CrossMark

Endogenous TRPV4 Expression of a Hybrid Neuronal Cell Line N18D3 and Its Utilization to Find a Novel Synthetic Ligand

Sungjae Yoo[1] · Seung-In Choi[1] · Seul Lee[2] · Jiho Song[2] · Chungmi Yang[1] ·
Sangsu Bang[1] · Seung Up Kim[3,4] · Kyung Hoon Min[2] · Sun Wook Hwang[1] (ID)

Abstract Primary sensory afferent neurons detect environmental and painful stimuli at their peripheral termini. A group of transient receptor potential ion channels (TRPs) are expressed in these neurons and constitute sensor molecules for the stimuli such as thermal, mechanical, and chemical insults. We examined whether a mouse sensory neuronal line, N18D3, shows the sensory TRP expressions and their functionality. In Ca^{2+} imaging and electrophysiology with these cells, putative TRPV4-mediated responses were observed. TRPV4-specific sensory modalities including sensitivity to a specific agonist, hypotonicity, or an elevated temperature were reproduced in N18D3 cells. Electrophysiological and pharmacological profiles conformed to those from native TRPV4 of primarily cultured neurons. The TRPV4 expression in N18D3 was also confirmed by RT-PCR and Western blot analyses. Thus, N18D3 cells may represent TRPV4-expressing sensory neurons. Further, using this cell lines, we discovered a novel synthetic TRPV4-specific agonist, MLV-0901. These results suggest that N18D3 is a reliable cell line for functional and pharmacological TRPV4 assays. The chemical information from the novel agonist will contribute to TRPV4-targeting drug design.

Introduction

Immortalized neuronal cell lines have contributed to rapid explorations of questions in the neuroscience field. Convenience in handling these cell lines may facilitate their popularity. More importantly, these neuronal cell lines mimicking expression profiles or functional properties of their origins would be useful to obtain the physiological and pharmacological parameters that reflect the native cellular and molecular behaviors. One of them is N18D3, a mouse dorsal root ganglion (DRG) neuron and mouse neuroblastoma N18TG2 hybrid cell line (Sanfeliu et al. 1999). During more than a decade, the cell line has been studied in toxicological aspects (Park et al. 2000; Park et al. 2008; Koh et al. 2004; Kim et al. 2005). Although the N18D3 is of sensory neuronal origin, no sensory properties have been examined with this cell.

ND8 and F-11, which are also hybrid cell lines derived from rat DRG neurons fused with the mouse neuroblastoma, were shown to have activities of sensory ion channels, TRPV1 and TRPV2, respectively (Wood et al. 1990; Bender et al. 2005). Sensory TRP channels serve crucial roles in recognizing tactile changes and painful insults. Thus, the utility of these cell lines may be promising for studies on sensory physiology and pain therapeutics targeting the TRP channels. In this context, we asked whether N18D3 exhibits sensory TRP channel-mediated properties. Furthermore, to examine

Sungjae Yoo and Seung-In Choi contributed equally to this work.

✉ Kyung Hoon Min
khmin@cau.ac.kr

✉ Sun Wook Hwang
sunhwang@korea.ac.kr

[1] Department of Biomedical Sciences, Korea University College of Medicine, #722 Moonsook Bldg., Inchon-Ro 73, Seongbuk-Gu, Seoul 02841, South Korea

[2] College of Pharmacy, Chung-Ang University, Heukseok-Ro 84, Dongjak-Gu, Seoul 06974, South Korea

[3] Medical Research Institute, Chung-Ang University School of Medicine, Seoul 06974, South Korea

[4] Division of Neurology, Department of Medicine, UBC Hospital, University of British Columbia, Vancouver, BC, Canada

pharmacological usefulness of N18D3, we tried to take advantage of this line to discover a novel ligand acting on its functional TRP.

TRPV4 is a sensory TRP channel known to play a role in mechanical and chemical nociception (Suzuki et al. 2003; Alessandri-Haber et al. 2005; Alessandri-Haber et al. 2006; Bang et al. 2012). As a result of this study, we showed that TRPV4 is a major TRP component expressed in N18D3 cells. Moreover, MLV-0901, a newly synthesized compound previously untested for its biological effect, is found to activate TRPV4 in our N18D3 experiments.

