and pain
6 [3], we hypothesized that Cav3.2 channels could be involved in the mechanism of the analgesic
7 action of paracetamol. In this study, we have investigated the involvement of Cav3.2 channels in this
8 effect using genetic (Cav3.2 knock-out mice) and pharmacological (Cav3 antagonist, TTA-A2)
9 strategies in rodents submitted to various nociceptive tests and pathological conditions.

10 Interestingly, Zygmunt et al. [56] have shown that AM404 is also a TRPV1 agonist. We
11 previously demonstrated that brain TRPV1 was an essential actor for the analgesic action of
12 paracetamol [34]. The suspected dual involvement of brain TRPV1 receptor and Cav3.2 channels in
13 paracetamol action led us to investigate the interaction between these two protagonists. This
14 potential interaction was studied with a behavioral approach by using genetically modified mice
15 (Cav3.2 or TRPV1 knock-out mice) and a pharmacological strategy (TTA-A2). We have also used
16 electrophysiological and calcium imaging technics to investigate the mechanism of the functional
17 interaction between Cav3.2 channels and TRPV1 receptors.

18 Overall, our results demonstrate, for the first time, that the supraspinal Cav3.2 T-type
19 calcium channel works with TRPV1 receptor to support the analgesic action of paracetamol and
20 consequently highlights them as potential targets for the development of new analgesics.

21

22 **2. Materials and methods**

23 *2.1 Animals*

24 Cav3.2 knock-out mice (*Cav3.2*^{-/-}, 20-25 g, male), TRPV1 knock-out mice (*TRPV1*^{-/-}, 20-25 g,
25 male), originally generated by Chen CC et al. [13] and Caterina et al. [11] respectively, and their wild
26 type littermates (*Cav3.2*^{+/+}, *TRPV1*^{+/+}, 20-25 g, male) were used. All animals and experiments were
27 performed with approval by the Committee for Research and Ethical Issues of the IASP and the
28 Institutional Ethic Committee for animal experiments (CEMEA Auvergne; nr: CE 53 - 12, CE 112 - 12,

1 CE 24 - 11, CE 13 - 10). Animals were housed under controlled environmental conditions (21 - 22°C;
2 55% humidity) and kept under a 12/12h light/dark cycle. Food and water were available *ad libitum*.
3 Animals were euthanized by cervical dislocation or CO₂.

4 *2.2 Behavioral Studies*

5 Animals were habituated to the testing environment before baseline testing. The
6 experimenter and drug administration were blinded to the genotype of the mice and the treatments.
7 Drug administration was performed a person other than the experimenter. In all experiments, drugs
8 were orally or subcutaneous administered at 10 ml/kg.

9 *2.3 Actimetry test*

10 For assessment of locomotor activity, mice were placed in actimetry boxes (Actisystem,
11 Apelex, Passy, France) and spontaneous motor activity was assessed by determining the number of
12 crossings of light beams during 15 min. The test was performed 10 min after TTA-A2 or vehicle
13 administration.

14 *2.4 Intracerebroventricular and intrathecal injections*

15 Injections were carried out on mice kept under gas anesthesia (isoflurane). The
16 intracerebroventricular injection was made with a syringe and calibrated needle with a guide so that
17 the needle diameter was 4 mm. The injection volume was 2 µl per mice. The anesthetized rat was
18 held in one hand by the pelvic girdle and a 25-gauge X 1-inch needle connected to a 25 µl Hamilton
19 syringe was inserted into the subarachnoid space between lumbar vertebrae L5 and L6, until a tail
20 flick was elicited. The syringe was held in position for few seconds after the injection of a volume of 2
21 µl per mice.

22 *2.5 Tail and paw immersion tests*

23 Mice were habituated to handling for three days a week prior to testing. The mouse tail or
24 paw was immersed in warm water (46°C). Latency to respond to the heat stimulus with a tail or paw
25 vigorous flexion were measured three times and averaged. The cutoff time was 30 seconds, after
26 which the tail was removed from the bath regardless of response. Tests were performed 45 minutes
27 after paracetamol or morphine administration.

28 *2.6 Formalin test*

29 Mice were allowed to acclimate to a Plexiglas chamber (30 cm x 30 cm x 30 cm) for at least
30 30 minutes before testing. Formalin (20 µl, 2.5% formalin in saline) was injected into the plantar
31 surface of one hind paw. Spontaneous pain behavior (licking) was recorded during the two typical
32 nociceptive phases: from 0 to 5 min (phase I) and from 15 to 40 min (phase II) after formalin injection
33 as previously described [34]. For AM404 experiments, only phase 1 was studied due to the fast
34 degradation of AM404 [34]. Paracetamol or morphine administrations were performed 20 minutes
35 before formalin injection. Capsaicin, AM404 or TTA-A2 were injected 10 minutes before formalin
36 test.

37 *2.7 Von Frey test*

1 Mice were placed in individual compartments on top of a wire surface and allowed to
2 acclimatize for one hour before testing. Withdrawal thresholds were assessed with the 1.4 g
3 calibrated von Frey filament (Bioseb, France). The latter was pressed perpendicularly five times
4 against the mid paw and held for 3 seconds. A positive response was noted if the paw was withdrawn
5 or licked and a pain response score (from 0 to 5) was determined. Test was performed 45 minutes
6 after paracetamol or morphine administration or 10 minutes after AM404 or capsaicin injection.

7 *2.8 Monoarthritis Induction*

8 Five microliters of complete Freund adjuvant (CFA, DIFCO Laboratories, Detroit, USA) [10]
9 were injected into both sides of the left ankle joint of mice under brief isoflurane anesthesia.
10 Thermal and mechanical paw thresholds were determined before and 7 days after CFA or vehicle
11 injection. Baselines for behavioral tests were performed at day 0 and day 7 before experimental
12 treatments.

13 *2.9 Cell culture and electrophysiological recordings*

14 Lumbar DRGs with attached roots were dissected from adult male C57BL/6J mice and single
15 cell suspension was obtained following an enzymatic and mechanical dissociation. Whole cell patch-
16 clamp recordings were performed 3–28 h after plating on medium sized DRG neurons with a
17 “rosette” phenotype as previously described [24]. For recording calcium current, the extracellular
18 solution contained (in mM): 2 CaCl₂, 100 TEACl, 2 NaCl, 1 MgCl₂, 40 Choline Cl, 5 Glucose, 5 4AP (pH
19 to 7.4 with TEAOH~330 mOsM). Pipettes with a resistance of 1-1.5 Mohm were filled with an internal
20 solution containing (in mM): 110 CsCl, 3 MgCl₂, 10 EGTA, 10 HEPES, 3 Mg-ATP, 0.6 GTP (pH to 7.,4
21 with CsOH, ~300 mOsM). All recordings were filtered at 5 kHz using an Axopatch 200B amplifier
22 (Axon instrument). Data was recorded using a pClamp10 (Axon Instrument) and Graphpad Prism
23 software. Currents were recorded from a holding potential of -75 mV. This maintains the T-type
24 channels in a partially inactivated state that is often preferentially sensitive to pharmacological
25 modulations[3,24].

