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

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1. Introduction

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

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

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

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

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

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

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

2. Materials and methods

2.1 Animals

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

CE 24 - 11, CE 13 - 10). Animals were housed under controlled environmental conditions (21 - 22°C; 55% humidity) and kept under a 12/12h light/dark cycle. Food and water were available *ad libitum*. Animals were euthanized by cervical dislocation or CO_2.

2.2 Behavioral Studies

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

2.3 Actimetry test

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

2.4 Intracerebroventricular and intrathecal injections

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

2.5 Tail and paw immersion tests

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

2.6 Formalin test

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

2.7 Von Frey test

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

2.8 Monoarthritis Induction

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

2.9 Cell culture and electrophysiological recordings

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

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

2.10 Calcium imaging

Human embryonic kidney cells, HEK293T, were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) human serum, 2 mM L-glutamine, 2 units/ml penicillin and 2 mg/ml streptomycin at 37°C in a humidity controlled incubator with 10% CO_2. HEK293T cells were transiently transfected with human TRPV1 using Mirus TransIT-293 (Mirus Corporation; Madison, WI, USA). To determine if the cells were transfected, capsaicin (1 µM) was used at the end of each experiment. Cells which did not-respond to capsaicin were used as controls.

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

software (Olympus). Bath solution contained (in mM) 145 NaCl, 5 KCl, 2 $CaCl_2$, 1.5 $MgCl_2$, 10 HEPES, 10 D-Glucose, pH = 7.4 buffered with NaOH.

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

2.11 Statistical Analysis

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

3 Results

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

We then demonstrated that, in *Cav3.2*$^{+/+}$ mice, a supraspinal injection (10 µg i.c.v.) of capsaicin (which needed TRPV1 to induce analgesia, see Supplementary Fig. S4) elicited an analgesic effect in the first (vehicle: 88 ± 9 s and Cap: 42 ± 13 s, $P < 0.01$) and second phase (vehicle: 231 ± 24 s 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 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). Conversely, the analgesic effect of i.c.v. administered TTA-A2 was maintained in TRPV1 knock-out mice (*TRPV1*$^{-/-}$) in the first (vehicle: 92 ± 11 s and TTA-A2: 34 ± 7 s, $P < 0.001$) and second (vehicle: 145 ±17 s and TTA-A2: 58 ± 9 s, $P < 0.01$) phase of the formalin test (Fig. 5c). Taken together, these results revealed that the analgesic effect induced by an activation of supraspinal TRPV1 receptors needs Cav3.2 calcium channels and suggests that these two protagonists could be involved sequentially, Cav3.2 being located downstream of TRPV1 and inhibited after TRPV1 activation, e.g. by AM404.

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

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

4 Discussion

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

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

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

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

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

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

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

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

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

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

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

Conflicts of interest statement

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

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Author contributions: Conceived and designed the experiments: NK CM AF MB AE EB. Performed the experiments: NK AF CM MB EB JC. Analyzed the data: NK CM AF EB MB AA JC. Wrote the paper: NK CM TV MB EB AE

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LEGENDS FOR FIGURES

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

Figure 2 I Supraspinal Cav3.2 channels are involved in paracetamol analgesic effect. The supraspinal Cav3.2 calcium channels involvement in paracetamol was investigated specifically by Cav3.2 block in 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 mg/kg *p.o.*), morphine (Mor; 1 mg/kg *s.c.*) and co-administrations effect were assessed in: (**a, c**) formalin and (**b**) von Frey (1.4 g) tests. Data are presented as mean SEM (n = 6–8). *, $P < 0.05$; compared to vehicle group; †, $P < 0.05$; compared to TTA-A2 + Mor co-administration group.