Materials and Methods

Cell Cultures

The N18D3 cells and HEK293T cells were maintained in DMEM containing 10% FBS and 1% penicillin/streptomycin (Invitrogen) (Park et al. 2008). For experiments, the cells were plated onto poly-L-ornithine-coated glass coverslips. Experiments were performed 24–72 h after plating. DRG neurons of adult ICR mice were cultured as previously (Bang et al. 2010a; Bang et al. 2010b). Cells were then plated onto poly-L-ornithine-coated glass coverslips in DMEM/F12 containing 10% FBS and 1% penicillin/streptomycin. For nerve growth factor (NGF) experiments, media for N18D3 cells or DRG neurons were supplemented with 50 ng/ml 2.5S NGF for 24–48 h after plating. For NGF supplementation, culture media were replaced every 24 h with ones supplemented with fresh NGF. HEK cells plated on coverslips were transiently transfected with 3 μg of individual TRP plasmid DNA per 35 mm dish and 600 ng/well of green fluorescent protein (GFP) complementary DNA (cDNA) in pCDNA3 using Fugene 6 (Roche Diagnostics). After 16–48 h, cells were subjected to drug treatments. Transiently transfected HEK cells were identified by GFP fluorescence. In vitro transfection of N18D3 cells with 5.3 μg/ml small interfering RNA (siRNA) was performed according to the above protocols for the HEK293T cells. mTRPV4-siRNAs were purchased from Bioneer (Daejeon, Korea). Target sequences for siRNA1 (si1), siRNA2 (si2), and siRNA3 (si3) are as follows: si1 5′-GUGAAAUCUACCAGUACUA-3′, si2 5′-GAGUGAAAUCUACCAGUAC-3′, and si3 5′-GACAUGAGGCGACAGGACU-3′. All the experiments were performed at least in triplicate with separate batches of cultures.

Ca²⁺ Imaging and Electrophysiology

Ca²⁺ imaging experiments for intracellular Ca²⁺ measurement were carried out as previously (Kim et al. 2008; Bang et al. 2011a). Briefly, cells were loaded with Fluo-3 AM (5 μM; at 37 °C for 1 h) in the bath solution containing 0.02% pleuronic acid (Invitrogen). The bath solution was composed of (in mM) 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, and 10 HEPES, adjusted to pH 7.4 with NaOH. Ca²⁺ imaging was performed with an LSM5 Pascal confocal microscope (Carl Zeiss, Germany) and time-lapse images (488-nm excitation/514-nm emission) were recorded every 3 s using Carl Zeiss ratio tool software. To test extracellular Ca²⁺-free conditions, the EGTA-including bath solution was used and composed of (in mM) 140 NaCl, 2 MgCl₂, 5 EGTA, and 10 HEPES, adjusted to pH 7.4 with NaOH. The threshold of intracellular Ca²⁺ increase was defined as 20% increase from the basal Ca²⁺ level (Story et al. 2003; Ryu et al. 2010). Whole-cell voltage clamp recordings were performed (Bang et al. 2007a; Bang et al. 2011b). Briefly, the same bath solution for the Ca²⁺ imaging was used for the extracellular solution. The pipette solution consisted of (in mM) 140 CsCl, 5 EGTA, 10 HEPES, 2 MgATP, and 0.2 NaGTP titrated to pH 7.2 with CsOH. For a voltage ramp protocol, hold at −60 mV for 250 ms, then ramp from −80 to +80 mV over 325 ms, and return to −60 mV for 250 ms after the ramp were continuously repeated with no intersweep interval. For the TRPV4-hypotonicity test, 100 mM NaCl was used instead of 140 mM to adjust the osmolality of the bath solution to 219 mOsm/kg (Ryu et al. 2010). For the TRPV4-temperature test, the bath temperature was monitored and controlled using a digital temperature controller and its temperature probe (CL-100 and TA-29, respectively; Warner Instruments, Hamden, CT, USA). All the experiments were performed at room temperature. Paired data were analyzed using the two-tailed Student's t test (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$) and shown as means ± SEM. For the comparison of the effects of six TRP channel activators, the responses from six TRP gene-transfected cells, or the time-dependent effects of NGF incubation, one-way analysis of variance (ANOVA) with Bonferroni post hoc test was performed.