26 HEK cells stably expressing the human Cav3.2 sequence were used as previously described
27 [24] and were transfected with GFP alone or with a mix of TRPV1 and GFP expression plasmids using
28 JetPEI. Application of capsaicin (1 μM) or AM404 (3 μM) was achieved with a gravity driven WAS-2
29 perfusion system allowing control of the bath temperature [22]. For AM404 application experiments
30 were performed at 33-35°C.

31 *2.10 Calcium imaging*

32 Human embryonic kidney cells, HEK293T, were grown in Dulbecco’s modified Eagle’s medium
33 (DMEM) containing 10% (v/v) human serum, 2 mM L-glutamine, 2 units/ml penicillin and 2 mg/ml
34 streptomycin at 37°C in a humidity controlled incubator with 10% CO₂. HEK293T cells were
35 transiently transfected with human TRPV1 using Mirus TransIT-293 (Mirus Corporation; Madison, WI,
36 USA). To determine if the cells were transfected, capsaicin (1 μM) was used at the end of each
37 experiment. Cells which did not-respond to capsaicin were used as controls.

38 Cells were loaded with 2 μM Fura-2 acetoxymethyl ester for 30 minutes at 37°C. The
39 intracellular concentration was monitored as the ratio of fluorescence signals measured upon
40 alternating illumination at 340 and 380 nm, using an MT-10 illumination system and the Cell^{AM}

1 software (Olympus). Bath solution contained (in mM) 145 NaCl, 5 KCl, 2 CaCl₂, 1.5 MgCl₂, 10 HEPES,
2 10 D-Glucose, pH = 7.4 buffered with NaOH.

3 As HEK cells express FAAH, an enzyme [37] which degrades AM404, the experiments of figure
4 4a have been performed in the presence of a FAAH inhibitor (PMSF, 10 μM). Experiments were
5 performed at 35-37°C.

6 2.11 Statistical Analysis

7 Data are expressed as mean ± SEM and recorded using SigmaStats 3.5 software. Data were
8 tested for normality and for equal variance. Multiple measurements were compared by one-way or
9 two-way ANOVA or by Kruskal-Wallis / Friedman test in the case of data that were not normally
10 distributed. The post hoc comparisons were performed by the Bonferroni method. Values of $P < 0.05$
11 were considered statistically significant.

12

13 3 Results

14 3.1 Paracetamol fails to produce an analgesic effect in Cav3.2 knock-out mice

15 In wild type littermates mice (*Cav3.2*^{+/+}), paracetamol increased the tail withdrawal latency in
16 the tail immersion test (vehicle: 7.7 ± 0.6 s and paracetamol: 16.0 ± 0.5 s; $P < 0.001$) (Fig. 1a).
17 Moreover, paracetamol decreased the paw withdrawal incidence (PWR) in the von Frey test (vehicle:
18 3.4 ± 0.2 PWR and paracetamol: 2.6 ± 0.2 PWR; $P < 0.01$) (Fig. 1b) and the licking time of the first
19 phase (vehicle: 135 ± 11 s and paracetamol: 72 ± 12 s; $P < 0.001$) and the second phase (vehicle: 241
20 ± 31 s and paracetamol: 61 ± 18 s; $P < 0.001$) of the formalin test (Fig. 1c). In the same tests
21 performed in Cav3.2 knock-out mice (*Cav3.2*^{-/-}), paracetamol was not able to modify scores obtained
22 in control animals (Fig. 1a, b and c). A similar result was obtained in a model of chronic inflammatory
23 pain induced by a peri-articular injection of Complete Freund Adjuvant in the left hindpaw (CFA
24 model). Both thermal (vehicle: 3.9 ± 0.23 s and paracetamol: 10.0 ± 0.8 s; $P < 0.05$) (Fig. 1d) and
25 mechanical hyperalgesia (vehicle: 4.5 ± 0.2 PWR and paracetamol: 2.1 ± 0.5 PWR; $P < 0.01$) (Fig. 1e)
26 were reduced by paracetamol in *Cav3.2*^{+/+}, while it did not produce any effect in *Cav3.2*^{-/-} mice.

27 Deletion of the Cav3.2 channel attenuates reactions to several noxious stimuli (here mainly
28 heat and mechanical ones) (Fig. 1). Thus, the analgesic effect of paracetamol might be masked by the
29 *Cav3.2*^{-/-} mouse hypoalgesic phenotype, introducing a bias in our experiments. We verified whether

1 another analgesic drug (morphine, 2 mg/kg s.c.) could be effective in *Cav3.2*^{-/-} mice. Indeed,
2 morphine produced a significant analgesic effect in *Cav3.2*^{-/-} mice in all tests used (Fig. 1) either in
3 naive mice (tail immersion, von Frey and formalin tests) or in the CFA model (paw immersion and von
4 Frey tests).

5 All these findings identify the novel role of Cav3.2 calcium channels in supporting the
6 analgesic action of paracetamol.

7 *3.2 Supraspinal Cav3.2 calcium channels are needed for paracetamol-induced analgesia*

8 The first observation was that TTA-A2 (5 µg/mouse), administered by intracerebroventricular
9 (i.c.v.) route, induced an antinociceptive effect in the first phase (vehicle: 109 ± 8 s and TTA-A2: 72 ±
10 7 s; *P* < 0.01) and the second phase (vehicle: 193 ± 18 s and TTA-A2: 93 ± 20 s; *P* < 0.01) of the
11 formalin test (Fig. 2a) and in the von Frey test (vehicle: 3.9 ± 0.4 PWR and TTA-A2: 2.6 ± 0.2 PWR; *P* <
12 0.01) (Fig. 2b) without inducing a sedative effect (Supplementary Fig. S1).

13 Secondly, in mice submitted to the formalin (Fig. 2a) or the von Frey (Fig. 2b) tests, co-
14 administration of TTA-A2 (i.c.v.) and paracetamol (200 mg/kg, *p.o.*) produced an effect not
15 significantly different compared to the effect of paracetamol or TTA-A2 alone. On the contrary, co-
16 administration of TTA-A2 (i.c.v.) and morphine (1 mg/kg, *s.c.*) produced a significantly more
17 pronounced analgesic action than the effect of the same drugs administered alone (Fig. 2a, b).

18 In parallel, specific action of TTA-A2 on Cav3.2 subtype channels was tested by assessing the
19 analgesic effect of TTA-A2 i.c.v.-injected in *Cav3.2*^{-/-} mice in the formalin test. No analgesic action of
20 TTA-A2 was observed in the first phase (vehicle: 68 ± 9 s and TTA-A2: 56 ± 14 s) nor in the second
21 phase (vehicle: 106 ± 12 s and TTA-A2: 109 ± 23 s) (Supplementary Fig. S2), confirming the specific
22 action of this compound on Cav3.2.

