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Cellular permeation of large molecules mediated by TRPM8 channels

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Abstract

While most membrane channels are only capable of passing small ions, certain non-selective cation channels have been recently shown to have the capacity to permeate large cations. The mechanisms underlying large molecule permeation are unclear, but this property has been exploited pharmacologically to target molecules, such nerve conduction blockers, to specific subsets of pain-sensing neurons (nociceptors) expressing the heat-gated transient receptor potential (TRP) channel TRPV1. However, it is not clear if the principal mediator of cold stimuli TRPM8 is capable of mediating the permeation large molecules across cell membranes, suggesting that TRPM8-positive nerves cannot be similarly targeted. Here we show that both heterologous cells and native sensory neurons expressing TRPM8 channels allow the permeation of the large fluorescent cation Po-Pro3. Po-Pro3 influx is blocked by TRPM8-specific antagonism and when channel activity is desensitized. The effects of the potent agonist WS-12 are TRPM8-specific and dye uptake mediated by TRPM8 channels is similar to that observed with TRPV1. Lastly, we find that as with TRPV1, activation of TRPM8 channels can be used as a means to target intracellular uptake of cell-impermeable sodium channel blockers. In a neuronal cell line expressing TRPM8 channels, voltage-gated sodium currents are blocked in the presence of the cell-impermeable, charged lidocaine derivative QX-314 and WS-12. These results show that the ability of somatosensory TRP channels to promote the permeation of large cations also includes TRPM8,

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Authors' contributions

Daniel McCoy, Ph.D., designed research and performed the experiments, analyzed the data and wrote the manuscript; Radhika Palkar, performed the experiments and analyzed the data; Yuening Yang, performed the experiments and analyzed the data; Serra Ongun, performed experiments; David McKemy, Ph.D., P.I. in this study, designed research, wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that there are no financial or non-financial competing interests associated with this study.

thereby suggesting that novel approaches to alter cold pain can also be employed via conduction block in TRPM8-positive sensory neurons.

Introduction

The extensively used local anesthetic lidocaine promotes pain relief via inhibition of nerve conduction by blocking voltage-gated sodium (Na^+) channels at an intracellular binding site comprised of pore regions of the channel in subunits I, III, and IV [32]. The cytosolic location of this inhibitory domain means that in order for lidocaine to be effective it must cross the plasma membrane, which lidocaine does as it largely exists in an uncharged, hydrophobic form in physiological settings. However, being that lidocaine is readily membrane permeable, it gains intracellular access to not only pain fibers (nociceptors) but also autonomic and motor neurons, making it an indiscriminant blocker of all neural activity. This ubiquitous nerve block has been an obvious negative side effect in clinical use of lidocaine as an anesthetic.

Non-selective cation channels, such as the transient receptor potential (TRP) channels TRPV1 and TRPA1, and purinergic P2X receptors, were first shown to permeate large cations in a time-dependent manner with robust stimulation of channel activity [3, 7, 12, 13, 21, 31, 43, 47]. This effect was initially believed to be due to an apparent change in these channels' pore properties such that the size of the ion permeation pathway increased, altering ion selectivity to allow large molecules to permeate. These intriguing results, along with similar analyses of P2X and TRPA1 channels, altered the predominant view that ion selectivity of the channel pore was an inflexible property [5].

However, recent studies have provided compelling evidence that this effect is not due to actual changes in the size of the pore [25, 37]. For example, inward currents were recorded in TRPV1-expressing cells stimulated with capsaicin when the cell-impermeable, cationic lidocaine derivative QX-314 (N-ethyl-lidocaine) was the only cation in external recording solutions [37]. This current had similar temporal properties as those recorded for small ions and, moreover, it's rapid time course suggested that the TRPV1 pore did not need to undergo dilation in order to allow this large molecule (265 daltons (da)) to permeate. Moreover, Li et al. recently reported for P2X receptors that the time-dependent shift in equilibrium potential that is considered an indicator of pore dilation was in fact due to changes in ion concentration inside the cell, not an apparent alteration of the ion permeation pathway [25]. Nonetheless, these studies show that these channels have large pores and the capacity to permeate large molecules across an otherwise impermeable cell membrane [5].

This emergent property of these channels was elegantly capitalized by Binshtok et al. when they demonstrated that silencing of heat nociceptors could be achieved by selectively targeting QX-314 to TRPV1-expressing neurons [7]. As it is a cation, QX-314 is essentially membrane impermeant and thus needs a transmembrane pathway to gain access to the intracellular binding site on Na^+ channels. In these studies, it was shown that stimulation with the agonist capsaicin caused TRPV1 channels to allow QX-314 to enter the cell and block nerve firing *in vitro* [7]. Furthermore, when QX-314 was co-administered with capsaicin *in vivo*, acute mechanical and thermal pain were attenuated with no effects on

motor and tactile responses [7]. Thus, this process serves as a cell-specific mechanism for targeted cell access of membrane impermeant molecules that have potential therapeutic relevance.

The irritant receptor TRPA1 has also been shown to allow permeation of large molecules [12, 43], yet it is unclear if other TRP channels can similarly permeate large molecules. For instance, TRPM8, a cold- and menthol-sensitive channel that is the principal mediator of cold stimuli in mammals [4, 24, 30] was initially reported to not promote the permeation of large molecules, such as the large cationic dye Yo-Pro-1 (357 da) or the smaller charge carrier $NMDG^+$ (195 da) upon stimulation with 100 μM menthol, when expressed in heterologous cells [12]. Similarly, Nakagawa et al., TRPM8 activation did not lead to permeation of QX-314 when concluded that large molecules did not permeate TRPM8 channels when they observed no change in heat pain responses in vivo, or uptake of fluorescently labeled QX-314 after hind paw of QX-314 with menthol [34]. In contrast, Banke et al. reported that the super cooling agent icilin, a highly potent and effective TRPM8 that also activates TRPA1 channels in vitro [30, 42, 50], promoted Yo-Pro-1 uptake in heterologous cells expressing canine TRPM8 channels [2]. The underlying reason for these contradictory results is unclear [17] and, moreover, it is unclear if TRPM8 channels can be used as means for cell-specific nerve block via entry of QX-314.

Here, we have re-examined TRPM8-mediated uptake of large cations, finding that, in our hands, the large cationic dye Po-Pro3 (351 da), a red-shifted variant of Yo-Pro-1, is able to permeate in both heterologous cells and sensory neurons expressing TRPM8 channels. Permeation can be blocked with a TRPM8-specific antagonist and under conditions which inhibit channel function. More relevant to the main utility of large molecule permeation, using a neuronal cell-line endogenously expressing voltage-gated Na^+ channels, we find that co-application of TRPM8 agonists with QX-314 blocks Na^+ currents when these cells are expressing TRPM8 channels. Thus, our results show that TRPM8 channels do enable the permeation of large cations, and that they can be potentially employed for cell-specific targeting of molecules, such as QX-314 and targeted nerve block in vivo, as has been elegantly done via TRPV1 and TRPA1 channels.