Analyses of Messenger RNA and Protein Expression of TRPs

The extraction of total RNA from N18D3 cells was carried out using Power cDNA Synthesis Kit (iNtRON Biotechnology, Korea). Reverse transcriptase PCR (RT-PCR) was performed using a PCR thermal cycler (TaKaRa, Japan). Reverse transcription was performed using the amfiRivert single-step RT-PCR kit (GenDEPOT, Barker, TX, USA) according to the manufacturer's protocol. PCR primers were as follows: mouse TRPV1, 5′-aactccacccccacactgaag-3′ and 5′-tcgcctctgcaggaaatact-3′; mouse TRPV4, 5′-acaacacccgagagaacacc-3′ and 5′-cccaaacttacgccacttgt-3′; and mouse TRPA1, 5′-cacgtgtgccaaagacaaca-3′ and 5′-agggctggctttcttgtgat-3′. The PCR products were electrophoresed on agarose gels and stained with ethidium bromide. To examine the TRPV1, TRPV4, and

TRPA1 protein expressions of N18D3 cells, the cells were lysed in RIPA cell lysis buffer on ice (Elpis biotech, Korea) containing 1 mM phenylmethylsulfonyl fluoride. The cell lysate was centrifuged at 15,000×g for 15 min and was followed by SDS-PAGE for Western blotting (Bio-Rad, Hercules, CA, USA). The transferred membranes were probed with mouse monoclonal anti-TRPV1 antibodies (1:500; Abcam, Cambridge, UK), rabbit polyclonal anti-TRPV4 antibodies (1:500; Alomon Labs, Israel), rabbit polyclonal anti-TRPA1 antibodies (1:500; Abcam), and with rabbit polyclonal anti-β-actin (1:2000; Bethyl, Montgomery, USA), followed by HRP-conjugated goat anti-rabbit antibodies (1:2000; Bethyl) before visualizing the proteins using ECL system (GE Healthcare, Buckinghamshire, UK).

Chemicals

All chemicals were purchased from Sigma-Aldrich unless otherwise described. Cinnamaldehyde was purchased from MP Biomedicals (Solon, OH, USA). MLV-0901 was synthesized in the laboratory. Ten to hundred millimolar stock solutions were made using water, DMSO, or ethanol and were diluted with test solutions before use. Final DMSO or ethanol concentrations were controlled not over 0.5%.

Results

Putative TRPV4 Responses in N18D3 Cell Line

Sensory neurons are known to express sensory TRP ion channels. We first examined whether the N18D3 cells express these TRPs in the plasma membrane using pharmacological experiments. Agonist-induced opening of the channels present in the plasma membrane causes an intracellular Ca^{2+} rise because the sensory TRPs are Ca^{2+}-permeable (Bang et al. 2010b). In the Fluo-3 Ca^{2+} imaging, 4-α-phorbol 12,13-didecanoate (4αPDD), the TRPV4 activator elicited robust increases in cytoplasmic Ca^{2+} level in N18D3 cells (Fig. 1a–c). Because the Ca^{2+} increases were detected in 2-mM extracellular Ca^{2+} condition but not in the extracellular Ca^{2+}-free condition, 4αPDD appears to activate TRPV4 located in the plasma membrane of the cells (Fig. 1a). The 4αPDD may nonspecifically induce Ca^{2+} influx via other unknown molecular components (Alexander et al. 2013). RN1734, the TRPV4-specific antagonist, readily inhibited 4αPDD-evoked Ca^{2+} influx in the 4αPDD-responsive cells, indicating that the Ca^{2+} influx is mediated by TRPV4 activation (Fig. 1a). To ask whether other sensory TRPs are functional, five agonists, some of which are promiscuously act on multiple TRPs but are known to be inert to TRPV4 activity, were applied. As a result, such robust Ca^{2+} response was not detected in the cells (Fig. 1a, c). These data indicate that TRPV4 is dominantly expressed in the plasma membrane of N18D3 among TRPs tested and that it is functionally active. We also checked the number of responders to each sensory TRP agonist. Of the N18D3 cells, 68.2% (n = 88/129 cells) were significantly responsive to 4αPDD, further suggesting that the dominant population of the cell line has functional TRPV4. In a smaller population, putative TRPV3 responses to camphor were also observed by Ca^{2+} imaging (34.7%, n = 25/72 cells).