23 A possible involvement of the spinal Cav3.2 calcium channels in paracetamol action has been
24 investigated by assessing its analgesic action in wild type mice submitted to the formalin test and
25 treated with TTA-A2. TTA-A2 (5 µg), administered intrathecally (i.t.), induced an analgesic effect in
26 the first phase (vehicle: 106 ± 12 s and TTA-A2: 65 ± 9 s, *P* < 0.001) and the second phase (vehicle:

1 255 ± 19 s and TTA-A2: 119 ± 29 s, $P < 0.001$) of the formalin test (Fig. 2c). Interestingly, co-
2 administration of TTA-A2 (5 µg, i.t.) and paracetamol (200 mg/kg, *p.o.*) induced a significantly higher
3 analgesic effect than both drugs administered alone. The same result was found with morphine (1
4 mg/kg, *s.c.*) and TTA-A2 (5 µg, i.t.) co-administration (Fig. 2c).

5 All together, these results demonstrate that supraspinal but not spinal Cav3.2 channels are
6 essential for the analgesic effect of paracetamol. Moreover, they show that specific supraspinal or
7 spinal inhibition of Cav3.2 calcium channels induce analgesia.

8 *3.3 AM404, the active metabolite of paracetamol, required Cav3.2 channels to mediate its analgesic*
9 *effect*

10 Intracerebroventricular injection of AM404 induced an analgesic effect in *Cav3.2^{+/+}* mice in
11 the first phase of the formalin test (vehicle: 85 ± 10 s and AM404: 47 ± 10 s; $P < 0.01$) (Fig. 3a) and in
12 the von Frey test, 7 days after peri-articular CFA injection (vehicle: 4.9 ± 0.1 PWR and AM404: 2.8 ±
13 0.3 PWR; $P < 0.01$) (Fig. 3b). In the same tests, no analgesic action of i.c.v. administered AM404 was
14 observed in *Cav3.2^{-/-}* mice (Fig. 3).

15 We further examined in DRG neurons the action of AM404 on Cav3.2 current in a whole-cell
16 patch clamp method. Contrary to paracetamol and *p*-aminophenol which did not inhibit Cav3.2
17 current, AM404 weakly inhibited Cav3.2 current, with a high $EC_{50} = 13.7$ µM (Fig. 3c, d), compared to
18 the antagonist, TTA-A2 ($EC_{50} = 9.0$ nM, result not shown).

19 *3.4 TRPV1 receptor and Cav3.2 channel: evidence of a functional relationship*

20 We first showed that AM404 (10 µM) induced a large intracellular calcium increase
21 dependent on the TRPV1 receptor (Fig. 4a). AM404 (10 µM) was as efficient as capsaicin (0.5 µM)
22 and elicited a response in all capsaicin responding cells. In these experimental conditions, we further
23 demonstrated that *p*-aminophenol (10 µM) induced a calcium mobilization, albeit much smaller and
24 slower than AM404 or capsaicin (Fig. 4b). This effect resulted from the action of locally produced
25 AM404 (transformation of *p*-aminophenol by HEK expressed FAAH). Indeed effect of *p*-aminophenol

1 application was lost after a treatment with a FAAH inhibitor (PMSF, 10 μ M; Fig. 4b). As for direct
2 application of exogenous AM404, the observed intracellular calcium mobilization depends on TRPV1,
3 as demonstrated by the use of the TRPV1 antagonist (capsazepin, CAPZ, 20 μ M; Fig. 4b,
4 Supplementary Fig. S3) or the lack of response in cells that were not transfected with the TRPV1
5 receptor (Fig. 4b). These experiments confirm that AM404 is able to activate TRPV1 receptors and
6 that p-aminophenol needs to be metabolized by FAAH (into AM404) in order to activate these
7 receptors.

8 We then demonstrated that, in *Cav3.2^{+/+}* mice, a supraspinal injection (10 μ g i.c.v.) of
9 capsaicin (which needed TRPV1 to induce analgesia, see Supplementary Fig. S4) elicited an analgesic
10 effect in the first (vehicle: 88 ± 9 s and Cap: 42 ± 13 s, $P < 0.01$) and second phase (vehicle: 231 ± 24 s
11 and Cap: 127 ± 30 s, $P < 0.05$) of the formalin test (Fig. 5a) and in the von Frey test (vehicle: 4.9 ± 0.1
12 PWR and Cap: 2.5 ± 0.5 PWR, $P < 0.01$) (Fig. 5b) while it had no effect in *Cav3.2^{-/-}* mice (Fig. 5a,b).
13 Conversely, the analgesic effect of i.c.v. administered TTA-A2 was maintained in TRPV1 knock-out
14 mice (*TRPV1^{-/-}*) in the first (vehicle: 92 ± 11 s and TTA-A2: 34 ± 7 s, $P < 0.001$) and second (vehicle: 145
15 ± 17 s and TTA-A2: 58 ± 9 s, $P < 0.01$) phase of the formalin test (Fig. 5c). Taken together, these results
16 revealed that the analgesic effect induced by an activation of supraspinal TRPV1 receptors needs
17 Cav3.2 calcium channels and suggests that these two protagonists could be involved sequentially,
18 Cav3.2 being located downstream of TRPV1 and inhibited after TRPV1 activation, e.g. by AM404.

19 Accordingly, we demonstrated, in HEK cells stably expressing recombinant Cav3.2 channels,
20 that capsaicin, which had a limited and fully reversible inhibitory effect on Cav3.2 channel activity in
21 the absence of co-expressed TRPV1 (Fig. 5d), potently inhibited Cav3.2 mediated currents when
22 TRPV1 channels were co-expressed (Fig. 5e). Furthermore, the inhibition was almost irreversible
23 even upon capsaicin washout. TRPV1 activation by capsaicin led to a linear current/voltage
24 relationship and augmentation of inward and outward currents at -75 and +75 mV when TRPV1 was
25 expressed (Supplementary Fig. S5a, b). Pooled experiments showed that capsaicin mediated Cav3.2
26 inhibition depends on TRPV1 activation (Fig. 5f). In similar experimental conditions, application of

1 AM404 activated TRPV1 channels (Supplementary Fig. S5d). As for native T-type current, AM404 has
2 also a small inhibitory effect on Cav3.2 (Fig. 5g and Supplementary Fig.S 5c). Corroborating the effect
3 of capsaicin, AM404-induced TRPV1 activation resulted in a nearly complete inhibition of Cav3.2
4 activity (Fig. 5h). This latter effect was significantly more pronounced than in HEK cells only
5 expressing Cav3.2 (Fig. 5i).