Materials and Methods

Heterologous cell culture

Mammalian expression vectors containing cDNA clones of rTRPM8, rTRPA1, and rTRPV1 (a gift from David Julius) were transfected into the human embryonic kidney cell line 293 (HEK293t) or the neuronal cell-line Neuro2A using TransIT-LT1 reagent (Mirus) following manufacturer's instructions. Cells were split on to round matrigel-coated (BD) coverglass 24 hrs after transfection and used for recordings within 48 hrs of splitting. Cells were maintained in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin inside a 37°C, 5% CO2 humidified incubator.

Dye uptake experiments

All experiments were carried out in standard calcium imaging buffer lacking calcium (CIB^-) unless otherwise noted. CIB^- contains: 136 mM NaCl, 5.4 mM KCl, 1 mM $MgCL_2$, 10 mM HEPES, 10 mM glucose and 0.33 mM NaH_2PO_4 adjusted to pH 7.4. CIB buffer is the same as CIB^- buffer only it contains 2 mM $CaCl_2$. After acclimating HEK293t cells transiently transfected with rTRPM8/V1/A1 or sensory neurons in CIB^- at room temperature for approximately 20 mins, cells were first perfused with CIB^- containing 1 μM PO-PRO3 (Life Technologies) for 2 mins to identify dead cells. Subsequent incubations were then carried out in solutions containing the various experimental compounds tested and 1 μM PO-PRO3. All drugs were dissolved in 100% ethanol to make stock solutions and diluted into CIB^- buffer at greater than 1–1000 dilutions. Thus, the vehicle for all experiments was CIB^- with less than 0.1% ethanol.

Cell images of Po-Pro3 fluorescence (Ex: 540 nm, Em: 570 nm) were acquired on an Olympus IX70 fluorescent microscope with Sutter Lambda LS light source, Roper CoolSnap ES camera, and the MetaImaging Software suite, and measured every 15 sec. The data was plotted as normalized fluorescence (F/F_0) with F_0 set as the initial fluorescence at the time of agonist addition for all experiments.

Electrophysiology

Voltage clamp recordings in Neuro2A cells were performed as described [14, 22]. Standard bath solution for whole-cell recordings contained (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 glucose, and pH 7.4 (adjusted with NaOH). Patch pipettes (2–3 MΩ) were pulled from borosilicate glass capillaries (Sutter Instruments, CA) and filled with internal solution for whole-cell recordings containing (in mM) 140 CsCl, 2 EGTA, 2 MgATP, 10 HEPES, pH 7.4 (adjusted with CsOH). Recordings were performed using an Axopatch 200B amplifier (Molecular Devices, Inc., Sunnyvale, CA) and Digidata 1320 data acquisition board (Molecular Devices, Inc., Sunnydale, CA) with pCLAMP 10 software (Molecular Devices, Inc., Sunnyvale, CA). Solutions were exchanged by gravity fed tubes connected to an 8-channel perfusion valve solution controller (Warner Instruments, Hamden, CT) and fed into a recording chamber (Warner Instruments, Hamden, CT). All drugs used in our experiments were stored and handled following the manufacturer's instructions.

Neuron culture and imaging

All experiments were approved by the USC Institutional Animal Care and Use Committee (protocol# 20158) and performed in accordance with the recommendations of the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Newborn transgenic mice (<P14) engineered to express GFP in $TRPM8^+$ sensory neurons (see Takashima et al., 2007 for details [45]) were deeply anaesthetized under isoflurane and then euthanized by cervical dislocation. Trigeminal ganglia were dissected then dissociated with 0.25% collagenase Type 1 (Roche) in a solution of 50% DMEM and 50% F-12 at 37°C for 30 mins. The ganglia were then pelleted and resuspended in 0.05% trypsin and incubated at 37°C for 2 mins. After washing in DMEM/F12 and pelleting, cells were triturated gently with a fire-polished Pasteur pipette in culture medium (DMEM/F-12 with 10% FBS and penicillin-streptomycin). Cells were then spun through a percol gradient consisting of 60%

percol overlaid by 30% percol in culture media. Enriched sensory neurons were then collected from the interphase between the 30% and 60% layers, washed, pelleted and resuspended in culture medium supplemented with nerve growth factor 7S (Invitrogen) (100 ng/ml) and plated onto coverslips coated with Matrigel (BD) (20 μl/ml). Cultures were then used 12–24 hrs after plating for dye uptake experiments.

To identify live functioning neurons in these cultures, cells were tested via calcium imaging as described [14]. Neurons were preloaded for 1 hr at room temperature with 10 μM Fura-2-AM, a cell permeable fluorescent dye that is differentially excited by 340 nm and 380 nm light based on whether or not it is bound to calcium. Positive Po-Pro3 dye uptake was defined as fluorescence exceeding a threshold of 3 standard deviations above baseline measurements carried out on non-responding neurons over a 10-minute window. The rate of dye uptake in functionally identified sensory neurons was determined over an 8-minute period post agonist application using a linear fit calculated with Microcal Origin software. After Po-Pro3 dye uptake experiments had concluded cells were perfused with CIB for 10 mins followed by a brief 15 s pulse of CIB containing 50 mM KCl to depolarize all electrically active cells. Subsequent changes in intracellular calcium were measured via ratiometric imaging and used to identify live functioning neurons for quantification purposes.

Results

Influx of large cationic molecules mediated by TRPM8 channels

To determine if agonist activation of TRPM8 channels leads to cellular uptake of large molecules we transiently transfected HEK293t cells with rat TRPM8 (rTRPM8) and measured agonist-evoked uptake of the cell-impermeable cationic Po-Pro3 (ThermoFisher, mw: 351.5 da). In nominally calcium-free (Ca^{2+}) solutions [12, 13], little to no uptake of Po-Pro3 (1 μM) was observed when cells were exposed for over 12 min to dye alone with vehicle (0.1% EtOH, see Fig. 1c, 2a). Similarly, no uptake was observed when empty-vector (pcDNA3) transfected cells were exposed to TRPM8 agonists at all concentrations tested herein. We then applied increasing concentrations of menthol, and similar to Chen et al. [12], observed little uptake when cells were exposed to menthol concentrations of 100 μM and below (Fig. 1a,c). However, when cells were stimulated with a saturating menthol concentration of 1 mM [30] we observed robust dye uptake in TRPM8 expressing cells (Fig. 1b,c), data demonstrating that, like TRPV1 and TRPA1, TRPM8 channels do allow uptake of larger cations.

Menthol is a low-potency TRPM8 agonist (EC50: 80 μM) and is poorly selective for TRPM8 channels at high concentrations [27, 30]. Thus, to further explore TRPM8-mediated cellular uptake of large molecules we employed the TRPM8-specific agonist WS-12 which, unlike menthol, has sub-micromolar channel potency (EC50: 193 nM) and has been found to be highly selective for TRPM8 [8, 27]. As above, no uptake was detected in the presence of 1 μM Po-Pro3 alone (Fig. 2a,c), whereas we observed elevated levels of intracellular Po-Pro3 fluorescence as the concentration of WS-12 was increased with uptake saturating at approximately 2 μM WS-12 (Fig. 2b,c). These data are consistent with the higher potency of WS-12 in comparison to menthol and, along with the noted selectively of WS-12 for

TRPM8, we used this agonist for our following characterization of TRPM8-mediated permeation of large cations.