We further confirmed TRPV4 activity in N18D3 in whole-cell voltage clamp electrophysiology. Pronounced increases in current were generated upon extracellular 4αPDD application (Fig. 1c). The 4αPDD-evoked currents were outwardly rectifying and the reversal potential was near 0 mV, which are typical features of a TRPV4-mediated current (Bang et al. 2010a). TRPV4 is activated by a hypotonic environment and by warm temperatures (Liedtke et al. 2000; Strotmann et al. 2000; Guler et al. 2002; Watanabe et al. 2002). Similar outwardly rectifying electrical responses to these two stimuli were also detected in the N18D3 cells (Fig. 1d, e). The responses observed in the electrophysiology were all inhibited by a sensory TRP blocker, ruthenium red at a concentration of 20 μM (data not shown). Therefore, these data suggest that TRPV4 is functioning normally in terms of its stimulus-dependent activation in N18D3 line.
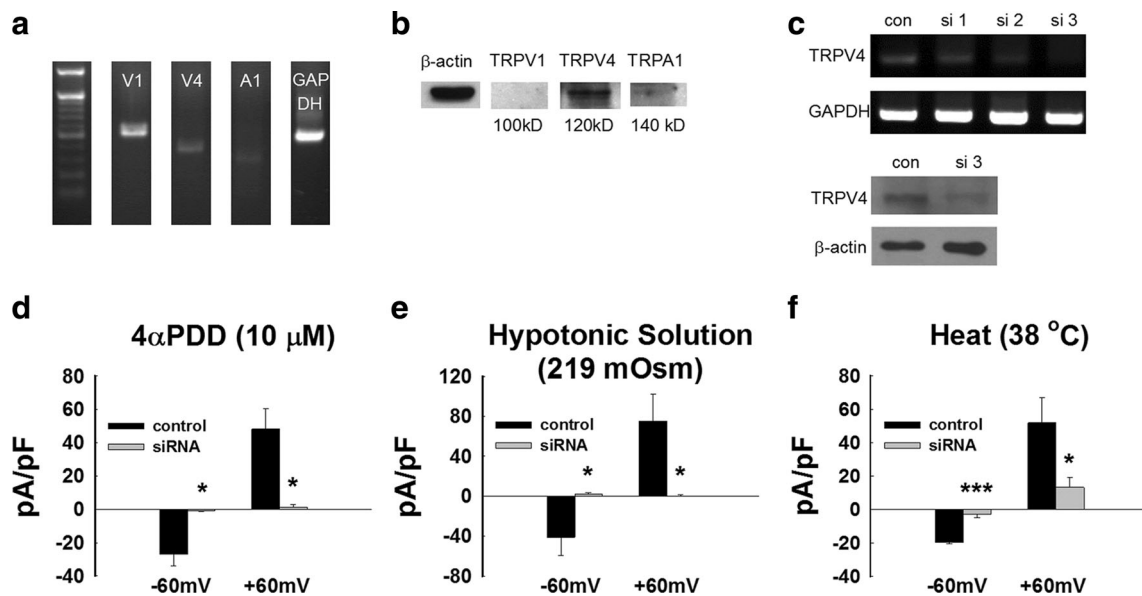
TRPV4 Expression in N18D3 Cell Line

Using RT-PCR and Western blot analysis, TRPV4 expression is determined. Because nociceptive function of the sensory neurons is generally mediated by TRPV1 and TRPA1 (Hwang and Oh 2007; Bang and Hwang 2009), we also checked the expression of these two TRPs in the N18D3 line. Consistent with the above functional analyses, TRPV4 was detected both in the messenger RNA (mRNA) and protein observations (Fig. 2a, b). Interestingly, TRPV1 mRNA was also present and that of TRPA1 was faintly detected. However, only TRPV4 protein was abundantly expressed in the Western blot analysis (Fig. 2b). Relatively high occurrence of TRPV4 protein and the dominant function of TRPV4 observed above in N18D3 cells might be, at least in part, dependent on transcriptional and translational regulations. Unexpectedly, no putative TRPA1 responses (Ca^{2+} influx in response to the TRPA1 agonist, cinnamaldehyde) were detected in the functional study (Fig. 1a, b) despite the presence of TRPA1 protein (Fig. 2b).

