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RESEARCH ARTICLE

# Physiological, pharmacological and behavioral evidence for a TRPA1 channel that can elicit defensive responses in the medicinal leech

**Torrie Summers, Yanqing Wang, Brandon Hanten and Brian D. Burrell***

## ABSTRACT

Transient receptor potential ankyrin subtype 1 (TRPA1) channels are chemosensitive to compounds such as allyl isothiocyanate (AITC, the active component of mustard oil) and other reactive electrophiles and may also be thermodetectors in many animal phyla. In this study, we provide the first pharmacological evidence of a putative TRPA1-like channel in the medicinal leech. The leech's polymodal nociceptive neuron was activated by both peripheral and central application of the TRPA1 agonist AITC in a concentration-dependent manner. Responses to AITC were inhibited by the selective TRPA1 antagonist HC030031, but also by the TRPV1 antagonist SB366791. Other TRPA1 activators – *N*-methylmaleimide (NMM) and cinnamaldehyde (CIN) – also activated this nociceptive neuron, although HC030031 only inhibited the effects of NMM. The polymodal nociceptive neurons responded to moderately cold thermal stimuli (<17°C) and these responses were blocked by HC030031. AITC sensitivity was also found in the pressure-sensitive sensory neurons and was blocked by HC030031, but not by SB366791. AITC elicited a nocifensive withdrawal of the posterior sucker in a concentration-dependent manner that could be attenuated with HC030031. Peripheral application of AITC *in vivo* also produced swimming-like behavior that was attenuated by HC030031. These results suggest the presence of a TRPA1-like channel in the medicinal leech nervous system that responds to cold temperatures and may interact with the leech TRPV-like channel.

**KEY WORDS: Leech, TRPA1, Invertebrate, Nociception**

## INTRODUCTION

Transient receptor potential (TRP) channels are a family of ion channels involved in both the detection and modulation of a variety of sensory inputs and can be found in both vertebrates and invertebrates (Damann et al., 2008). The most well-known TRP channel is the transient receptor potential vanilloid 1 (TRPV1) channel which detects noxious thermal (>40°C) and chemical stimuli (e.g. capsaicin and $H^+$) (Damann et al., 2008). Recently, we published evidence of a capsaicin-sensitive TRPV-like channel in the medicinal leech (Summers et al., 2014). Another TRP channel involved in nociceptive signaling is the transient receptor potential ankyrin subtype 1 protein (TRPA1). The TRPA1 channel is a non-selective cation channel that can be activated by a variety of molecules including polygodial, formalin, anandamide, tetrahydrocannabinol and the reactive electrophiles AITC (the TRPA1-activating component of mustard oil), NMM, and CIN (Jordt et al., 2004; Laursen et al., 2014). TRPA1 may also have thermosensitive properties, but whether it is directly activated by cold (<17°C) or contributes to the development of cold hypersensitivity is still being debated (Karashima et al., 2009; Vilceanu and Stucky, 2010; Laursen et al., 2014; Moparthi et al., 2014).

Invertebrate TRPA1 channel homologs have been studied extensively in ecdysozoans (e.g. *Drosophila* and *Caenorhabditis elegans*) where they exhibit many of the roles typically associated with the mammalian TRPV1 channel, such as detection of noxious thermal and mechanical stimuli (Kang et al., 2010; Neely et al., 2011). However, a recent and highly detailed bioinformatics study has identified TRPA1 channels in lophotrochozoans (i.e. mollusks and annelids), specifically the mollusk *Lottia gigantea* and the polychaete annelid *Capitella teleta* (Peng et al., 2015). Furthermore, these lophotrochozoan TRPA1 channels appear to be more closely related to the vertebrate TRPA1 than to the TRPA channels found in other invertebrate phyla. Thus, there is a compelling need to study the physiological function of the TRPA1 channel in this group of invertebrates.

The medicinal leech (*Hirudo verbana*) is a lophotrochozoan of particular interest for the study of TRP function because of the similarity of the leech's somatosensory neurons to those found in mammals (Smith and Lewin, 2009). The leech possesses specific sensory neurons that detect light touch (T cells), sustained pressure (P cells) and both mechanical and polymodal nociceptive stimuli (N cells) (Nicholls and Baylor, 1968; Blackshaw et al., 1982; Pastor et al., 1996). Previous studies have found that the polymodal lateral N cells (lN cells) respond to capsaicin, $H^+$ and noxious heat and that this response can be blocked with the selective TRPV1 antagonist SB366791, suggesting the presence of a TRPV-like channel in the leech (Pastor et al., 1996; Summers et al., 2014).

Here, we show the first pharmacological evidence for a TRPA1-like channel in the medicinal leech. Both nociceptive and non-nociceptive afferents that respond to AITC have been identified and this activity can be inhibited by the TRPA1 antagonist HC030031. Responses to other reactive electrophiles, specifically NMM and CIN, have also been observed. Additional evidence has been obtained showing that TRPA1 may mediate responses to moderate cold in the leech. Finally, *in vivo* behavioral responses elicited by AITC activation of the presumed TRPA1 channel have been characterized.

## RESULTS

### TRPA1-like receptor in the polymodal N cells

First, the peripheral and central effects of AITC were examined. Peripheral effects were examined using a body-wall preparation (Fig. 1B), which consisted of a section of leech periphery

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(skin+muscle) pinned flat to the bottom of a Sylgard™-lined recording chamber and still connected to the CNS (1–3 ganglia) via segmental nerve roots that project from the ganglia to the periphery (Nicholls and Baylor, 1968). Central effects were examined using isolated ganglia. Activity of afferents was measured prior to and then during treatment with 10 µmol $l^{-1}$ to 2 mmol $l^{-1}$ AITC (Fig. 1A), which was added to either the body-wall preparations or isolated ganglia using a manual rapid solution exchange system. Responses to AITC were compared with responses in vehicle control experiments consisting of saline plus ascending levels of DMSO (0.0001%, 0.001%, 0.0025%, 0.005%, 0.01% or 0.02%). Topical application of AITC to the external surface of the skin in these body wall preparations elicited action potential firing in the polymodal, lateral nociceptive (lN) cell in a concentration-dependent manner (Fig. 2A) based on a two-way ANOVA that detected a significant effect of AITC versus vehicle ($F_{1,47}$=501.867, $P$<0.001), a significant concentration effect ($F_{5,47}$=28.463, $P$<0.001) and a significant interaction effect indicating an effect of increasing concentrations of AITC, but not increasing levels of the vehicle, DMSO ($F_{5,47}$=29.192, $P$<0.001). Central application of AITC produced a similar concentration-dependent increase in lN cell activity (Fig. 2A; treatment effect, $F_{1,97}$=845.508, $P$<0.001; concentration effect, $F_{5,97}$=42.613, $P$<0.001; interaction effect, $F_{5,97}$=43.319, $P$<0.001). The ability of both centrally and peripherally applied AITC to elicit lN activity is a novel finding in the leech, and the effective concentration range observed in these experiments is comparable to what is reported in both mammals and other invertebrates (Jordt et al., 2004; Kang et al., 2010; Neely et al., 2011; Weller et al., 2011).