WS-12-mediated Po-Pro3 uptake is TRPM8-specific

Next, to determine if uptake was dependent upon TRPM8 channel activity, we first determined if the TRPM8-specific antagonist PBMC could prevent dye uptake [22]. As above, 2 μM WS-12 induced robust dye uptake, but in the presence of 25 nM PBMC, application of WS-12 did not lead to an increase in Po-Pro3 fluorescence (Fig. 3a). We also tested whether or not dye uptake initiated by WS-12 could be arrested by subsequent addition of antagonist. 2 μM WS-12 induced a robust increase in fluorescence that continued after the co-application with vehicle, but was halted and reached a steady state after addition of 25 nM PBMC (Fig. 3a). Thus, WS-12 mediated dye uptake requires TRPM8 channel activity.

We and others have shown that TRPM8 channels desensitize rapidly due to Ca^{2+} influx and the subsequent cleavage of the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) by Ca^{2+}-sensitive phospholipases [14, 40]. In addition, divalent cations such as Ca^{2+} and Ba^{2+} serve as partial pore blockers of TRPM8 channels [14, 19]. Moreover, uptake of similar cationic dyes via TRPV1 channels has been shown to be inhibited in the presence of Ca^{2+}, presumably due to the known Ca^{2+}-dependent desensitization of this channel [10, 13]. Therefore, we determined if TRPM8-mediated uptake of Po-Pro3 was also Ca^{2+}-sensitive, finding that WS-12-induce dye uptake was reduced in the presence of physiological Ca^{2+} concentrations (2 mM) compared to the robust fluorescence observed in standard Ca^{2+}-free Po-Pro3 recording solutions (Fig. 3b) [13]. Thus, these results suggest that factors regulating large molecule permeation via TRPM8 channels are consistent those that effects TRPV1 [1, 2, 31, 33].

Prior studies have shown that TRPA1, another sensory neuron-specific channel that permeates large cations and implicated in cold transduction [23, 42], can be activated by menthol in a species-specific manner [11, 20]. As WS-12 is a menthol derivative [49], we next tested this agonist on cells expressing TRPA1 to determine its specificity. First, to confirm that TRPA1 channels can permeate large molecules, we exposed rTRPA1-transfected cells to Po-Pro3 as described above, first observing no uptake when these cells were exposed to vehicle. When the TRPA1 agonist allyl-isothiocyanate (AITC; 300 μM) was applied we observed robust dye uptake (Fig. 4a, d), but found that even at super saturating concentrations of 10 μM, WS-12 did not induce dye uptake (Fig. 4b, d). The subsequent addition of AITC following WS-12 did induce uptake in these cells (Fig. 4c, d), confirming functional TRPA1 responses and that TRPA1 channels can permeate large cations.

Lastly, we compared Po-Pro3 dye uptake in HEK293T cells transiently transfected with TRPV1, TRPA1, or TRPM8 to determine how relative influx differed between these three channels when stimulated with their specific agonists. As shown in Fig. 5, all three channels were able to mediate the passage of a large cation such as Po-Pro3 with TRPV1 and TRPA1 channels showing qualitatively more robust uptake than TRPM8. Nonetheless, taken together these data support TRPM8-specific dye uptake resulting from administration of the potent agonist WS-12.

WS-12 stimulates Po-Pro3 dye uptake in sensory neurons expressing TRPM8

With TRPM8-specific dye uptake seen in heterologous cells using WS-12 and menthol, we next turned our focus on whether these same results could be replicated in sensory neurons expressing TRPM8. Trigeminal ganglia from the $Trpm8^{GFP}$ mice, a well characterized mouse line in which the reporter green fluorescent protein (GFP) is expressed via the *trpm8* transcriptional promoter [45], were used to analyze Po-Pro3 uptake via TRPM8 channels. In these animals, GFP serves a faithful marker of $TRPM8^{+}$ neurons (Fig. 6a), and ganglia were dissected from neonatal $Trpm8^{GFP}$ mice with primary cultures of individual neurons prepared as described [45]. Next, we perfused 1 μM Po-Pro3, observing extensive background fluorescent labeling after 2 min of exposure (Fig. 6b), presumably labeling dead or dying cells in this acute culture. Importantly, viable neurons, including those labeled with GFP (arrowheads), identified via depolarization-induced (50 mM KCl) Ca^{2+} responses (see [45]), were dye-negative. When these cells were perfused with 2 μM WS-12 we found an increase in Po-Pro3 fluorescence in 87.4 ± 5.0% of GFP^{+} neurons (Fig. 6c,d) (threshold at 3 standard deviations above baseline; n=363 cells). Moreover, we only observed dye uptake in 1.9% of GFP^{-} cells, results consistent with electrophysiological and Ca^{2+}-microfluorimetric studies [14, 45]. Therefore, as with heterologously expressed channels, WS-12 is able to promote the uptake of large molecules via TRPM8 channels expressed in native cells.

Of note, our analysis of neuronal uptake of Po-Pro3 observed two distinct populations of $TRPM8^{+}$ neurons with comparatively different rates of dye uptake. One population was distinguished by a fast rate of dye uptake (0.43±0.1 (F/F_0)/min, r^2=0.99, n=5 exp.) with a second cohort of neurons with a comparably slow rate (0.15±0.002 (F/F_0)/min, r^2=0.99, n=8 exp; Fig. 6e). Prior functional assays of TRPM8 neurons have identified two distinct subpopulations that are differentiated by their sensitivity to capsaicin [28, 30, 38, 46, 51], with the capsaicin-insensitive cohort characterized as having higher TRPM8 current density in comparison to capsaicin-sensitive cells [28, 51]. Consistent with these results, we found that a cohort of the GFP^{+}-slow cells showed an increase in dye uptake when capsaicin (10 μM) was applied ~8 min after stimulation with WS-12 (Fig. 6f). Capsaicin sensitivity was never observed in GFP^{+}-fast cells (n>35), results suggesting that the detected difference in Po-Pro3 uptake is due to differential TRPM8 channel expression [6, 29].

Na^{+}-channel block by TRPM8-mediated uptake of QX-314

The primary utility of large molecule uptake via channel activation is the ability to specifically target molecules, such as QX-314, to cells involved in nociception [39]. With our Po-Pro3 results indicating that TRPM8 channels can also promote uptake of large molecules when stimulated, we asked whether or not Na^{+} currents could be blocked by TRPM8-mediated uptake of QX-314. To this end we used the mouse neuroblastoma cell line Neuro2A (N2A), which express voltage-gated Na_v channels, but not TRPM8, allowing control of TRPM8 expression via transient transfections. To determine if WS-12 and QX-314 had any effects on these Na^{+} currents in the absence of TRPM8 expression we performed a single voltage step to 10 mV (from a holding potential of −80 mV), observing large and rapidly adapting Na^{+} currents that were also present after 5 min bath application of 5 mM QX-314 with 2 μM WS-12 (Fig. 7a). Thus, WS-12 does not lead to QX-314 influx in the absence of TRPM8 channels. Next, we performed similar experiments in N2A cells

transiently transfected with rTRPM8, observing a decrease in the peak Na^+ current over time (peak currents reduced by 87.6 ± 4.6% after >120 sec exposure to WS-12 and QX-314, n=5) suggesting slow build-up of QX-314 into these cells (Fig. 7b), similar to that observed for TRPV1 channels [7]. Thus, these data show that TRPM8 channels can be used to target other large molecules such as QX-314, and employed in a strategy to target molecules to sensory neurons that express TRPM8.