6 **4 Discussion**

7 Using different *in vitro* (patch clamp, calcium imaging) and *in vivo* (different pain tests and
8 models) experiments we demonstrated that: 1) supraspinal Cav3.2 channels were involved in
9 paracetamol analgesic action; 2) supraspinal Cav3.2 channels modulate pain perception and 3) a
10 functional relationship between TRPV1 receptors and Cav3.2 channels mediates the analgesic action
11 of paracetamol.

12 The loss of paracetamol effect in *Cav3.2*^{-/-} mice regardless of the experimental conditions,
13 suggests that Cav3.2 channels are needed for the analgesic action of paracetamol not only in acute
14 nociception but also in inflammatory situations. This involvement might be questioned due to the
15 reduced response of *Cav3.2*^{-/-} to noxious stimuli, however, we have demonstrated by using morphine
16 that an additional analgesia could happen in these mice. Thus the loss of the paracetamol effect can
17 clearly be linked to Cav3.2 channel deletion and, paracetamol and morphine mediated analgesia can
18 be clearly distinguished. However these results are not informative in localizing, within the CNS, the
19 channels involved in the effect of paracetamol.

20 The site of action of paracetamol is still a matter of debate. Some authors showed a
21 peripheral action site [19,32,36] while central one was shown by others [7,18,34,35,44,45]. We
22 previously demonstrated that spinal [35] and brain [34] receptors were involved in the action of
23 paracetamol in animals and possibly in healthy volunteers too [45]. Cav3.2 channels are widely
24 located in the body. Notably, Cav3.2 channels are expressed in the spinal cord [37,50] and in
25 different nuclei in the brain: hypothalamus, thalamus, amygdala, hippocampus, midbrain, and cortex
26 [50]. Various T-type channel antagonists such as ethosuximide [14,39], mibefradil [33,48], NNC [38]

1 have been used to explore the role of these channels but none of them are highly specific. Recently,
2 a new class of molecules has been designed, called TTA compounds, which presents major specificity
3 for T-type channels [46,52] and a body of evidence placed TTA-A2 as the most specific antagonist of
4 Cav3.2 subtype channels. We previously demonstrated that its effect on T-type channels was greater
5 on Cav3.2 than on Cav3.1 and Cav3.3 (EC_{50} Cav3.1: 100 nM ; Cav3.2: 9 nM ; Cav3.3: 30 nM) [24] and
6 that its analgesic effect (after systemic administration) was lost in *Cav3.2*^{-/-} mice [24]. Using i.c.v.
7 injections, we showed that Cav3.2 inhibition in the CNS mediated analgesic effects. Based on this
8 data, the loss of an effect of orally administered paracetamol after i.c.v. injection of TTA-A2
9 demonstrated an involvement of supraspinal Cav3.2 channels but not spinal channels, as shown by
10 the lack of an occluding effect of i.t. TTA-A2 on paracetamol-induced analgesia. This demonstration
11 of a supraspinal site of action of paracetamol agrees with our previous work showing the
12 involvement of supraspinal TRPV1 receptors [34] in paracetamol-induced analgesia. It also agrees
13 with results obtained in healthy volunteers showing an activation of inhibitory bulbospinal pathways
14 by paracetamol [44].

15 Incidentally, it is interesting to note that TTA-A2, administered alone either supraspinally or
16 spinally, induced analgesia, without affecting locomotion. These results, in line with the analgesia
17 observed in *Cav3.2*^{-/-} mice (present and Choi et al. study [15]), confirmed that Cav3.2 calcium
18 channels are tonically involved in nociception at these two sites to modulate pain.

19 Regarding the mechanism of the supraspinal involvement of Cav3.2 by paracetamol, previous
20 results have shown that the central action of paracetamol involves metabolites [25], namely p-
21 aminophenol, transformed in the brain into AM404. AM404, i.c.v.-injected, produced an analgesic
22 effect in *Cav3.2*^{+/+} but not in *Cav3.2*^{-/-} mice in the two tests used. These results demonstrate a
23 functional link between AM404 and Cav3.2 channels, which has been similarly shown for
24 lipoaminoacids such as anandamide, some polyunsaturated fatty acids or N-acyl ethanolamide,
25 which are T-type calcium channel blockers [12,20,49], and for endogenous N-arachidonoyl glycine
26 (NAGly) a more potent Cav3.2 inhibitor [3]. Interestingly, NAGly promotes an analgesic effect in

1 animals [3,26] which is abolished in *Cav3.2*^{-/-} mice [3], suggesting that an inhibition of Cav3.2 could
2 support its analgesic effect. The same mechanism could be evoked for AM404. Indeed, AM404
3 induced an inhibition of native Cav3.2 mediated T-type currents in D-hair DRG neuron subtypes.
4 However, this inhibition was weak compared to that induced by other lipoaminoacids such as NAGly
5 [3], which questions the involvement of this direct inhibitory effect in the analgesic action of AM404
6 observed *in vivo*.

7 A recent work has shown that AM404-induced activation of brain TRPV1 channels was
8 needed for the analgesic action of paracetamol [34] and p-aminophenol, its first metabolite [4].
9 Accordingly, we showed here that p-aminophenol inhibited calcium currents only when FAAH
10 enzyme and TRPV1 were functional confirming the role of AM404 as an active metabolite and its
11 activation of TRPV1. We confirmed, using intracellular calcium imaging and a whole cell patch-clamp
12 recordings, the ability of AM404 to activate TRPV1 (Supplementary fig. S5d) as previously
13 demonstrated by Zygmunt et al. [56]. The ability of TRPV1 activation to inhibit Cav3.2 channels was
14 then demonstrated by showing that capsaicin and AM404 (devoid of or inducing a limited direct
15 inhibitory effect on Cav3.2 channels, respectively) strongly inhibited Cav3.2 currents when HEK cells
16 were co-transfected with the channels and TRPV1 receptors. This effect was very sensitive since the
17 basal tonic activity of TRPV1 was also effective in reducing Cav3.2. This effect is reminiscent of
18 findings in rat sensory neurons, known to express Cav3.2, describing an inhibition of T-type currents
19 by TRPV1 mediated action [16]. We similarly demonstrated this pathway here by demonstrating the
20 minimal necessity of the two channels being expressed together, likely located in a close vicinity, to
21 observe the regulation. Importantly, we further showed that this inhibition can be obtained with
22 physiological levels of extracellular calcium, in contrary to the previous report from Comunanza et al.
23 [16], suggesting that this modulation is likely to occur *in vivo*. The hypothesis stated to explain the
24 mechanism involved in this modulation is a calcium regulation. Indeed, massive influx of calcium ions
25 after TRPV1 activation could inhibit Cav3.2 channels through the activation of kinases (for review

1 [55]), such as PTK [27], PKA [29,53,54], PKC [9] and ROCK [28] and therefore decrease neuronal
2 excitability.