To further confirm whether the putative electrophysiological responses to the above three stimulations are TRPV4-specific phenomena, we used siRNA transfection of N18D3 cells. The 48-h transfection with mTRPV4 siRNA caused decreases in both the TRPV4 mRNA and protein levels (Fig. 2c). We then statistically compared the current responses at ±60 mV in the whole-cell voltage clamp with transfected and untransfected N18D3 cells. In the siRNA-transfected N18D3 cells, the
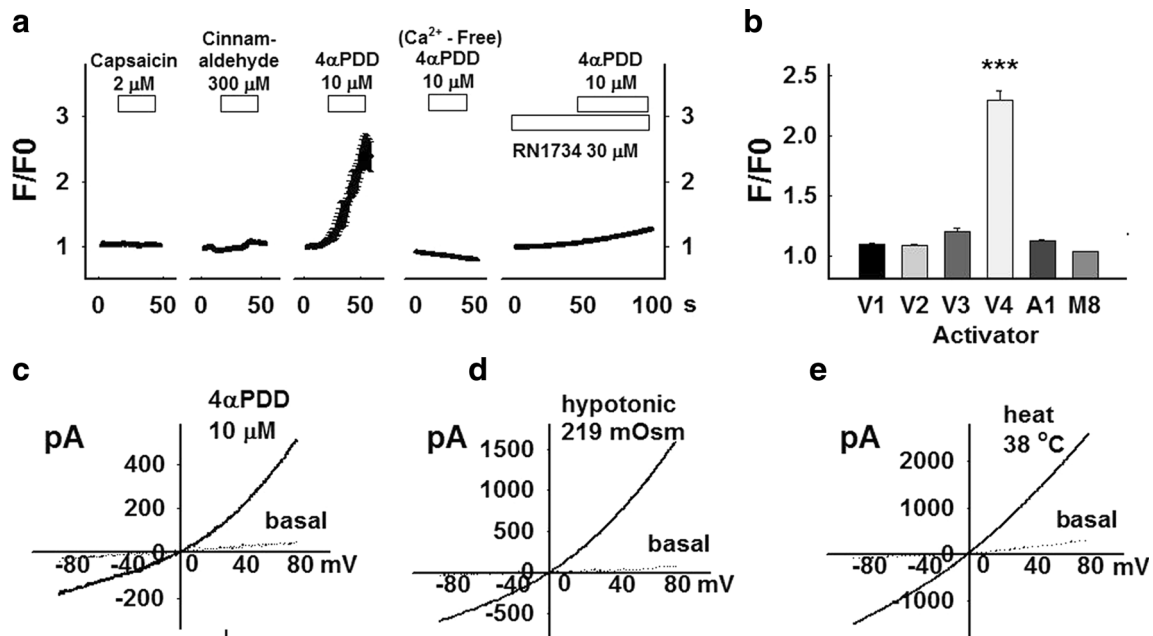
Fig. 1 TRPV4 is functionally active in N18D3 cells. **a** 4αPDD and elevated intracellular Ca^{2+} levels in N18D3 cells ($n = 88$) in Fluo-3 Ca^{2+} imaging experiments. In the extracellular Ca^{2+}-free condition or with the presence of RN1734, 4αPDD failed to elevate the intracellular Ca^{2+} levels ($n = 52$ and $n = 146$, respectively). Capsaicin (TRPV1 agonist) and cinnamaldehyde (TRPA1 agonist) failed to elevate the intracellular Ca^{2+} levels ($n = 61$ and $n = 86$, respectively). **b**, **c** TRPV4 is prominently active in N18D3 cells relative to other sensory TRP channels. For each sensory TRP agonist, the following drugs were used: capsaicin (2 μM) for TRPV1 ($n = 61$), probenecid (100 μM) for TRPV2 ($n = 61$), camphor (4 mM) for TRPV3 ($n = 72$), 4αPDD (10 μM) for TRPV4 ($n = 129$), menthol (300 μM) for TRPM8 ($n = 86$), and cinnamaldehyde (300 μM) for TRPA1 ($n = 86$). **c–e** Whole-cell current-voltage relationships of the current response of N18D3 to 4αPDD ($n = 4$) (**c**), hypotonic buffer ($n = 7$) (**d**), or heat ($n = 6$) (**e**). Abbreviations: *A1, TRPA1; V1, TRPV1; V2, TRPV2; V3, TRPV3; V4, TRPV4; M8, TRPM8*