Discussion

Here we present evidence that TRPM8 channels can allow large cationic molecules to pass across cell membranes in response to agonist stimulation, and that the cell-impermeant Na_V channel blocker QX-314 can block voltage-gated Na^+ channels in cells expressing TRPM8. Specifically, we have shown that the cationic dye Po-Pro3 is taken up by heterologous cells transfected with TRPM8 when stimulated by WS-12 and menthol. It is unclear why prior studies did not observe large cationic dyes permeating TRPM8, but this may be due to methodological differences [12, 17, 34]. For example, Chen et al. used a high throughput FLIPR based assay in which aggregate fluorescence of a lawn of cells was summed for an overall measurement of dye uptake, whereas we measured dye uptake in individual cells, thus lowering signal to noise [12]. Therefore, these assays may have been less sensitive due to the unknown transfection efficiency of the cells being measured, as well as had higher background due to variations in cell confluency and its inclusion of non-specific dye uptake in dead or dying cells. However, Banke et al. used a similar assay and did find dye uptake in the presence of TRPM8 channels using the more potent icilin as an agonist [2], suggesting that dye uptake via TRPM8 requires robust channel stimulation.

Consistent with these findings, by robustly stimulating TRPM8 with the super-potent agonist WS-12 (EC50 193 nM) and controlling for sensitivity and background issues by measuring individual cells, we were able to definitively show dye uptake in TRPM8-transfected heterologous cells. Furthermore, we showed that the TRPM8-specific antagonist PBMC could be used to both prevent and block the progression of WS-12-mediated dye uptake. Lastly, WS-12 also allowed Po-Pro3 to permeate sensory neurons expressing TRPM8 channels, establishing that like TRPV1 and TRPA1, TRPM8 does allow the permeation of large molecules [3]. What now remains to be determined is the biophysical basis of this process and if it occurs via a mechanism similar to TRPV1, TRPA1, and P2X channels [5].

A second, and more translationally relevant line of evidence for large molecule permeation is our observations that voltage-gated Na^+ currents are abolished in N2A cells expressing TRPM8 and exposed to QX-314 in the presence of WS-12. This fundamental observation is key in that cold-sensitive nerve fibers and nociceptors can now be targeted for nerve condition block similar to what has been performed for heat nociceptors through TRPV1 channels [7]. Again our data does contradict previous findings regarding QX-314. For example, Nakagawa et al. (2013) concluded that QX-314 did not permeate TRPM8 channels as they failed to observe uptake of fluorescently labeled QX-314 in neurons after an intraplantar injection with menthol [34]. It is unclear why they obtained this negative result, but we speculate that the levels of intracellular QX-314 needed to block Na^+ currents in N2A cells is likely to be considerably lower than that needed to observe a fluorescently

labeled form in vivo. Moreover, menthol's low potency should also be considered. While not definitive, our comparison of Po-Pro3 uptake in heterologous cells expressing TRPV1, TRPA1, and TRPM8 does suggest that permeation via TRPM8 channel activation is not as robust.

Furthermore, the in vivo versus in vitro permeation conditions may also have had a significant impact on permeation. This study also measured heat sensitivity after injection of QX-314 and menthol as an in vivo read out of nerve conduction block, observing no effects under their experimental conditions. However, TRPM8 is expressed in only a small subset of presumptive heat sensitive nerve fibers, as determined by co-expression with TRPV1, accounting for a small fraction of the total $TRPV1^+$ neuronal population [26, 45]. Therefore, it is unlikely that targeting QX-314-mediated nerve block to this small subset of TRPV1 neurons ($TRPM8^+$) would alter heat sensitivity, a postulate consistent with the absence of heat-pain deficits in mice lacking TRPM8 neurons [24].

Conclusions

Understanding the cellular basis for cold pain has clinical relevance as cold hypersensitivity occurs in both inflammatory and neuropathic pain conditions such as carpal tunnel syndrome (CTS), diabetic neuropathies, multiple sclerosis, complex regional pain syndrome (CRPS), a side-effect of many chemotherapeutics, and is prevalent after hand fractures or whiplash [9, 15, 16, 18, 35, 41, 44, 48]. Thus, chronic cold pain is as clinically widespread a condition as other pain modalities, yet the mechanisms underlying cold hypersensitivity are not known, nor are there effective treatments targeting cold pain. In rodent injury models, cold sensitivity is heightened and has been attributed to both TRPM8 and TRPA1 channels [24, 36]. Thus, the cellular basis for cold hypersensitivity is hypothesized to reside within a subpopulation of TRPM8-expressing cold nociceptors and/or in TRPA1 expressing nociceptors. While our data does not conclusively demonstrate that these molecules are permeating via the TRPM8 pore, our results do show that TRPM8 activation does lead to the influx of large molecules and, more relevantly, that voltage-gated Na^+ channels can be blocked in cells expressing TRPM8. Thus, each of these afferent populations is now pharmacologically accessible to nerve conduction block such that future studies can examine the utility of targeting cold pain by TRPM8 or TRPA1 mediated cellular uptake of membrane-impermeant analgesics or cell function modifiers.

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Highlights

- Large molecules permeate heterologous cells and sensory neurons expressing TRPM8 ion channels.
- Molecule permeation induced by the TRPM8 agonist WS-12 is channel-dependent.
- The cell-impermeant lidocaine derivative QX-314 blocks voltage-gated Na^+-channels in cells expressing activated TRPM8.
- Along with prior analysis of TRPV1 and TRPA1 channels, these data show that pain and somatosensory TRP channels can permeate large molecules.