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

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

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

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

Figure-1 (KERCKHOVE)

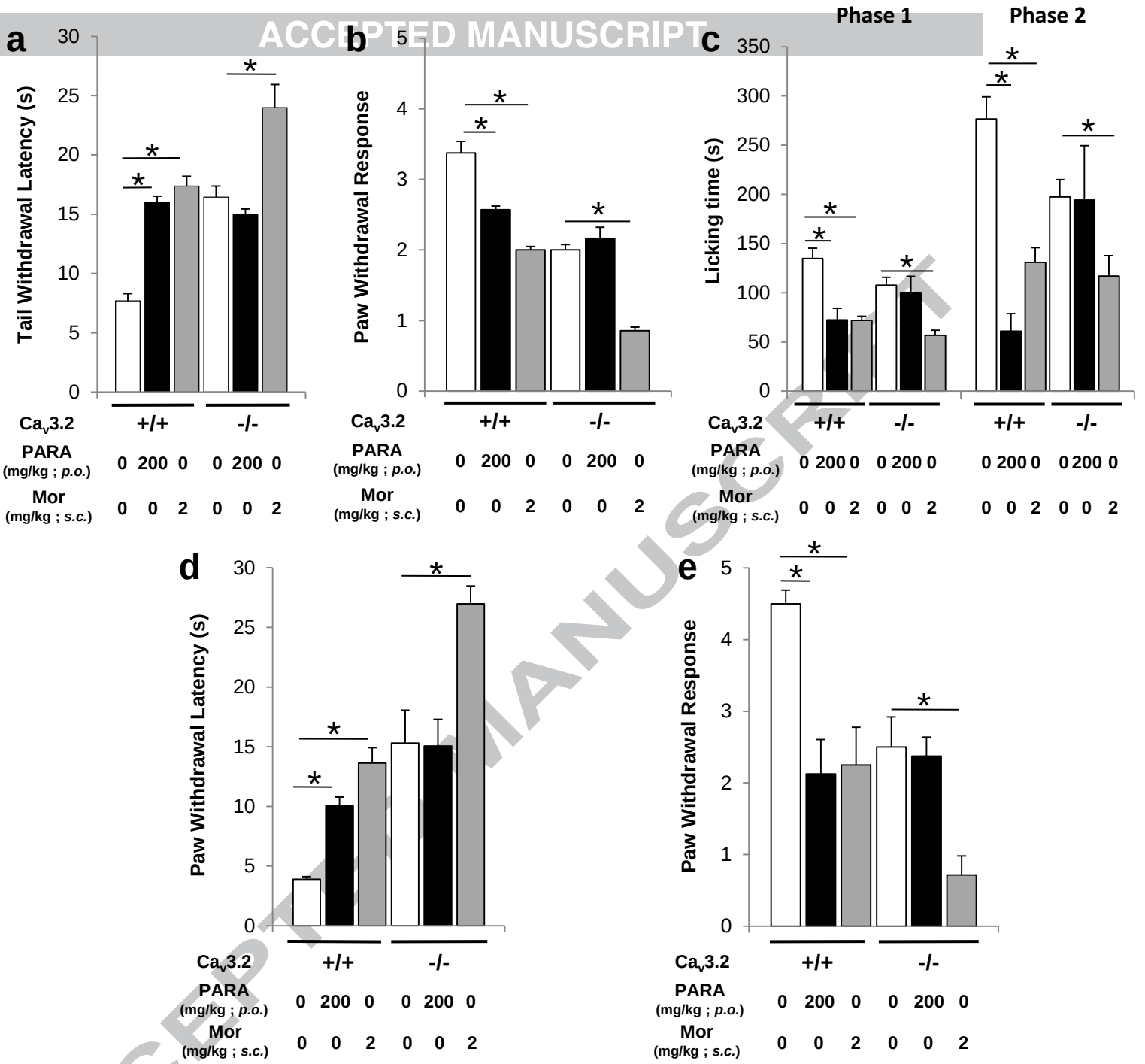


Figure-2 (KERCKHOVE)