When 100 µmol $l^{-1}$ AITC was co-administered with the TRPA1 antagonist HC030031 (10 µmol $l^{-1}$) (Laursen et al., 2014), both peripheral and central responses were significantly attenuated in the polymodal lN cells (Fig. 2B,C). This is consistent with the effects of AITC being mediated by the leech TRPA1 channel. Next, the selective TRPV1 antagonist SB366791 (10 µmol $l^{-1}$) was co-administered with AITC. Surprisingly, SB366791 could partially attenuate the responses to AITC. There was no effect on activity when SB366791 or HC030031 was applied alone. A one-way ANOVA detected significant treatment effects of the antagonists in both peripheral ($F_{3,24}$=15.781, $P$<0.001) and central ($F_{5,30}$=56.5, $P$<0.001) preparations with *post hoc* analysis detecting significant attenuating effects of HC030031 ($P$<0.001) and SB355701 ($P$<0.05) on AITC-induced activity in the lN cells.

Previous studies have found that noxious heat (>43°C) activates lN cells and that this heat-induced activity can be attenuated with the TRPV antagonist SB366791 (Pastor et al., 1996; Summers et al., 2014). Since it has been shown that some invertebrates use their TRPA1 channel homologs for thermodetection of noxious heat (Neely et al., 2011), we repeated and expanded these experiments with the use of heat and HC030031. In experiments in which saline heated to 43°C was applied to the patch of skin in the body wall preparation, activity in the lN cell was observed, consistent with previous findings (Pastor et al., 1996; Summers et al., 2014). This activity was not attenuated by HC030031, but surprisingly, co-application of this TRPA1 antagonist increased responsiveness to noxious heat (Fig. 3A,B; *t*-test $P$<0.04). It is possible that this increased response was due to the TRPA1 antagonist increasing the input resistance of the lN cell; however, no significant change in input resistance was observed following application of HC030031 alone (pre-test input resistance=23±6.6 MΩ versus post-test=25±8.6 MΩ; $N$=7, $t$=0.49, $P$>0.05).

Next, because TRPA1 in mammals has been implicated as a detector of moderate cold (<17°C) (Story et al., 2003; Karashima et al., 2009; Moparthi et al., 2014), these experiments were repeated using chilled saline. The lN cell was found to respond to chilled saline starting at approximately 17°C. Furthermore, this cold-induced activity was significantly attenuated with co-application of HC030031 (Fig. 3A,B; $P$<0.001). These results suggest that TRPA1-like channels in the lN cells have a thermosensitivity similar to mammalian TRPA1.

### Responses to AITC in other sensory cells

Next, the responses of other leech mechanosensory cells were tested for responses to AITC. The T cells and medial N cells (mN, which are mechano-nociceptors) did not respond to 100 µmol $l^{-1}$ AITC when applied centrally or peripherally. However, the P cells did respond when AITC was applied peripherally, but not when applied centrally (Fig. 4A). In previous experiments studying the putative TRPV channel in the leech, peripheral application of capsaicin was also found to activate the P cells (Summers et al., 2014). However, this capsaicin-elicited activity could be blocked by the AMPA receptor antagonist CNQX, indicating that capsaicin was activating an unknown TRPV-containing afferent that was driving P-cell activity via glutamatergic synaptic transmission. To test whether this was also the case for AITC-induced activation of the P cells, CNQX (50 µmol $l^{-1}$) was bath-applied to the CNS prior to

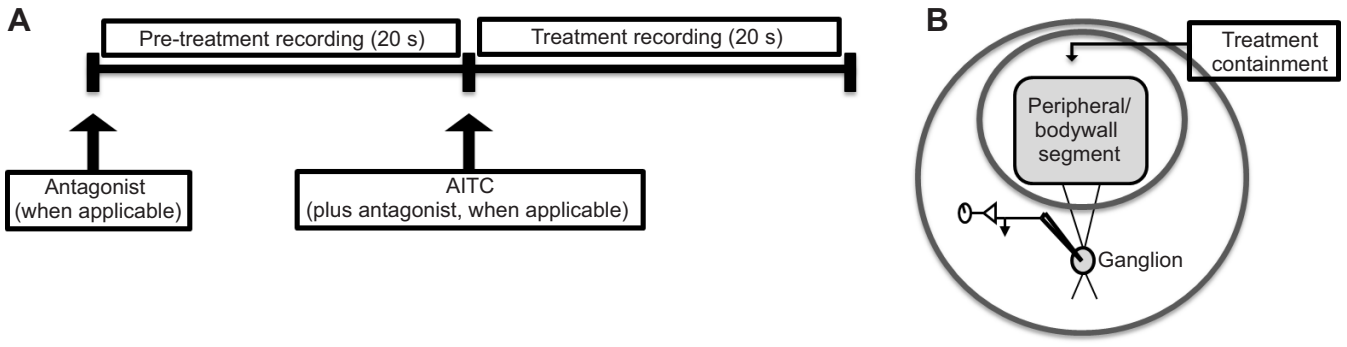


**Fig. 1. Set-up for electrophysiology experiments.** (A) Time frame of central and peripheral physiology experiments. Activity was measured 20 s prior to AITC treatment and then for 20 s during drug application. AITC-elicited activity was calculated as the difference between these two periods. In some experiments, the preparation was pre-treated with HC030031 or SB366791, prior to AITC treatment (antagonists were also included in the AITC solution). (B) Diagram of the body wall preparation. Intracellular recordings were made from the N, P or T cells within a ganglion that was still innervating a section of body wall. Treatments (AITC or thermal stimuli), TRPV1 antagonist (SB366791) or TRPA1 antagonist (HC030031) were applied directly to the body wall, which was isolated with a plastic containment ring and secured to the bottom of the dish with petroleum jelly.