3 The next step was to characterize this functional link in pain supraspinal modulation.
4 Intracerebroventricular injection of capsaicin induced analgesia in *Cav3.2^{+/+}* mice in all the
5 nociceptive tests. This effect was not observed in *Cav3.2^{-/-}* mice. Cav3.2 channels are thus needed for
6 the analgesic effect of the TRPV1 agonists, capsaicin or AM404. However, the reciprocal does not
7 apply, the analgesic action of i.c.v.-injected TTA-A2 was unchanged in *TRPV1^{-/-}* mice compared to
8 littermates. Taken together, these results demonstrate that the agonist of TRPV1 and AM404 induces
9 analgesia through a supraspinal TRPV1-dependent Cav3.2 current inhibition. That explains why the
10 antinociceptive effect of paracetamol was inhibited both by both an i.c.v. TRPV1 receptor antagonist
11 [34] and the Cav3.2 inhibitor TTA-A2. The involvement of supraspinal TRPV1 receptors are in line
12 with literature showing that activation of brain TRPV1 induces an analgesic effect (for review [40]).

13 To conclude, we have identified brain Cav3.2 channels as a novel targets for the analgesia
14 induced by paracetamol. We positioned these channels with two actors previously shown to be
15 involved in the action of paracetamol: AM404, its bioactive metabolite, and TRPV1 receptors. The
16 present results led us to complete the sequential events we previously proposed [34,35] to explain
17 the mechanism of action of paracetamol: metabolism of p-aminophenol (in the liver) and AM404 in
18 brain (thanks to FAAH) which induces an activation of brain TRPV1 [4] (linked with CB1 receptors)
19 which in turn induces a Cav3.2 inhibition presently shown to participate in the supraspinal analgesic
20 effect of paracetamol. Further studies are needed to elucidate the link between brain Cav3.2
21 inhibition and activation of inhibitory bulbospinal pathways involved in the analgesic effect of
22 paracetamol [7,35,44]. Finally, we highlighted supraspinal Cav3.2 calcium channels as an important
23 actor for pain modulation which appears as a promising and interesting target for the treatment of
24 pain.

25 **Conflicts of interest statement**

1 The authors declare that no competing financial interests concerning this work.

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12 experiments: NK AF CM MB EB JC. Analyzed the data: NK CM AF EB MB AA JC. Wrote the paper: NK
13 CM TV MB EB AE

14

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25

26

1

2 **LEGENDS FOR FIGURES**

3 **Figure 1 | Paracetamol fails to induce analgesic effect in Cav3.2 knock-out.** The analgesic effect of
4 paracetamol (PARA, 200 mg/kg *per os*) was assessed in: (a) tail immersion, (b, e) von Frey, (c)
5 formalin and (d) paw immersion tests in Cav3.2 knock-out (*Cav3.2^{-/-}*) and their wild-type littermates
6 (*Cav3.2^{+/+}*). Morphine (Mor, 2 mg/kg *subcutaneous*) groups have been used as positive control
7 groups. In all tests and conditions, paracetamol lacks its analgesic effect in *Cav3.2^{-/-}* mice. Data are
8 presented as mean SEM (n = 6–8). *, *P* < 0.05; compared to vehicle groups.

9 **Figure 2 | Supraspinal Cav3.2 channels are involved in paracetamol analgesic effect.** The supraspinal
10 Cav3.2 calcium channels involvement in paracetamol was investigated specifically by Cav3.2 block in
11 brain (a, b) and spinal cord (c). TTA-A2 (5 µg/2 µl i.c.v. and 5 µg/5 µl i.t.), paracetamol (PARA; 200
12 mg/kg *p.o.*), morphine (Mor; 1 mg/kg *s.c.*) and co-administrations effect were assessed in: (a, c)
13 formalin and (b) von Frey (1.4 g) tests. Data are presented as mean SEM (n = 6–8). *, *P* < 0.05;
14 compared to vehicle group; †, *P* < 0.05; compared to TTA-A2 + Mor co-administration group.

15 **Figure 3 | Cav3.2 calcium channels are inhibited by AM404 and essential for AM404-induced**
16 **analgesia.** AM404 and Cav3.2 calcium channels relationship was assessed by *in vitro* (whole cell
17 patch clamp) and *in vivo* (formalin and von Frey test) approaches. the AM404 analgesic effect (2 µg/2
18 µl i.c.v.) was assessed in (a) formalin phase 1 and (b) von Frey tests in *Cav3.2^{-/-}* and *Cav3.2^{+/+}* mice. (c)
19 Whole-cell calcium currents recorded from a DRG neurons expressing Cav3.2 channels (D-Hair
20 neuron) in the absence (control, Ctrl) and in the presence of 1 and 3 µM AM404. Currents were
21 elicited by a depolarization at -30 mV (200 ms duration) applied every 5 s from -75 mV. (d) Dose-
22 response curve of AM404 in Cav3.2 current (n = 6–8, open diamonds), paracetamol (filled circle) and
23 p-aminophenol (filled square). Currents were elicited by a depolarization at -30 mV (200 ms duration)
24 applied every 5 s from -75 mV. Data are presented as mean SEM (n = 6–8). *, *P* < 0.05; compared to
25 vehicle groups.

26 **Figure 4 | AM404 and FAAH dependent metabolism of p-aminophenol are TRPV1 activators.** (a)
27 Time course of intracellular calcium elevation in HEK cells transfected with (black traces, n = 18) or
28 without (gray traces, n = 17) TRPV1 receptors in response to application of capsaicin (0.5 µM) or
29 AM404 (10 µM). PMSF (10µM) was applied in all experiments to block the degradation of AM404 by
30 FAAH. Intracellular calcium elevation upon application of 50 µM ATP at the end of the protocol
31 shows that all the cells (Control and TRPV1 transfected) were effectively loaded with the Fura2
32 calcium sensitive probe. (b) Application of p-aminophenol evokes Fura2 340/380 ratio in HEK293
33 cells expressing TRPV1, but not in untransfected cells or in the presence of the FAAH antagonist,
34 PMSF (10 µM).