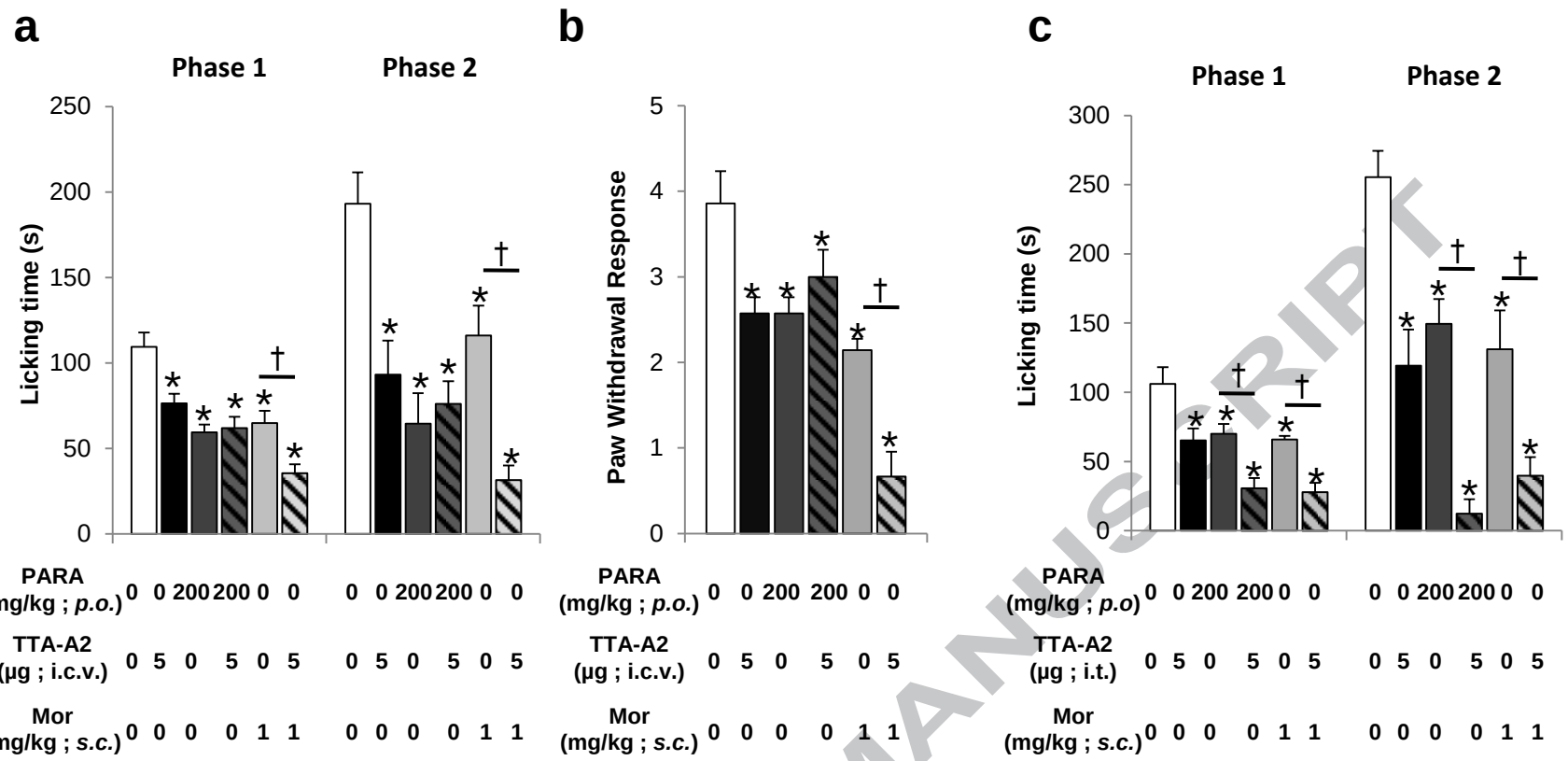


Figure-3 (KERCKHOVE)