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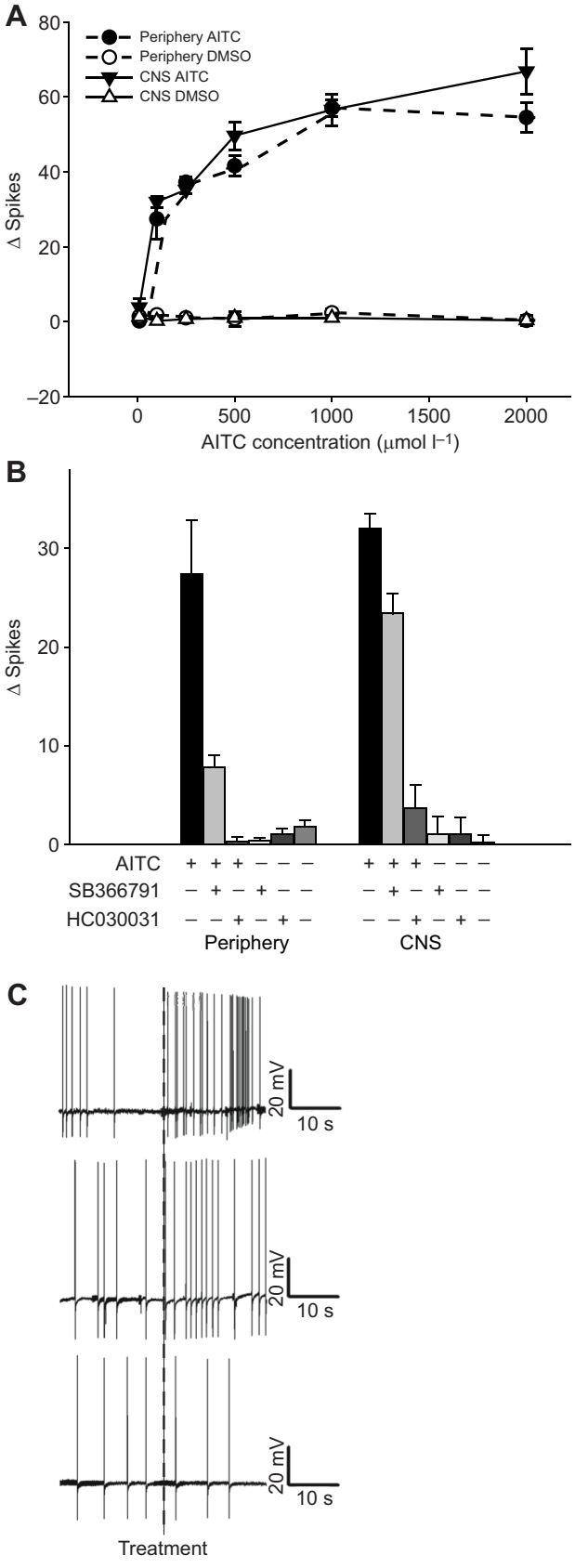


**Fig. 2. Effect of AITC and antagonism by either the TRPA1 antagonist HC030031 or TRPV1 antagonist SB366791 in polymodal N cells.** (A) Concentration-dependent response of lateral N cells (Δ spikes) to peripherally and centrally (CNS) applied AITC. Sample size (*N*) for the central preparations was as follows (DMSO control *N* is in parentheses); 10 μmol $l^{-1}$, *N*=5 (3); 100 μmol $l^{-1}$, *N*=5 (5); 250 μmol $l^{-1}$, *N*=4 (4); 500 μmol $l^{-1}$, *N*=3 (4); 1 mmol $l^{-1}$, *N*=5 (3); 2 mmol $l^{-1}$, *N*=6 (3). Sample size for the peripheral preparations was 10 μmol $l^{-1}$, *N*=4 (4); 100 μmol $l^{-1}$, *N*=5 (5); 250 μmol $l^{-1}$, *N*=5 (3); 500 μmol $l^{-1}$, *N*=5 (3); 2 mmol $l^{-1}$, *N*=4 (3); 2 mmol $l^{-1}$, *N*=4 (3). (B) Both HC030031 and SB366791 (10 μmol $l^{-1}$) blocked the response of lateral N to peripherally (AITC+HC030031, *N*=5; AITC+SB366791, *N*=5) and centrally applied AITC (100 μmol $l^{-1}$; AITC+HC030031, *N*=5; AITC +SB366791, *N*=5). Neither HC030031 (peripheral, *N*=3; central, *N*=4) nor SB366791 (peripheral, *N*=3; central, *N*=7) alone was able to affect lN cell activity. (C) Sample traces of the lateral N-cell activity during AITC treatment (top), AITC+SB366791 (middle) and AITC+HC030031 (bottom).

peripheral application of 100 μmol $l^{-1}$ AITC. CNQX has been successfully used in the leech to block glutamatergic transmission within the CNS (Wessel et al., 1999; Li and Burrell, 2008; Yuan and Burrell, 2010). Unlike the situation with capsaicin, CNQX did not block the AITC-induced activation of the P cells (Fig. 4B; one-way ANOVA $F_{1,10}$=0.402, $P$>0.05).

Since AITC activation of P cells appears to be a direct effect, further studies were conducted to characterize the putative TRPA1 channels within the P cells. First, a concentration series of peripherally applied AITC was conducted using the same methods described for the lN cell concentration series (Fig. 4C). A two-way ANOVA revealed that AITC-elicited activity in the P cells increased in a concentration-dependent manner, with a significant effect detected of AITC versus vehicle ($F_{1,65}$=126.251, $P$<0.001), a significant concentration effect ($F_{5,65}$=5.5, $P$<0.001) and a significant interaction effect indicating an effect of increasing concentrations of AITC, but not increasing levels of DMSO ($F_{5,65}$=5.7, $P$<0.001). A *post hoc* analysis revealed that the higher concentrations (500–2000 μmol $l^{-1}$ were not significantly different ($P$>0.05), but these higher concentrations were all significantly different from the lower concentrations (10–250 μmol $l^{-1}$; $P$<0.005). Next, the antagonists SB366791 or HC030031 (10 μmol $l^{-1}$) were co-applied with 100 μmol $l^{-1}$ AITC. When the antagonists were co-applied to the periphery, a one-way ANOVA revealed a significant effect of treatment (Fig. 4E; $F_{5,26}$=27.316, $P$<0.001). *Post hoc* analysis showed that AITC-induced P-cell activity was significantly reduced by HC030031 ($P$<0.001), but not SB366791 ($P$>0.05). These results indicate the presence of a TRPA1-like channel in the P cells that is functionally different from those identified in the capsaicin-sensitive lN cells since SB366791 did attenuate AITC-elicited responses in the lN cells, but not the P cells.

### Effects of other reactive electrophiles

In both vertebrates and invertebrates, activation of TRPA1 by AITC is mediated by direct covalent modification of the channel by this reactive electrophile (Macpherson et al., 2007; Kang et al., 2010). Therefore, the ability of other reactive electrophiles to stimulate the lN cell and the ability of the TRPA1 antagonist HC030031 to block this activity was tested. NMM (100 μmol $l^{-1}$ to 1 mmol $l^{-1}$) applied centrally elicited activity in the lN cell in a concentration-dependent manner (Fig. 5A; one-way ANOVA, $F_{3,12}$=4.39, $P$<0.05). lN cell activity elicited by 200 μmol $l^{-1}$ NMM was significantly reduced by pre-treatment with HC030031 (Fig. 5A,B; 50 μmol $l^{-1}$; $t$=4.32, $P$<0.05). Central application of CIN (0.5–3 mmol $l^{-1}$) also stimulated the lN cell with activity increasing in a concentration-dependent manner (Fig. 5C; one-way ANOVA, $F_{3,17}$=12.61, $P$<0.001). However, 200–300 μmol $l^{-1}$ HC030031, the maximum concentration that could be delivered with the antagonist remaining in solution, failed to reduce the activity elicited by 1 mmol $l^{-1}$ CIN (Fig. 5C,D; $t$=0.77, $P$>0.05). These findings provide partial support