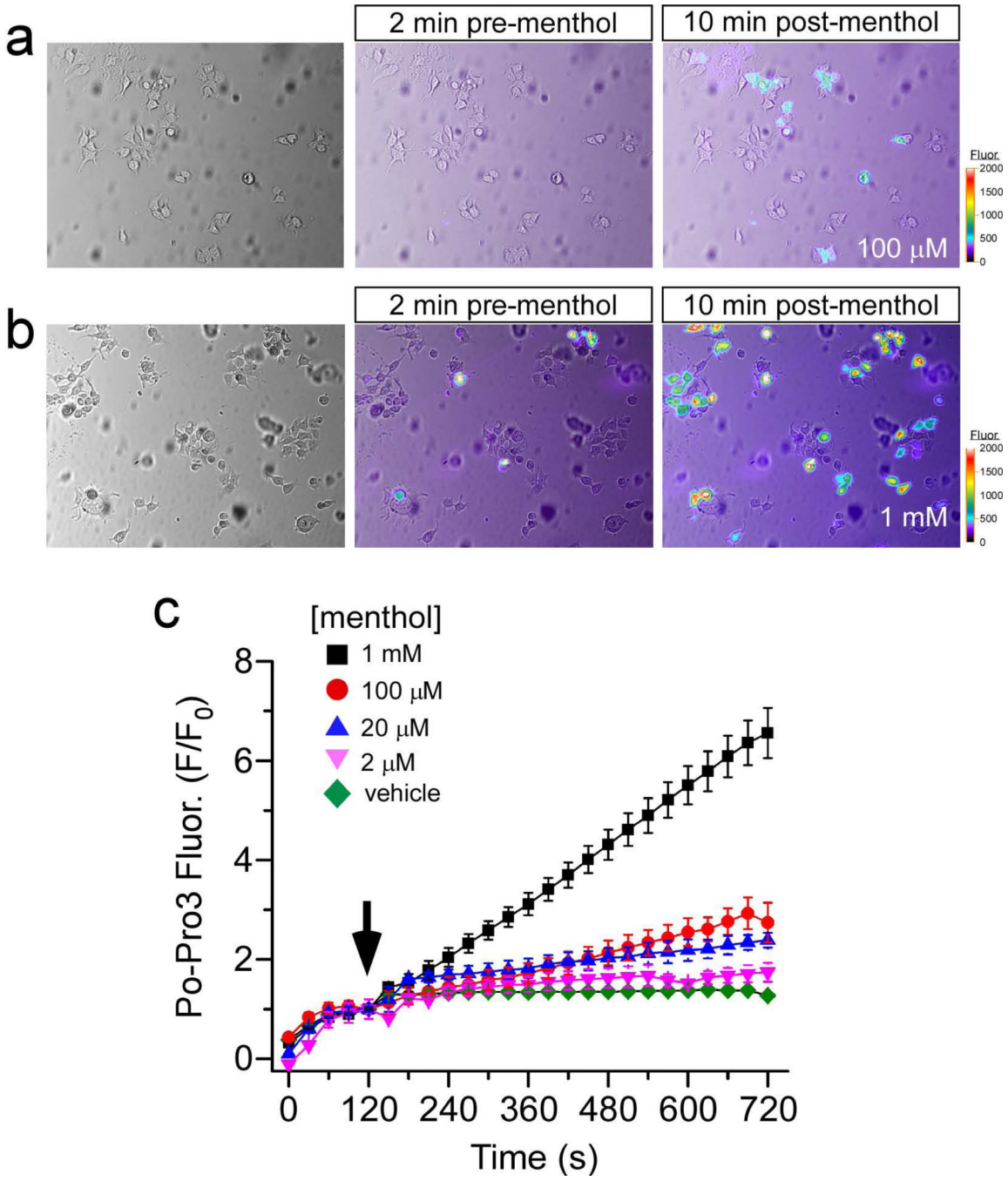

Fig. 1. Menthol stimulates Po-Pro3 dye uptake in TRPM8-transfected HEK cells

a) HEK293t cells transfected with rTRPM8 take up little dye in the presence 100 μM menthol but show robust fluorescence in the presence of 1 mM menthol (b). Cells were first perfused with 1 μM Po-Pro3 for 2 minutes, followed by 10 minutes of 1 μM Po-Pro3 plus agonist. c) Po-Pro3 dye uptake in TRPM8-transfected cells. Transfected cells exposed to vehicle showed no uptake and values (F/F_0) are expressed as fluorescence (F) normalized to initial fluorescence at the time of agonist application (F_0) ± standard error of the mean (SE; n>15 cells, 3 experiments).

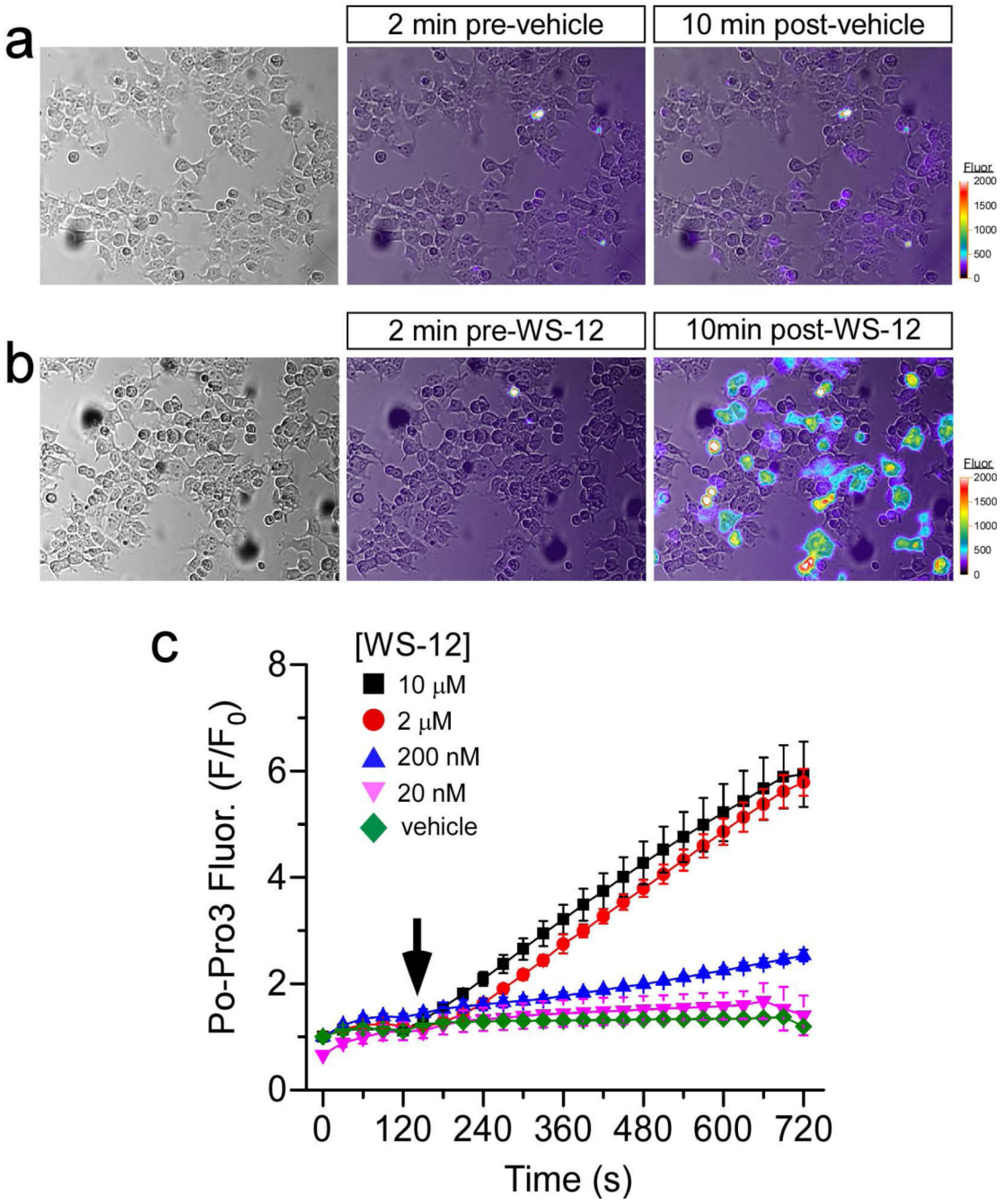

Fig. 2. Strong activation of TRPM8 channels leads to robust uptake of large molecules

a) rTRPM8 transfected HEK293t cells exposed to vehicle showed no uptake of Po-Pro3. b) 2 μM WS-12 induced robust Po-Pro3 uptake in cells transfected with rTRPM8, with dye uptake occurring in a concentration-dependent manner (c). Values are expressed as normalized fluorescence units ± SE (n>15 cells, 3 experiments).