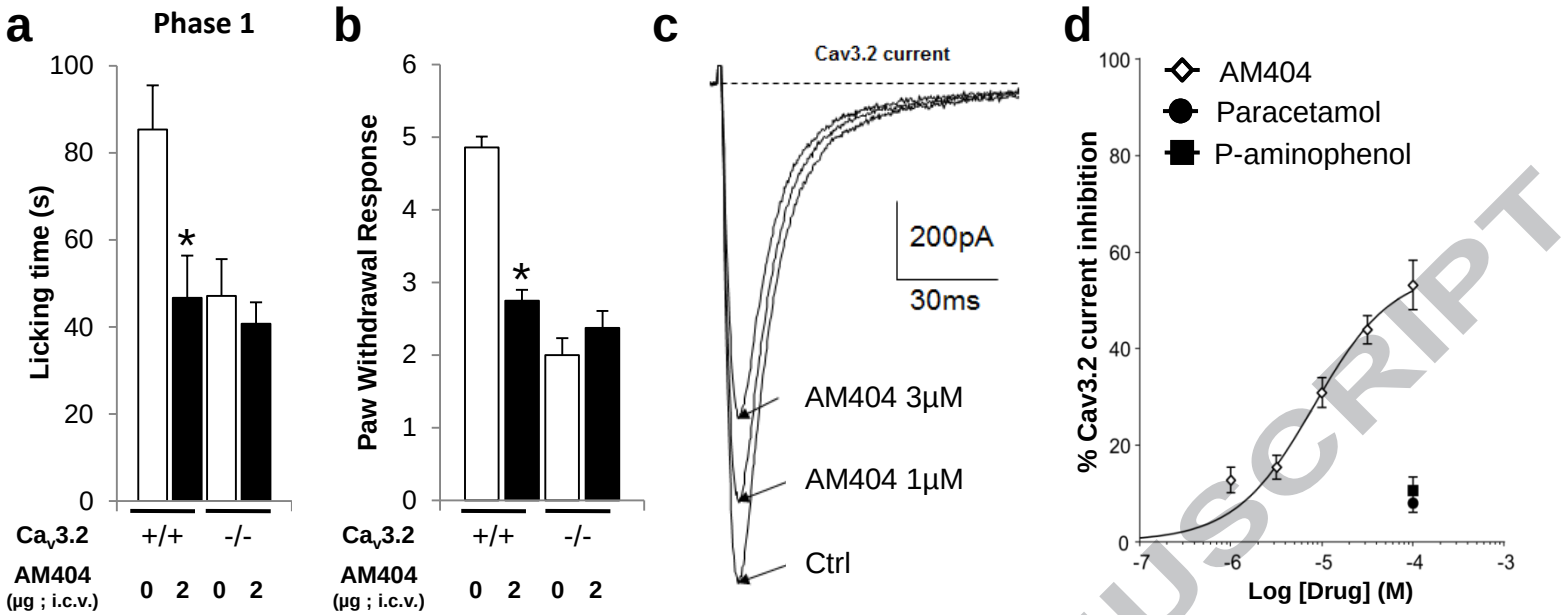
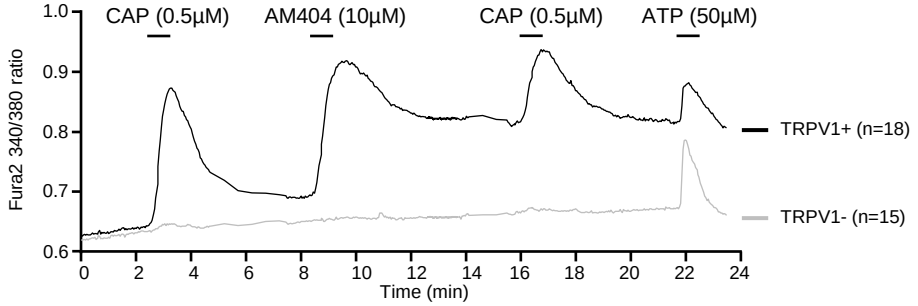


Figure-4 (Kerckhove)