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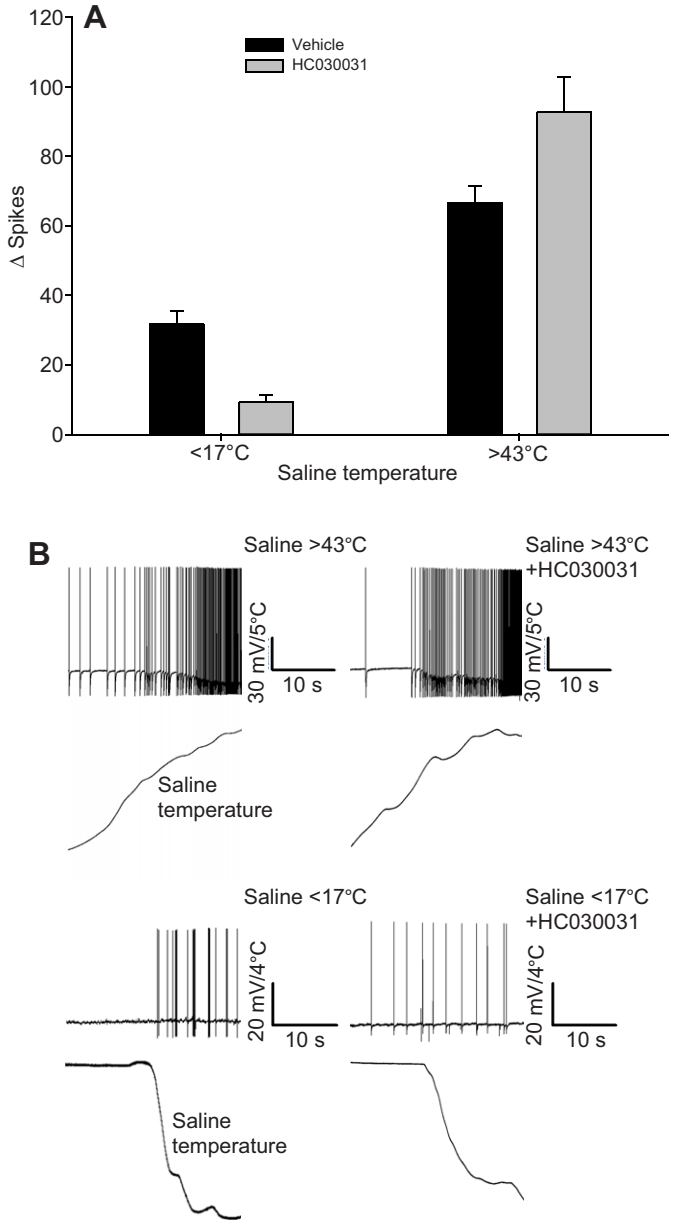


**Fig. 3. Effect of noxious heat and moderate cold stimuli on polymodal N cells from the medicinal leech.** (A) Application of saline chilled to <17°C elicited lN activity (*N*=6) that was blocked by HC030031 (*N*=8). Saline heated to >43°C also elicited activity (*N*=5) that was actually enhanced by HC030031 (*N*=4). (B) Traces of noxious heat activates the lN cells (top left), peripherally applied HC030031 enhances noxious heat activity in lN cells (top right), moderate cold activates lN cells (bottom left) and peripherally applied HC030031 reduces cold-induced activity in lN cells (bottom right).

for the presence of a TRPA1-like channel in the leech given that the reactive electrophiles NMM and CIN, along with AITC, are all able to activate the lateral N cell. However, it is unclear why HC030031 was able to inhibit activity elicited by AITC and NMM, but not by CIN.

Although not a reactive electrophile, menthol has been shown to directly activate human TRPA1 channels (Moparthi et al., 2014). However, 1–2 mmol $l^{-1}$ menthol failed to elicit any activity in the leech lN cell (data not shown). This suggests that, similar to *Drosophila* TRPA1 (Xiao et al., 2008), the TRPA1-like channel in the leech is insensitive to menthol.

### Behavioral effects of AITC

Next, the behavioral responses to increasing AITC concentrations (10–2000 µmol $l^{-1}$) were observed *in vivo*. Application of AITC (1 ml) to the posterior sucker of the leech produced a withdrawal response that decreased in latency with increasing AITC concentrations. The posterior sucker withdrawal was present starting at 100 µmol $l^{-1}$ (Fig. 6A), comparable to the threshold at which the lN cell was activated by AITC. Co-application of 100 µmol $l^{-1}$ HC030031 with AITC increased the latency to respond and decreased the number of animals responding to AITC (Fig. 6A). Two-way ANOVA detected a statistically significant effect of treatment ($F_{3,153}$=215.03, $P$<0.001), significant effect of concentration ($F_{5,153}$=92.10, $P$<0.001) and a significant interaction effect between the treatment and concentration ($F_{15,153}$=19.61, $P$<0.001). Subsequent *post hoc* test of the treatment effect confirmed that the AITC-treated group was significantly different from the AITC+HC030031 and DMSO groups ($P$<0.001) and that the AITC+HC030031 group was significantly different from the DMSO group ($P$<0.001).

Following posterior sucker withdrawal, the higher concentrations of AITC produced a sporadic swimming-like behavior, despite the fact that the leech was not immersed in water. Swimming is behaviorally characterized by the animal flattening its body, a flare of the posterior sucker and initiation of repeated traveling-wave undulations of the body (Kuffler, 1978). This swimming behavior could be seen for brief durations starting at 250 µmol $l^{-1}$ AITC (4/6 responded with swimming), but at 500 µmol $l^{-1}$ AITC, the behavior became more consistent (6/6 animals responded with swimming); the duration of swimming was highly variable between animals until 1000 µmol $l^{-1}$ (Fig. 6B). Because of the variability, these data failed tests for normality and were therefore analyzed using Kruskal–Wallis one-way ANOVA on ranks. This analysis revealed a significant difference between the treatment groups ($H$=56.89, $P$<0.001). *Post hoc* Mann–Whitney comparisons of swim duration following treatment with AITC versus AITC+HC030031 found significant differences at 2000 µmol $l^{-1}$ ($U$=6.00, $P$<0.025), 500 µmol $l^{-1}$ ($U$=4.50, $P$<0.025), 500 µmol $l^{-1}$ ($U$=4.00, $P$<0.01) and 250 µmol $l^{-1}$ ($U$=6.00, $P$<0.05).