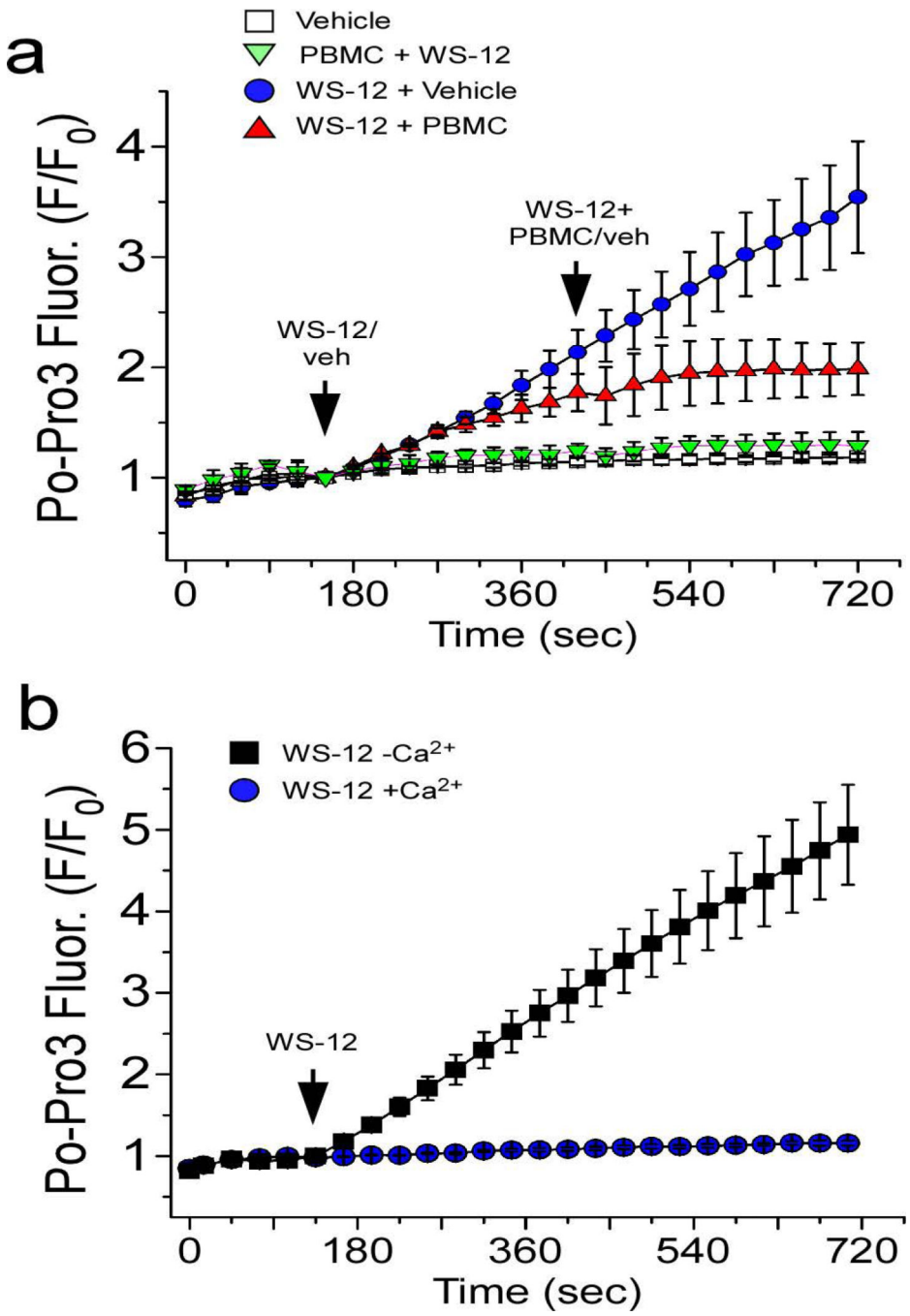

Fig. 3. WS-12 stimulated dye uptake is TRPM8-specific

a) HEK293t cells transfected with rTRPM8 take up Po-Pro3 in the presence of WS-12 (2 μM) but not if simultaneously incubated with the TRPM8-specific antagonist PBMC (25 nM), or if PBMC is added subsequent to WS-12. b) WS-12-mediated dye uptake is reduced in the presence of extracellular Ca^{2+}.