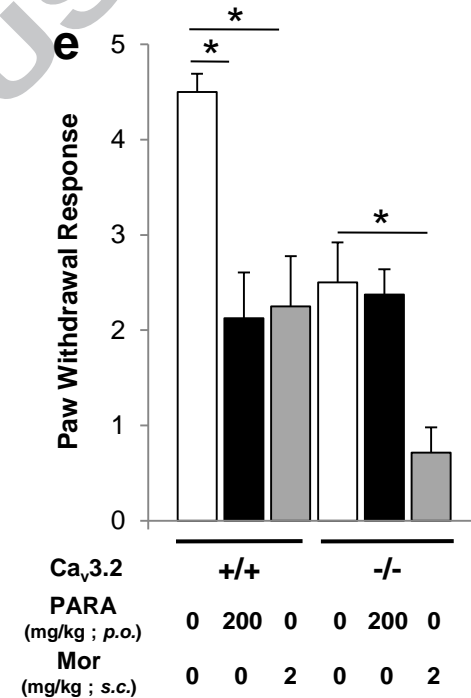
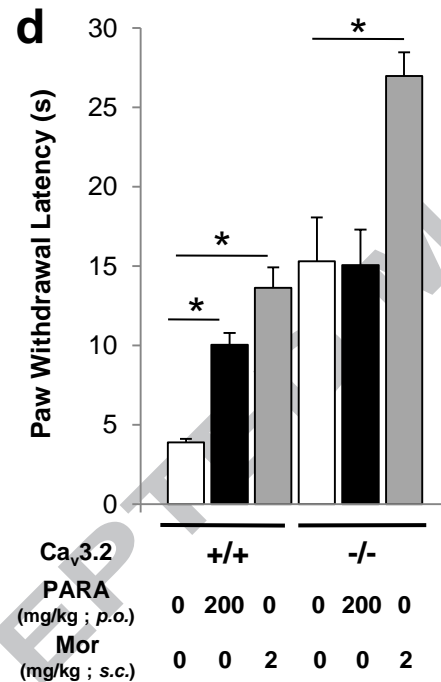
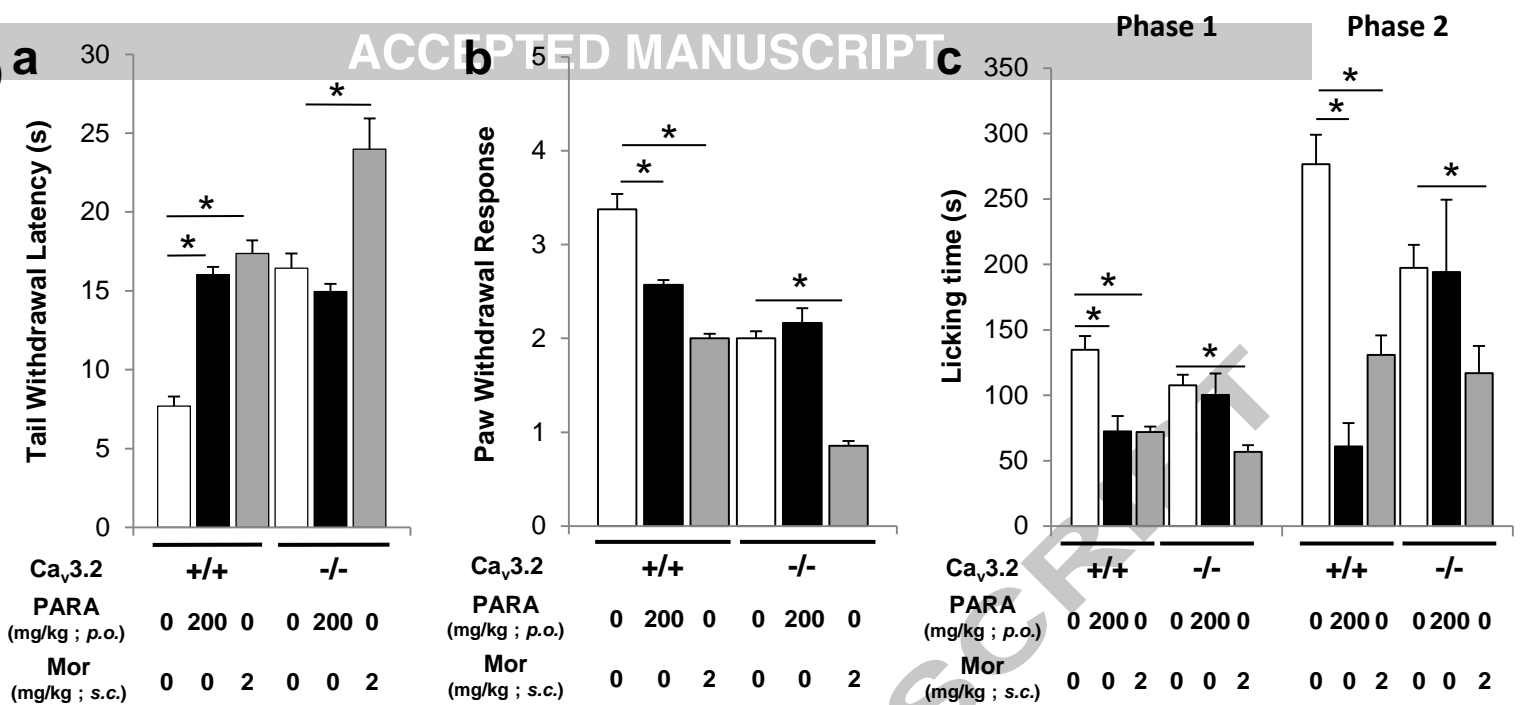
35 **Figure 5 | Relationship between Cav3.2 channel and TRPV1 receptor.** The capsaicin analgesic effect
36 (Cap, 10 µg/2 µl i.c.v.) was assessed in (a) formalin (2.5%) and (b) von Frey tests in *Cav3.2^{-/-}* and
37 *Cav3.2^{+/+}* mice. TTA-A2 analgesic effect (5 µg/2 µl i.c.v.) was assessed in (c) formalin test in TRPV1
38 knock-out mice (*TRPV1^{-/-}*) and their wild type littermates (*TRPV1^{+/+}*). (d) Representative Cav3.2
39 current traces evoked by 200 ms depolarization ramps from -75mV to +75mV, under control
40 conditions (black trace) and during the washout of 1 µM capsaicin (gray trace) on a typical HEK cell
41 expressing Cav3.2 alone. Evolution of the maximal inward current evoked by the depolarizing ramp

1 during the time course of the experiment is plotted on the bottom part. The time points labeled as
2 (1) and (2) correspond to the upper control and wash traces respectively. (e) Same representation as
3 in (d) for a typical HEK cell expressing Cav3.2 and TRPV1. Note that in this case the Cav3.2 mediated
4 current is nearly abolished after capsaicin stimulation of TRPV1. (f) Mean Cav3.2 inhibition mediated
5 after 1 μ M capsaicin application when TRPV1 is coexpressed (black bar) or not (open bar). Data are
6 presented as mean SEM (n = 6–8). (g,h) Evolution of the maximal inward current evoked by the
7 depolarizing ramp during the time course of a similar experiment as in (d,e) but using AM404. (i)
8 Mean Cav3.2 inhibition mediated after 3 μ M AM404 application when TRPV1 is coexpressed (black
9 bar) or not (open bar). Data are presented as mean SEM (n = 6–8). *, $P < 0.05$; compared to vehicle
10 groups (behavior) or with or without TRPV1 (electrophysiology).