## DISCUSSION

In this study, we have found the first pharmacological evidence for the presence of central and peripheral TRPA1-like channels in the medicinal leech. Peripheral or central application of AITC was able to activate the polymodal lN neurons in a concentration-dependent manner and this activity could be blocked with the selective TRPA1 antagonist HC030031. AITC is a reactive electrophile that activates both vertebrate and invertebrate TRPA1 channels as a result of covalent bonds with conserved cysteine and lysine residues (Macpherson et al., 2007; Kang et al., 2010). Other reactive electrophiles, specifically NMM and CIN, were also tested and did activate the lN cell in a concentration-dependent manner. However, HC030031 only inhibited NMM-elicited activity, despite the fact that this antagonist has been shown to inhibit CIN-mediated activation of TRPA1 (El Karim et al., 2011). The most parsimonious explanation is that the effects of CIN are not mediated by a TRPA1-like channel in the leech. This would suggest that the leech TRPA1-like channel has an altered response to reactive electrophiles. There are examples of TRPA channels in both *Drosophila* and *C. elegans* that are missing essential cysteine residues and are insensitive to reactive electrophiles (Kindt et al., 2007; Kang et al., 2010). It is possible that the leech version of TRPA1 represents an intermediate type that retains many, but not

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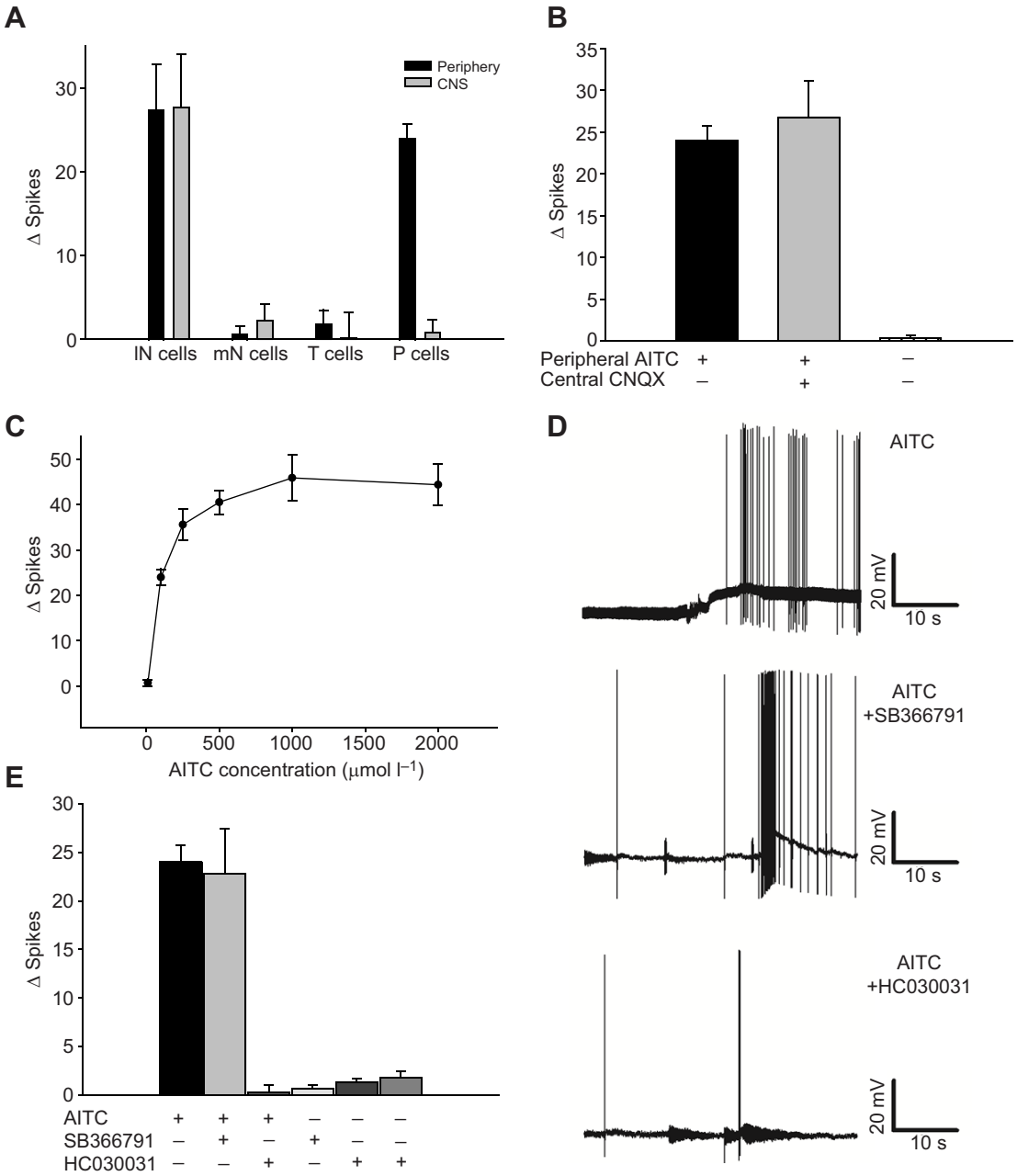


**Fig. 4. Effect of AITC in non-nociceptive neurons.** (A) Peripherally and centrally applied AITC (100 μmol $l^{-1}$) directly activates the lateral N cells (lN), but only activates the P cells when applied peripherally (peripheral, *N*=6; central, *N*=6). Neither central nor peripheral application of AITC activated the medial N (mN; peripheral, *N*=6; central, *N*=3) or T (peripheral, *N*=4; central, *N*=3) cells. (B) CNQX has no effect on the AITC-induced activity in the P cell, indicating that the effect of the TRPA1 activator is not driven by glutamatergic signaling (AITC, *N*=6; AITC+CNQX, *N*=5; DMSO, *N*=3). (C) Activity in P cells increases with increasing peripheral AITC concentration [10 μmol $l^{-1}$, *N*=4 (DMSO=3); 100 μmol $l^{-1}$, *N*=6 (5); 250 μmol $l^{-1}$, *N*=7 (4); 500 μmol $l^{-1}$, *N*=6 (4); 1 mmol $l^{-1}$, *N*=7 (3); 2 mmol $l^{-1}$, *N*=14 (3)]. (D) Traces of AITC induced P cell activity (top) and induced the effects of SB366791 (middle) and HC030031 (bottom) on this activity. (E) AITC-induced activity could be blocked with HC030031 (*N*=4), but not SB366791 (*N*=5). Neither HC030031 (*N*=3) nor SB366791 (*N*=3) by itself has any effect on P cell activity.

the full complement, of the residues that form covalent bonds with reactive electrophiles. Resolving these issues will require both molecular characterization of the leech TRPA1 channel and a more detailed electrophysiological examination of the stimuli that activate these putative TRPA1 channels in cultured sensory cells.