Fig. 2 TRPV4 expression in N18D3 cell line and its knockdown using siRNA. **a** RT-PCR results of mRNA of TRPV1, TRPV4, and TRPA1 in the N18D3 cells. **b** Western blot results from N18D3 cells. **c** RT-PCR (upper) and western blot (lower) results of TRPV4 mRNA and protein in the N18D3 cells when knocked down with siRNA transfection. siRNAs with three different target sequences were used (si1, si2, and si3), and among the three, si3 transfection showed the greatest knockdown effects. The TRPV4 target sequence for si3 was described in the "Materials and Methods" section. **d**, **e** Summary of N18D3 current responses at ±60 mV in whole-cell voltage clamp experiments using N18D3 cells transfected or untransfected with mTRPV4 siRNA (si3), upon 4αPDD application (**d**), hypotonic buffer perfusion (**e**), or heat stimulation (**f**). *con* untransfected control

putative TRPV4 responses to 4αPDD, hypotonicity, or heat were all robustly attenuated (Fig. 2d–f), which indicates that the three kinds of the responses of N18D3 cells are mediated by TRPV4 activation.

The 4αPDD Pharmacology of N18D3 TRPV4

If the TRPV4 of N18D3 sensory cell lines and that of native sensory neurons are similar in their pharmacological profiles, N18D3 cells would be useful for the identification and pharmacological characterization of TRPV4-acting drugs because the hybrid cells have functional TRPV4 activity. To test this, we composed concentration-response curves of 4αPDD in N18D3 cells and primarily cultured mouse DRG neurons using Fluo-3 Ca^{2+} imaging. As a result, responses of TRPV4 in the N18D3 cells showed lower potencies and efficacy than those of cultured DRG neurons ($EC_{50N18D3}$ = 14.6 μM versus EC_{50DRG} = 5.1 μM; saturated response$_{N18D3}$ = 2.15 versus saturated response$_{DRG}$ = 2.34; Fig. 3a, b). Acute primary culture may represent a mildly injured condition, and NGF, which is not only a growth factor but also an inflammatory mediator, was routinely supplemented in the culture medium for neuronal survival (Story et al. 2003; Zimmermann et al. 2009). Thus, higher TRPV4 expression via inflammation where NGF takes part may make a difference. We tested whether NGF incubation affects the TRPV4 responses of the N18D3 cells. Indeed, both efficacy and potency of the 4αPDD response were elevated to the levels observed in the native DRG neurons by N18D3 culture in the media including NGF for 48 h ($EC_{50N18D3}$ = 5.1 μM; saturated response$_{N18D3}$ = 2.50; Fig. 3b). Thus, changes in the receptor sensitivity to agonist and the number of receptors may cause this elevation. This effect was time-dependent and phosphoinositide 3-kinase (PI3K)-dependent (Stein et al. 2006). NGF incubation for 24 h failed to show a significant increase in Ca^{2+} influx in response to 4αPDD, and co-incubation with PI3K inhibitor wortmannin prevented the 48-h NGF effect (Fig. 3c). Collectively, TRPV4 expressed in N18D3 cells mimics that in the native DRG neurons, which suggest that N18D3 is a useful cell line for TRPV4 pharmacological study.

Identification of a Novel TRPV4 Agonist

Using experiments with the N18D3 line, we found a novel TRPV4 agonist, MLV-0901 (Fig. 4a). At micromolar ranges, MLV-0901 induced Ca^{2+} influx predominantly in the N18D3 cells (85.7% of cells tested; n = 30 cells; Fig. 4b, c). The whole-cell voltage clamp experiments with N18D3 also showed the agonistic activity of this chemical (Fig. 4d, g). Moreover, its current-voltage curve was outwardly rectifying. To confirm whether these responses were mediated by TRPV4 activation, we examined the responses of sensory TRP-transfected HEK293T cells and mTRPV4 siRNA-transfected N18D3 cells. Only TRPV4-expressing HEK cells showed intracellular Ca^{2+} elevation but siRNA-transfected N18D3 cells failed to show electrophysiological responses upon MLV-0901 application, suggesting that this chemical is a specific agonist for TRPV4 (Fig. 4e, g). According to the concentration-response curve from N18D3 Ca^{2+} imaging, MLV-0901 was found to have a similar potency, and a lower efficacy compared to the known TRPV4 agonist, 4αPDD, which indicates that MLV-0901 is a partial agonist (Figs. 3b and 4c). MLV-0901 concentration-response relationships from TRPV4-expressing HEK cells displayed a similar potency, indicating that the N18D3 may be a useful cellular system even when compared to a heterologous expression system transfected with a single receptor, in producing TRPV4 pharmacological profiles. Altogether, the data from the N18D3 cell line demonstrate that N18D3 is a reliable cell line for investigating TRPV4 functions and pharmacology.