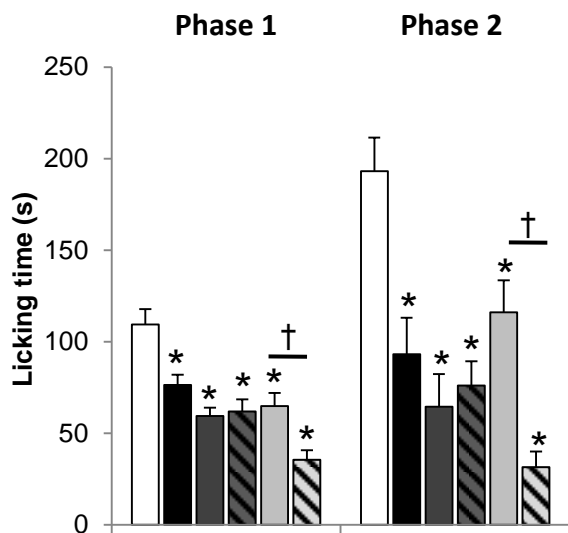
11

Figure-1

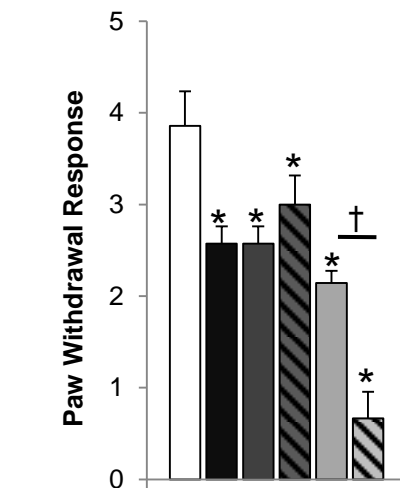
(KERCKHOVE)