Cold stimuli (<17°C) perfused onto the periphery also induced lN activity and this activity could be attenuated with HC030031. Peripheral application of AITC *in vivo* elicited a withdrawal of the posterior sucker and produced a spontaneous swimming behavior which both could be attenuated with HC030031. These results are consistent with properties of the mammalian TRPA1 channel, which is sensitive to AITC and can act as a thermodetector for moderately noxious cold (<17°C) stimuli (Karashima et al., 2009; Moparthi et al., 2014). Previously, we reported evidence for capsaicin sensitivity in the lN cells that can be blocked with the selective TRPV1 antagonist SB366791, indicating the presence of a TRPV-like channel in the leech (Summers et al., 2014). In the current study, it was observed that these same neurons responded to AITC. The sensitivity of the leech polymodal lN cells to both TRPV1 and TRPA1 agonists is consistent with the co-localization

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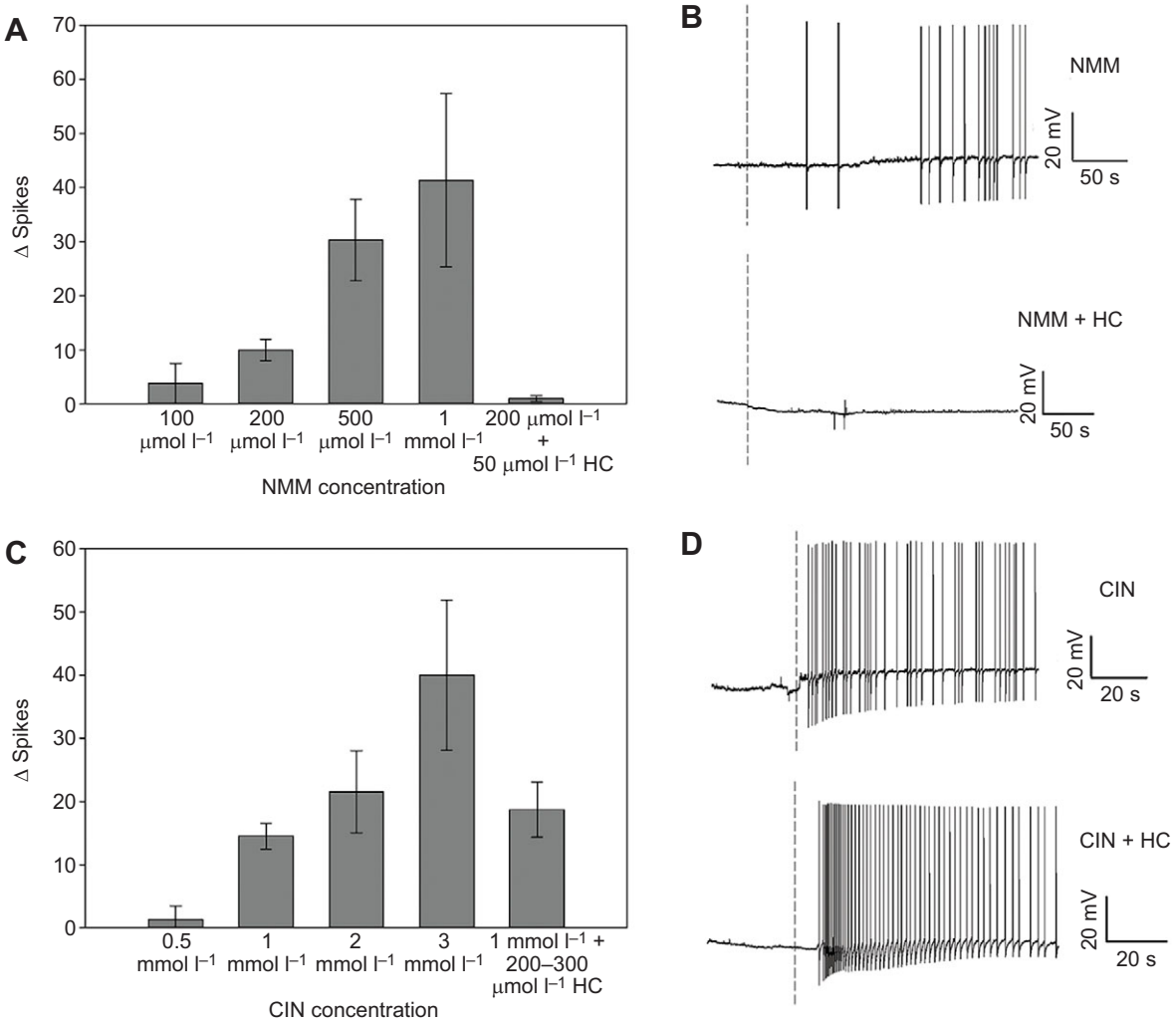


**Fig. 5. Effect of other reactive electrophiles on the leech polymodal nociceptive neuron.** (A) Concentration-dependent response of the lN cell to central application of NMM at 100 μmol $l^{-1}$ (*N*=4), 200 μmol $l^{-1}$ (*N*=3), 500 μmol $l^{-1}$ (*N*=3) and 1 mmol $l^{-1}$ (*N*=3). Application of 20 μmol $l^{-1}$ HC030031 inhibited the lN cell response to 200 μmol $l^{-1}$ NMM (*N*=3). (B) Sample traces of the lN response to 200 μmol $l^{-1}$ NMM alone (top) and with HC030031 (bottom). (C) Concentration-dependent response of the lN cell to central application of CIN at 0.5 (*N*=9), 1 (*N*=4), 2 (*N*=2) and 3 mmol $l^{-1}$ (*N*=3). Application of 200–300 μmol $l^{-1}$ HC030031 failed to inhibit the lN cell response to 1.0 mmol $l^{-1}$ CIN (*N*=4). (D) Sample traces of the lN cell response to 1.0 mmol $l^{-1}$ CIN alone (top) and with HC030031 (bottom).

of these two TRP channels in mammals (Salas et al., 2009; Malin et al., 2011). The range of AITC used on the leeches is well within the range of concentrations used experimentally in mammals and invertebrates for eliciting nocifensive responses (Jordt et al., 2004; Kang et al., 2010; Weller et al., 2011).

In our previous study, we found that the lN cells are sensitive to noxious heat and that this heat-induced activity can be attenuated with co-application of the TRPV1 antagonist SB366791. In the current study, we repeated these experiments and found that the lN cells also respond to moderately cold saline perfused onto the periphery. The lN response to cold, but not noxious heat, could be attenuated with HC030031. In insects, TRPA homologs such as painless in *Drosophila* and NvHsTRPA in *Nasonia vitripennis*, are known to mediate responses to noxious heat, but not cold (Matsuura et al., 2009; Neely et al., 2011). However, *C. elegans* do utilize a TRPA1-like channel to detect cold temperatures (<17°C) (Fischer et al., 2014). In mammals, TRPA1 is thought to be a thermodetector of cold (Karashima et al., 2009; Moparthi et al., 2014), but this has been questioned by at least one study proposing that the activation of TRPA1 by cold is an indirect mechanism (Zurborg et al., 2007).

A surprising result of the thermal stimulus studies in the leech is that sensitization of the lN cells to noxious heat by HC030031 treatment was observed. One possibility is that HC030031 has off-target effects on the putatively heat-sensitive leech TRPV. Alternatively, these findings could be evidence of an interaction between TRPA1 and TRPV channels. AITC can cause sensitization of responses to heat (Martin et al., 2004; Simons et al., 2004; Carstens and Mitsuyo, 2005; Merrill et al., 2008; Sawyer et al., 2009) and this is thought to be the result of TRPA1-mediated sensitization of TRPV1 (Jansen et al., 1978). Because HC030031 may operate as an allosteric modulator (Xiao et al., 2008), it is possible that this antagonist elicits a conformational change in TRPA1 that, while antagonizing channel function, is able to activate the biochemical pathways responsible for TRPV sensitization. Such allosteric modulation is observed in the effects of the AMPA-type glutamate receptor antagonist CNQX, which blocks gating of the ionotropic channel, but also activates biochemical pathways that elicit internalization of glutamate receptors and gap junction proteins (Lin et al., 2000; Li and Burrell, 2008).