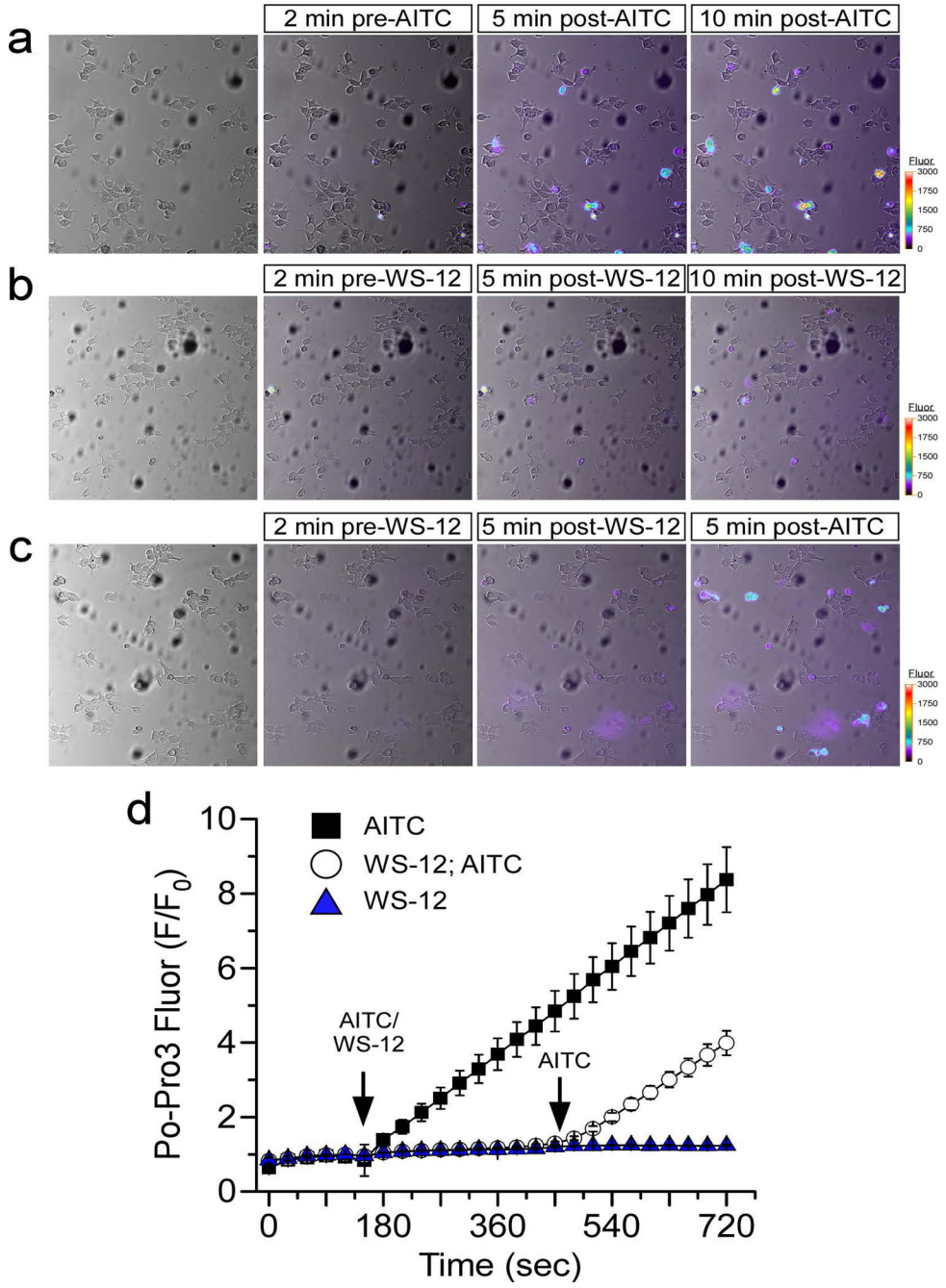

Fig. 4. AITC, but not WS-12, induced TRPA1-specific dye uptake

a) AITC does induce Po-Pro3 dye uptake in rTRPA1 transfected HEK293t cells. b) Saturating concentrations of WS-12 (10 μM) do not cause dye uptake in cells transfected with rTRPA1. c) rTRPA1-transfected cells that did not take up dye when exposed to 10 μM WS-12 (5 min) did show uptake with a subsequent addition of AITC. d) Comparisons of dye uptake under different conditions in a–c. Values are normalized fluorescence units ± SE (n>15 cells, 3 experiments)

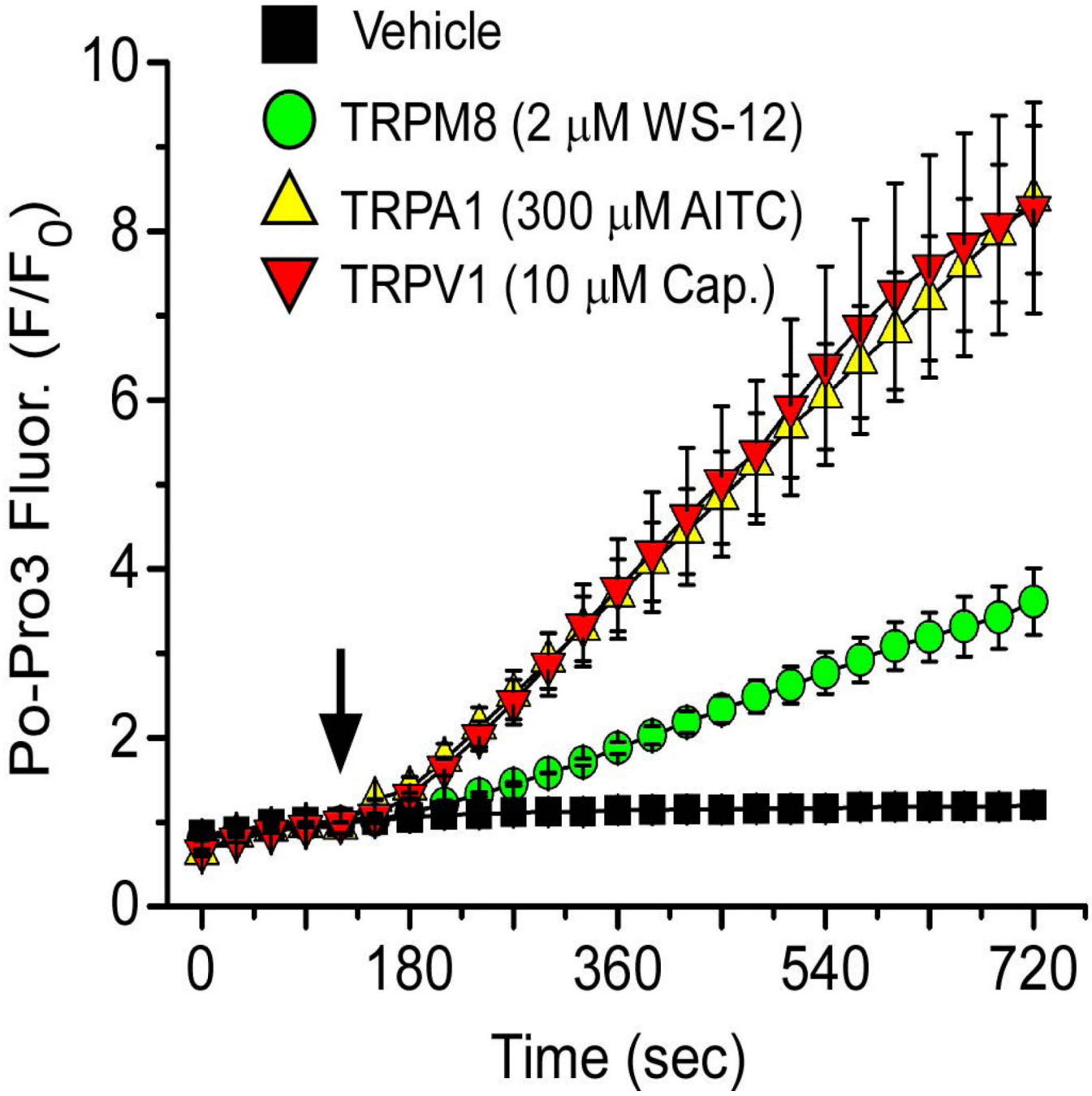

Fig. 5. Po-Pro3 dye uptake in rTRPM8, TRPA1 and TRPV1-transfected HEK cells

HEK293t cells transfected with rTRPM8, rTRPA1 and rTRPV1 can take up dye in the presence of WS-12, AITC and capsaicin (Cap), respectively. Cells were first perfused with 1 μM Po-Pro3 for 2 minutes (120 s), followed by 10 minutes (600 s) of 1 μM Po-Pro3 plus agonist (2 μM WS-12, 300 μM AITC or 10 μM CAP demarcated by arrow). Values are expressed as normalized fluorescence units ± SE (n>15 cells, 3 experiments).