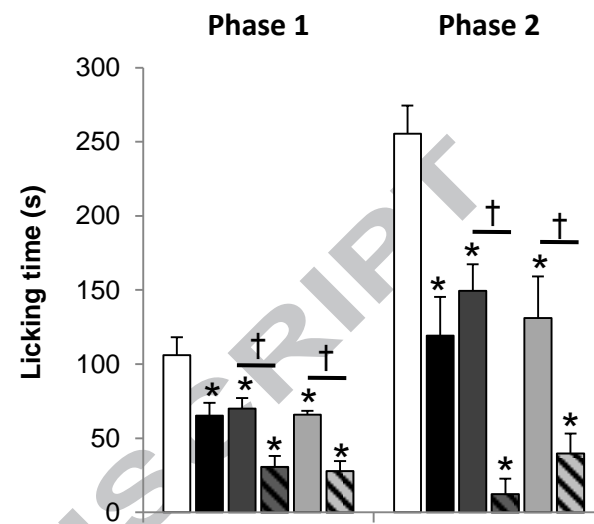
a



b



c



ACCEPTED MANUSCRIPT

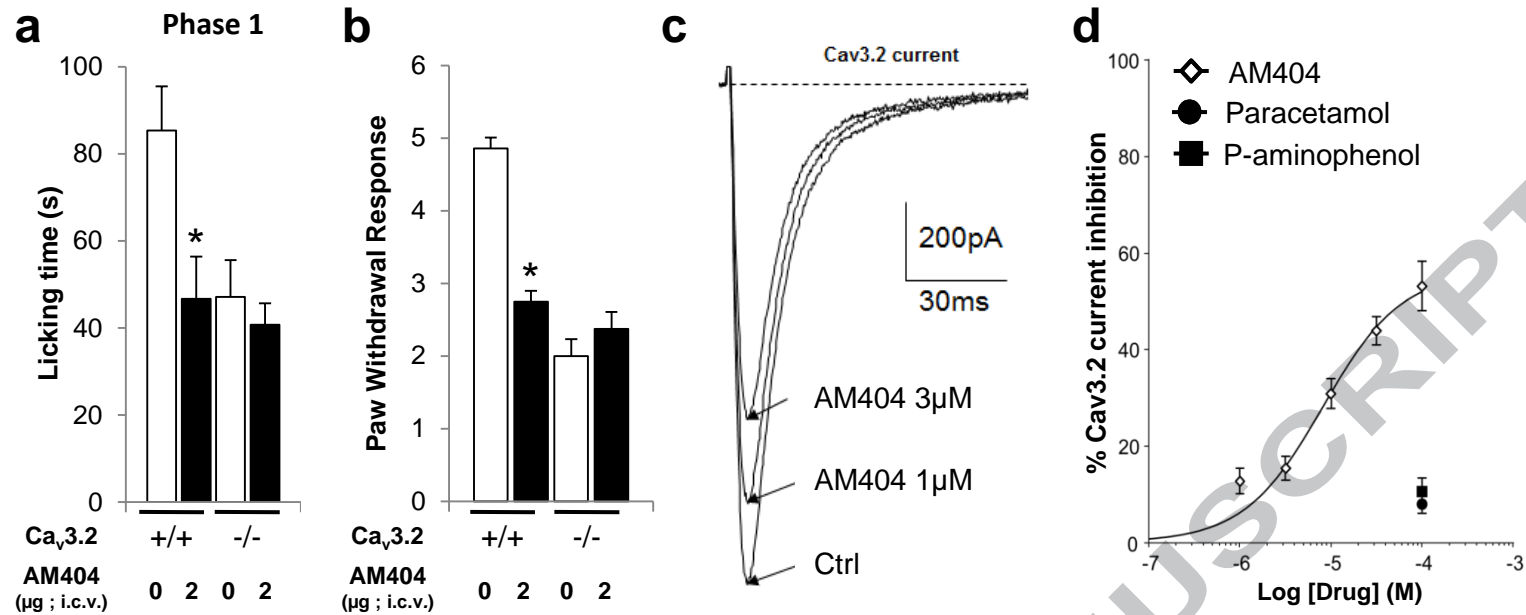
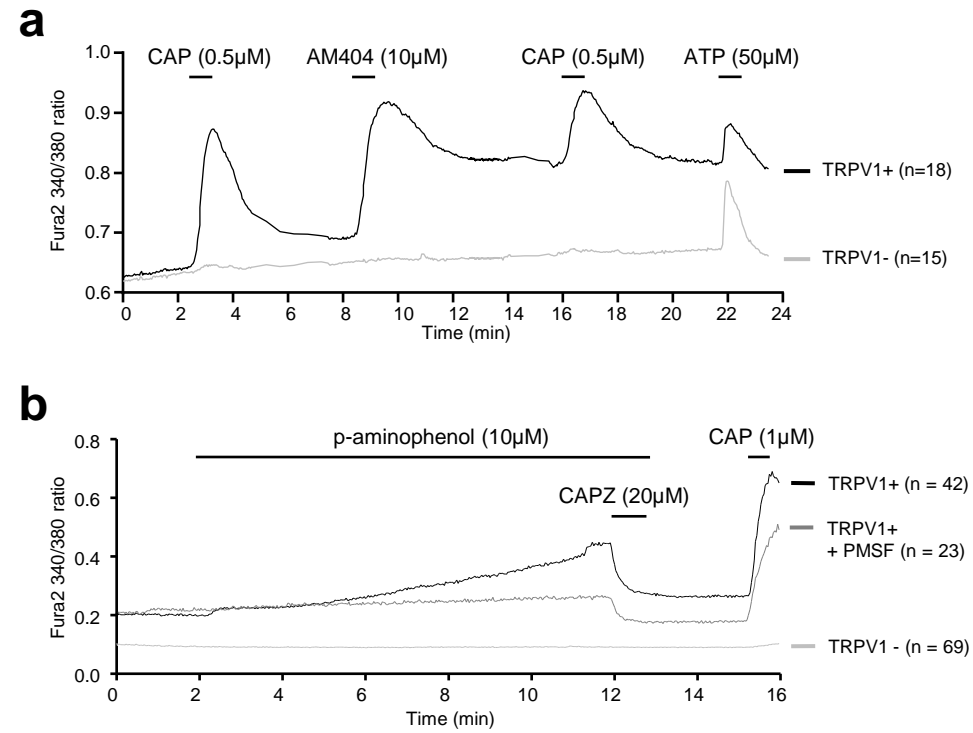
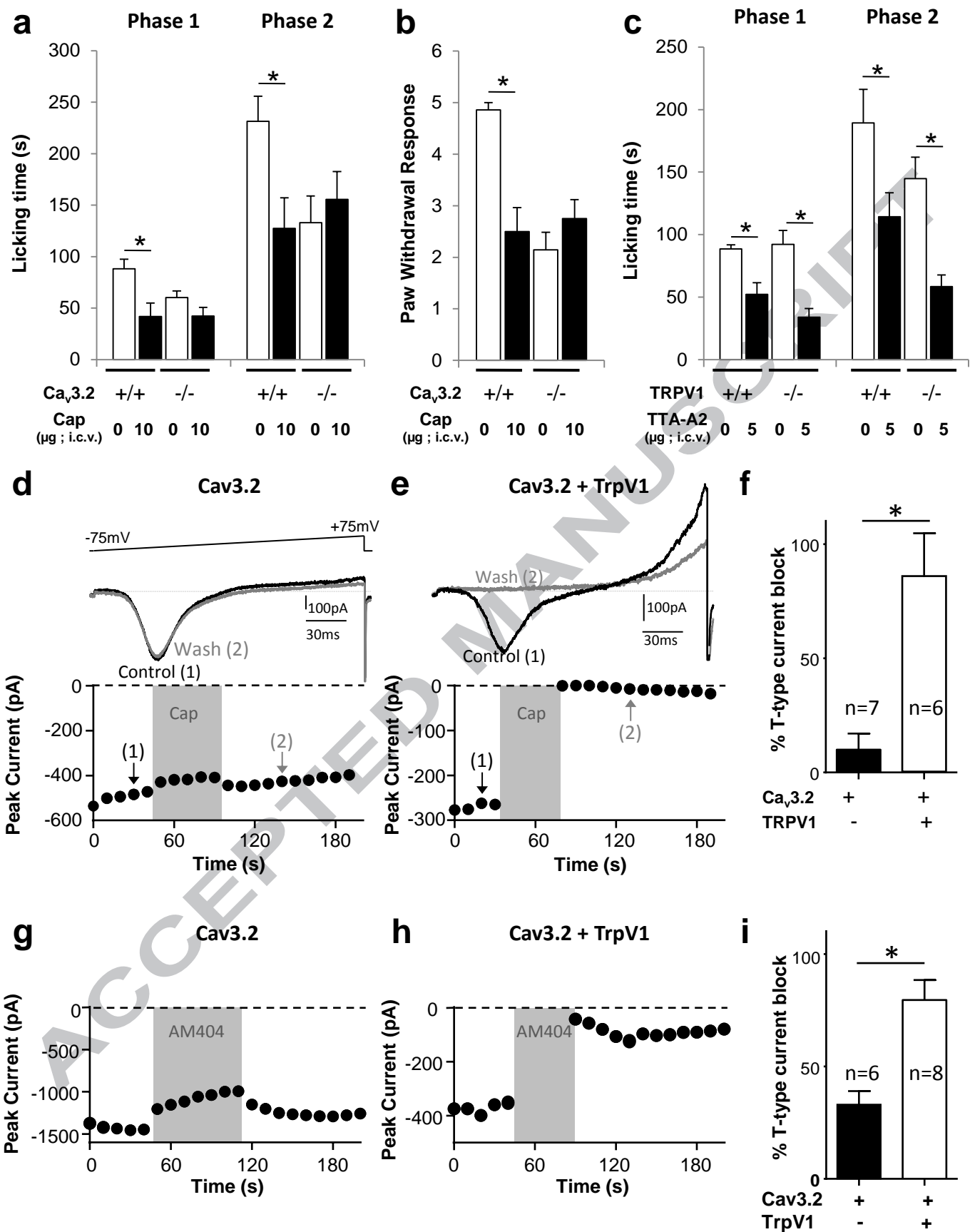


Figure-4
(Kerckhove)





1 **Summary**

2

3 The supraspinal Cav3.2 calcium channels are involved in analgesic effect of paracetamol,
4 through their inhibition following the activation of supraspinal TRPV1 receptors by AM404.

5

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1 **ABSTRACT**

2 To exert analgesic action, paracetamol requires complex metabolism to produce a brain specific
3 lipoaminoacid compound, AM404, which targets central TRPV1 receptors. Lipoaminoacids are also
4 known to induce analgesia through T-type channel inhibition (Cav3.2). In this study we show that the
5 antinociceptive effect of paracetamol is lost in mice when supraspinal Cav3.2 channels are inhibited.
6 Therefore, we hypothesized a relationship between supraspinal Cav3.2 and TRPV1, via AM404, which
7 mediates the analgesic effect of paracetamol. AM404 is able to activate TRPV1 and weakly inhibits
8 Cav3.2. Interestingly, activation of TRPV1 induces a strong inhibition of Cav3.2 current. Supporting
9 this, intracerebroventricular administration of AM404 or capsaicin produces antinociception that is
10 lost in *Cav3.2^{-/-}* mice. Our study, for the first time, (1) provides a molecular mechanism for the
11 supraspinal antinociceptive effect of paracetamol, (2) identifies the relationship between TRPV1 and
12 Cav3.2 and (3) discloses supraspinal Cav3.2 inhibition as a potential pharmacological strategy to
13 alleviate pain.

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