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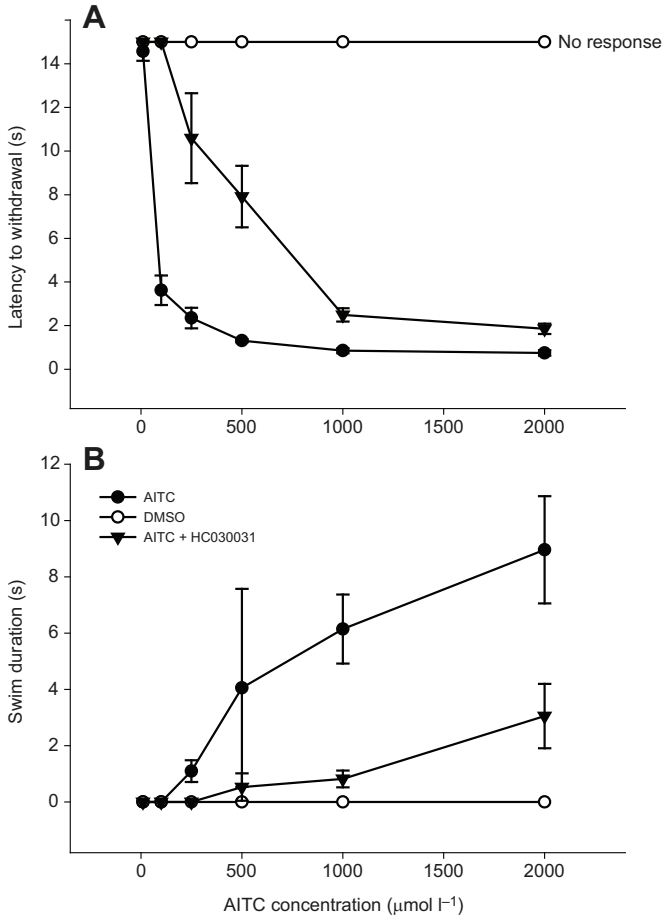


**Fig. 6. AITC elicited nocifensive responses in leeches with increasing concentration that were blocked or reduced when pre-treated with HC030031.** (A) The latency to initiate withdrawal of the posterior sucker decreased with increasing AITC concentration [10 μmol $l^{-1}$, *N*=5 (DMSO *N*=6); 100 μmol $l^{-1}$, *N*=7 (7); 250 μmol $l^{-1}$, *N*=7 (6); 500 μmol $l^{-1}$, *N*=6 (6); 1 mmol $l^{-1}$, *N*=7 (6); 2 mmol $l^{-1}$, *N*=9 (7)]. Co-application of HC030031 with AITC increased the latency to withdraw to a given AITC concentration (10 μmol $l^{-1}$, *N*=3; 100 μmol $l^{-1}$, *N*=6; 250 μmol $l^{-1}$, *N*=6; 500 μmol $l^{-1}$, *N*=9; 1 mmol $l^{-1}$, *N*=6; 2 mmol $l^{-1}$, *N*=6). Animals that did not respond with a full withdrawal within 15 s of AITC application were recorded as having a 15 s response. (B) Duration of AITC-induced spontaneous swimming. Swimming behavior started at 250 μmol $l^{-1}$ AITC and increased with increasing concentration. Co-application of the HC030031 reduced swimming behavior at higher concentrations and blocked it at 250 μmol $l^{-1}$.

Another interesting finding of this study was that peripheral application of AITC induced concentration-dependent activation of both P cells. In our previous study, we found that peripheral application of capsaicin stimulated the P cells and that this activation was not a direct effect because central application of CNQX blocked this capsaicin-elicited activity (Summers et al., 2014). The current study found that CNQX had no effect on AITC-induced activity whereas HC030031 was successful at blocking this activity, suggesting that the TRPA1 activator is directly stimulating the P cell.

Although AITC-induced activity in the P cells was blocked by HC030031, it was not affected by the TRPV1 antagonist SB366791. This was not the case in the capsaicin-sensitive lN cells, where both HC030031 and SB366791 could significantly attenuate AITC-induced activity in both the periphery and CNS. Previous studies have demonstrated that SB366791 has no effect on TRPA1 channels (Andrade et al., 2008). One possibility is that SB366791 has an off-target effect, although the fact that the antagonist failed to block AITC-induced activity in the capsaicin-insensitive P cells suggests that this is not the case. A second possibility is that AITC directly activates leech TRPV, as has been observed with mammalian TRPV1 channels (Ohta et al., 2007; Everaerts et al., 2011; Gees et al., 2013). In this case, the partial effect of SB366791 on AITC-elicited activity represents inhibition of the TRPV-mediated component of the response. A third possibility is that there is an interaction between the lN cell TRPV1 and TRPA1 channel. Evidence of naturally occurring TRPV1/TRPA1 heteromeric channels is debatable at this time (although see Fischer et al., 2014), but other TRP channels can form heteromeric channels (Strübing et al., 2003; Cheng et al., 2007). Furthermore, there is evidence in mammals that TRPV1 and TRPA1 channels in sensory neurons interact in the plasma membrane (Salas et al., 2009; Spahn et al., 2014). The TRPA1 activators AITC (at millimolar concentrations) and $H_2O_2$ have both been shown to activate TRPV1 via intracellular signaling that was originally initiated by TRPA1 activation (Everaerts et al., 2011). Furthermore, both HC030031 and SB366791 can block ongoing nociception caused by $H_2O_2$ (Moparthi et al., 2014). The interaction between TRPV1 and TRPA1 viewed in the larger context of pain signaling is of interest to the findings of this study because inflammatory hyperalgesia is thought to be a product of both synergistic TRPV1 and TRPA1 channel activation (Spahn et al., 2014).

The final result of the study characterized the behavioral responsiveness to topical AITC treatment and found that this TRPA1 activator elicited a nocifensive withdrawal behavior that decreased in latency as the concentration was increased. The reliability of the AITC-induced withdrawal at a given concentration and the ability of HC030031 to attenuate these effects is important for the development of the leech as an animal model of nociceptive signaling and provides a basis for the use of AITC as an activator of nociceptive neurons in future behavioral experiments. In addition, our behavioral studies also found that AITC at high concentrations could elicit spontaneous flattening and undulation of the leech's body that closely resembled swimming and was attenuated with HC030031. This effect was not observed following capsaicin application, which reliably elicited crawling instead (Summers et al., 2014). Leeches have distinct neural circuits that mediate swimming as opposed to crawling behavior (Kristan et al., 2005) and it is possible that AITC is somehow selectively activating this 'swim initiation' circuit. The initiation of swim behavior in the leech is quite complex, but one possibility is that swimming is elicited via direct activation of the P cells by AITC. Previous studies have found that both P and lN cell activity can produce swimming behavior, although P cells may be more effective (Brodfuehrer and Friesen, 1986; Debski and Friesen, 1987). At 500 μmol $l^{-1}$ all of the animals produced a swim behavior, but there was a large variance in the duration of swimming elicited. The animals alternated between bursts of swimming and crawling indicating that a circuit level 'switch' is being activated while the animal attempted to regulate itself and crawl away from the noxious stimuli (Esch et al., 2002). Future experiments are needed to explore this circuit and investigate how AITC elicits swimming in a physiological preparation.