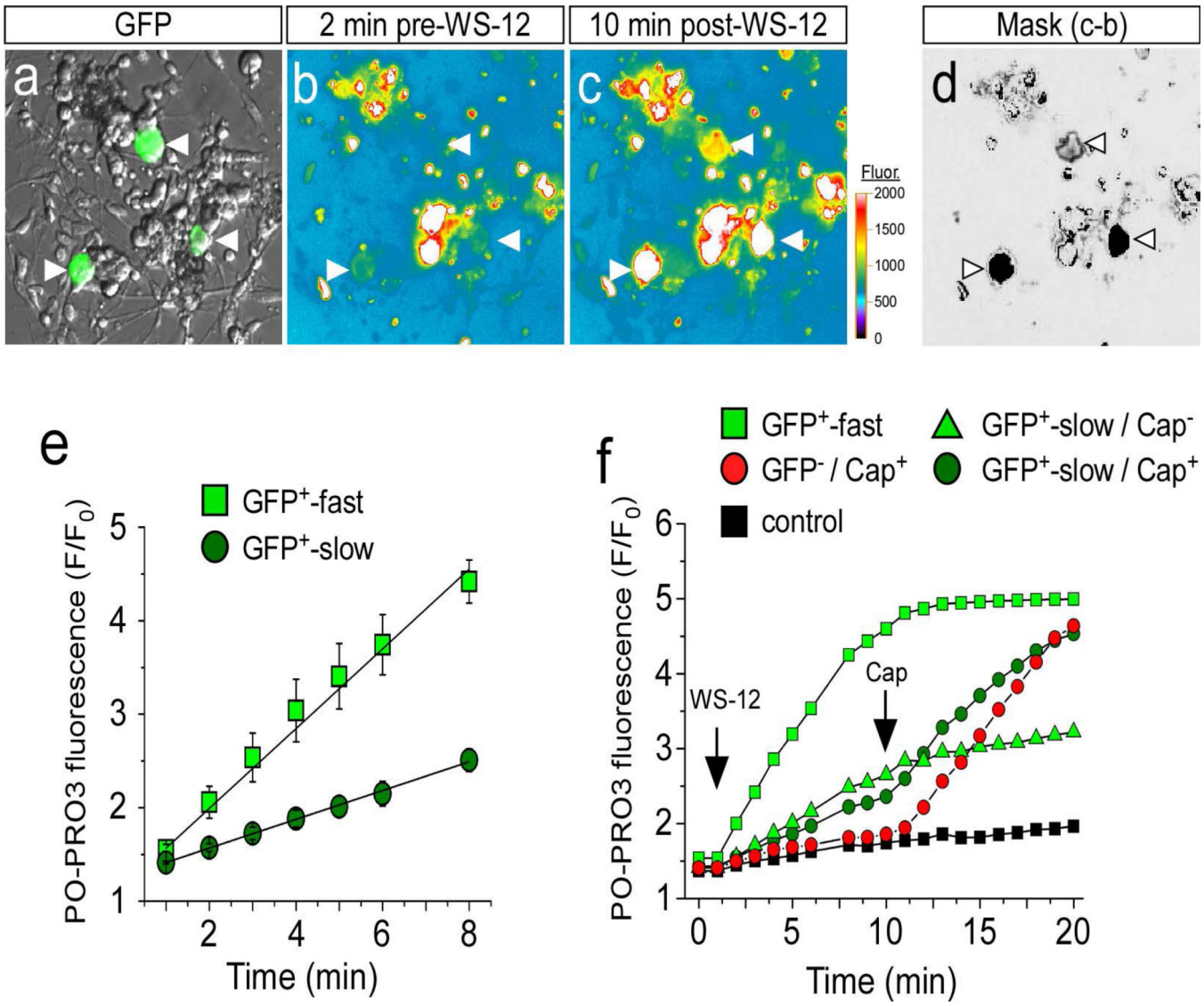

Fig. 6. WS-12-mediated dye uptake in TRPM8-expressing sensory neurons

a) Representative bright field and GFP channel superimposed image of trigeminal sensory neuron cultures from Trpm8^{GFP} mice. Arrowheads mark GFP+ neurons. b) Po-Pro3 fluorescence after 2 minutes alone shows dye incorporation in dead and dying cells. c) Robust fluorescence was observed in GFP+ neurons after a subsequent 10-minute incubation with Po-Pro3 and 2 μM WS-12. d) Mask of images in b and c shows dye uptake in GFP+ (arrowheads) neurons. e) Initial uptake rates in TRPM8+ neurons. f) Capsaicin sensitivity correlates with the slow uptake in GFP$^+$ sensory neurons. Data are mean F/F_0 values from 10–16 cells obtained from 3 different experiments.

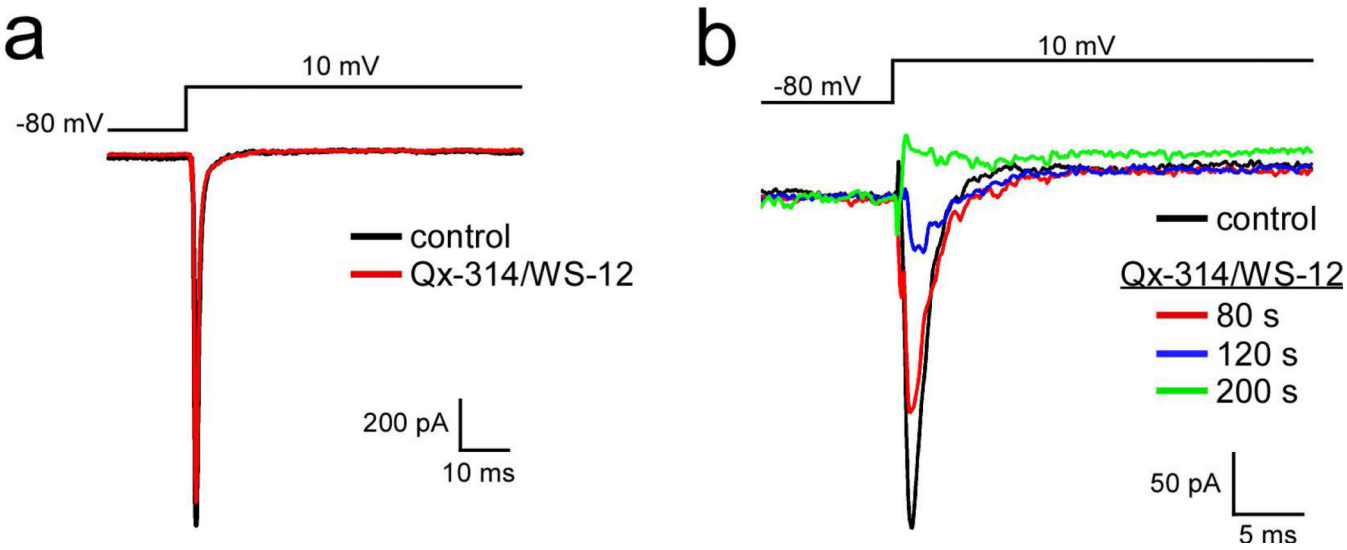

Fig. 7. QX-314 blocks voltage-gated Na^+-currents in cells expressing TRPM8

a) Effect on sodium currents in an N2A cell not expressing TRPM8 before (black line) and after a 5 min exposure to QX-314 (5 mM) and WS-12 (2 μM), compared to b) the progressive block of these currents in a TRPM8-expressing N2A cell (n=5).