a



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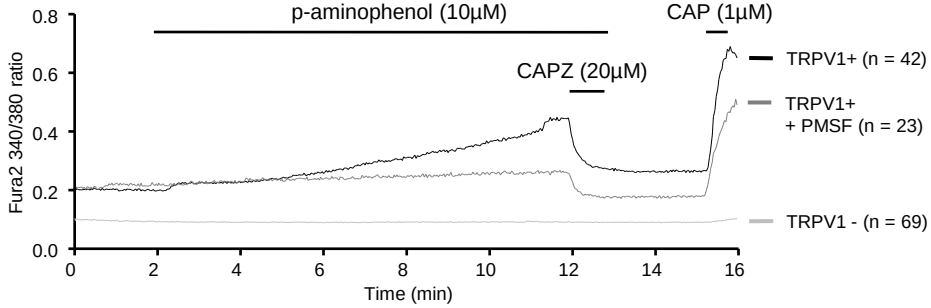
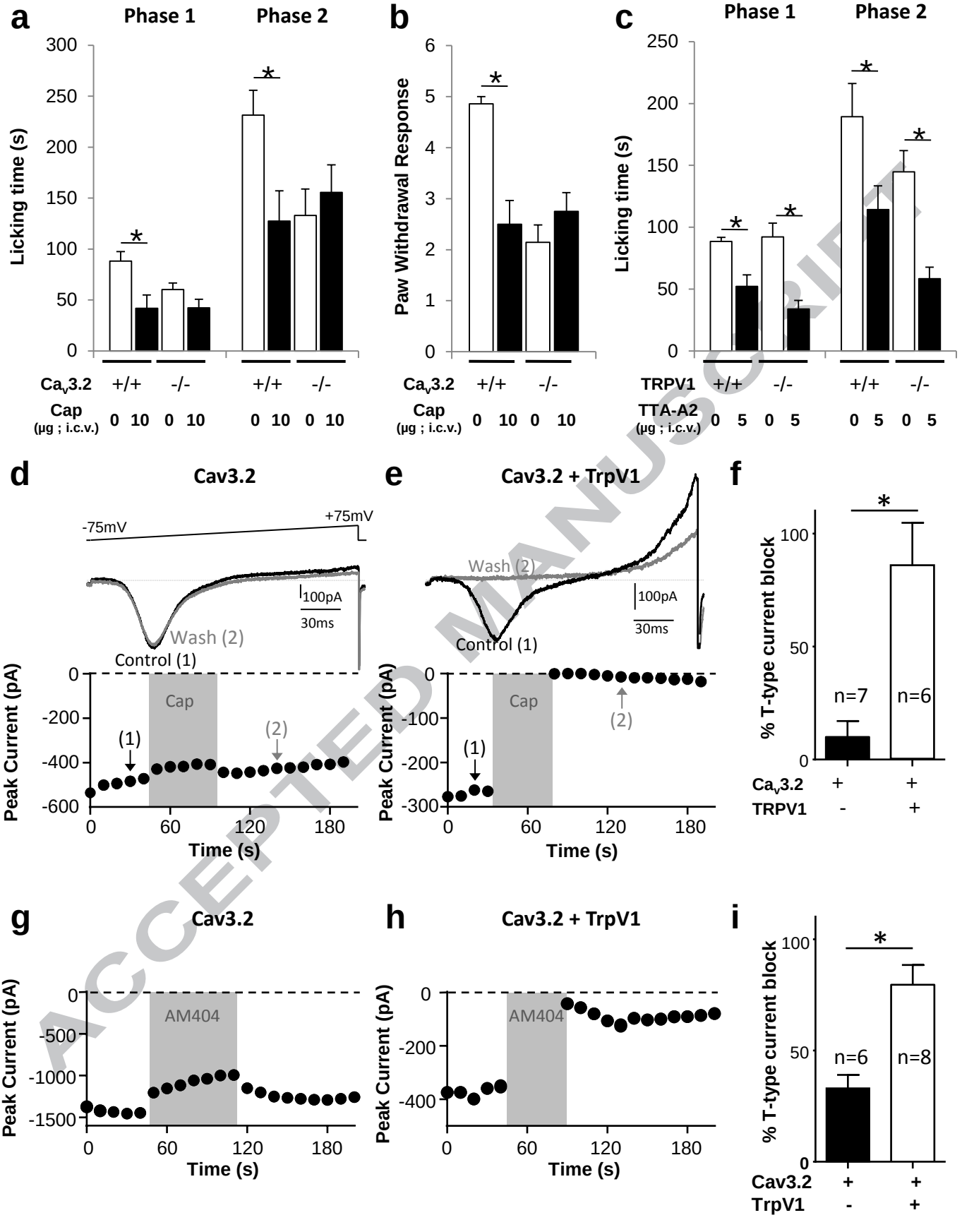


Figure-5 (KERCKHOVE)



Summary

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

ABSTRACT

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