The findings of this study have identified, for the first time, pharmacological evidence for a TRPA1-like receptor in the medicinal leech that is sensitive to both AITC and cold, similar to mammals. These TRPA channels were found in the same polymodal nociceptive neurons that have been previously identified as containing a TRPV-like channel. Our findings add to the body of literature indicating a significant interaction between the

TRPA and TRPV channels that could be responsible, in part, for the sensitization of nociceptive signaling pathways found in many chronic pain conditions. The presence of both a TRPV and TRPA1 channel in the leech is of particular importance for development of the leech as an animal model of pain signaling since the interactions we found between the channels closely resemble what has been observed in mammals.

## MATERIALS AND METHODS

### Animal preparation

Leeches (*Hirudo verbana* Carena 1820, 3 g) were obtained from a commercial supplier (Niagara Medicinal Leeches, Cheyenne, WY) and maintained in a vented plastic container (30 cm long, 21 cm wide, 9 cm deep) filled halfway with artificial pond water (0.52 g $l^{-1}$ $H_2O$ Instant Ocean, replaced every 2 days) on a 12 h:12 h light:dark cycle at 18°C. Animals were used within approximately a month of being received from the supplier and were not fed because feeding can elicit significant changes in behavioral responsiveness (Kristan et al., 2005). Prior to dissection, animals were placed in an ice-lined dissecting tray filled with ice-cold leech saline (in mmol $l^{-1}$: 114 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 NaOH and 10 HEPES, pH 7.4) and dissections were started when the animal ceased spontaneous movement. Dissections were carried out in ice-cold leech saline solution. For electrophysiological experiments using isolated ganglia, individual ganglia were dissected and placed within a recording chamber (≈2 ml volume). All pharmacological treatments were applied by rapid replacement of normal saline with treatment saline using a two-syringe manual fluid exchange system. For body wall experiments, (Fig. 1B), ganglia found posterior to the reproductive segments (segments 5–6) were dissected with lateral segmental nerves still connected to a portion of the body wall. All pharmacological treatments and thermal stimuli [leech saline cooled to 15°C using a heating/cooling perfusion pre-stage (ALA Scientific Instruments Inc., Westbury, NY)] were restricted to the peripheral body wall portion of the preparation using a Sylgard™ enclosure that was placed around the body wall and sealed to the bottom of the recording chamber with petroleum jelly (Fig. 1B). Drugs or heated saline were applied to the external surface of the body wall. For all pharmacological experiments, drugs were dissolved in leech saline from frozen stock solutions. Final concentrations were made from stock solutions just prior to the individual experiments. Control experiments were conducted using ascending concentrations of 0.0001%, 0.001%, 0.0025%, 0.005%, 0.01% or 0.02% dimethyl sulfoxide (DMSO). The following drugs were obtained from Sigma-Aldrich (St Louis, MO): capsaicin, 95% AITC, CNQX, DMSO, NMM and CIN (>95%). SB 366791 and HC 030031 were purchased from Tocris (Ellisville, MO).

### Electrophysiology

Current clamp (bridge-balanced) intracellular recordings were made using sharp glass microelectrodes (35–40 MΩ) fabricated from borosilicate capillary tubing (1.0 mm OD, 0.75 mm ID; FHC, Bowdoinham, ME) using a horizontal puller (Sutter Instruments P-97; Novato, CA). Each microelectrode was filled with 3 mol $l^{-1}$ $K^+$ acetate. Impalement of individual neurons was carried out using a manual micropositioner (Model 1480; Siskiyou Inc., Grants Pass, OR). Signals were recorded using a bridge amplifier (BA-1S; NPI, Tamm, Germany) and then digitally converted (Digidata 1322A A/D converter) for observation and analysis (Axoscope; Molecular Devices, Sunnyvale, CA).

Touch (T), lateral nociceptive (lN), medial nociceptive (mN), lateral pressure (lP) and medial pressure (mP) cells were identified based on their size, position within the ganglion and action potential shape (Muller et al., 1981). In these experiments, the ganglion was pinned ventral side up in the recording chamber. For AITC experiments, activity in these cells was recorded for 20 s in normal leech saline followed by 20 s in treatment agonist (Fig. 1A). For NMM and CIN experiments, 30 s pre- and post-treatment intervals were used. The agonist-elicited activity was determined by subtracting the amount of activity (number of action potentials) during the initial normal saline period from the activity during the agonist treatment period. Experiments using SB366791 or HC030031 involved pretreating the preparation immediately prior to recording (1 ml; 10 µmol $l^{-1}$ SB366791 or HC030031), followed by co-application of antagonist and agonist during the recorded treatment period.

### Behavior experiments

Intact animals (each weighing approximately 3 g) were placed in a plastic Petri dish (14.5 cm diameter, 165 $cm^2$ area) lined with filter paper saturated with pond water (0.5 g $l^{-1}$ Instant Ocean). This chamber was of sufficient size to permit the leeches, which are approximately 4 cm in length, ample room to locomote. All animals were acclimated to the arena for 20 min prior to the start of the experiments. AITC (1 ml) was applied to the posterior sucker. Experiments requiring an antagonist were pre-treated 5 s prior to the start of the experiment in addition to co-application of the antagonist with AITC. Each animal was only exposed to a single concentration of AITC to avoid the effects of desensitization. Behavioral observations were recorded using a digitized video camera (Sony Handycam HDR-CX580) and analyzed using Noldus Ethovision software. The behavior that was analyzed included the latency to withdraw the posterior sucker and the duration of swimming-like behavior. The withdrawal latency was measured as the period between the start of the AITC application (which could be observed in the video recordings) and the time at which the animal initiated a withdrawal from the noxious stimuli. Animals that failed to initiate a withdrawal within 15 s of the AITC application were scored as non-responsive.

### Statistics

Data are presented as means±s.e. Statistical analyses using a one-way analysis of variance (ANOVA) were performed to determine main effects with Newman–Keuls *post hoc* tests to confirm the ANOVA results. In the case of non-parametric statistical analysis, a Kruskal–Wallis one-way ANOVA of ranks was performed followed by a *post hoc* Mann–Whitney *U*-test for comparisons of pairs of treatment groups. All significance was determined at an alpha level of at least $P<0.05$.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

T.S. and B.D.B. designed the experiments and developed the experimental techniques utilized in this study. T.S., Y.W., B.H. and B.D.B. carried out the experiments and performed the subsequent data analysis. T.S. and B.D.B. wrote the manuscript.

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