Discussion

It has been suggested that immortalized neuronal cell lines may be useful in order to overcome the limitations of experiments with primarily cultured neurons, such as the low survival rate of the primarily collected cells and related needs for multiple animal sacrifices. Such neuronal cell lines, if they successfully reproduce the properties of their origins, would be valuable for a variety of investigations like pharmacological and toxicological screening as well as for basic characterization of cellular functions. Many neuronal cell lines have been developed. However, only a few sensory neuronal cell lines are currently utilized and the focus of the studies using them is mainly differentiation. Assessments of their sensory properties have been rarely reported. Wood et al. developed multiple subclones of a rat DRG cell line fused with N18TG2, and some of these had sensibilities to a TRPV1 agonist capsaicin or to an inflammatory mediator bradykinin (Wood et al. 1990). Capsaicin-evoked responses were observed in an immortalized human DRG cell line HD10.6 and an embryonic rat DRG cell line 50B11 (Raymon et al. 1999; Chen et al. 2007). Currently, F-11 cell lines are actively used for studies on sensory ion channels, receptors, and other biochemical signaling mechanisms (Jahnel et al. 2003; Bender et al. 2005; Rimmerman et al. 2008; Ruan et al. 2008), and TRPV2 seems to be a major sensory molecule in this line (Jahnel et al. 2003; Bender et al. 2005). Here, we demonstrate that another sensory hybrid cell line N18D3 expresses functional TRPV4, and thus is useful for studies on native TRPV4 properties and its pharmacological modulation.

The N18D3 lines were developed in the late 1990s (Sanfeliu et al. 1999). The cell line has been utilized in searching for mechanisms, whereby oxidative stress induces
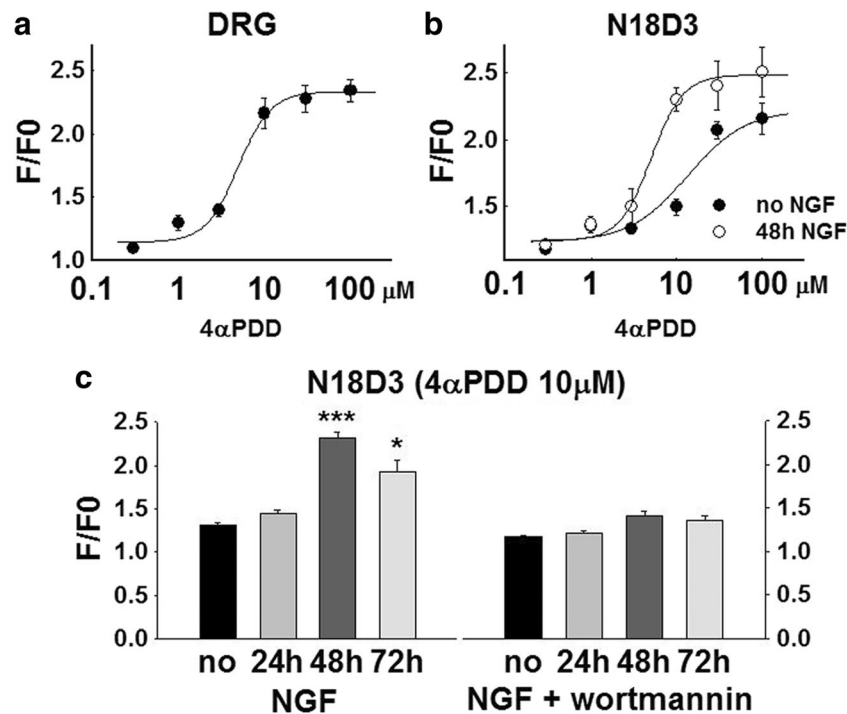
Fig. 3 Concentration-response relationships of intracellular Ca^{2+} elevation in the cultured mouse DRG neurons (**a**) and in N18D3 cells (**b**) in response to 4αPDD activation. The curves were generated from Hill plot. **a** Filled circles represent the mean responses at each concentration ($n = 5$–63 for each points) ($EC_{50} = 5.1$ μM). **b** Filled circles represent the mean responses ($n = 14$–35 for each point) from N18D3 cells cultured for 48 h without 2.5S NGF ($EC_{50} = 14.6$ μM). Open circles represent those ($n = 16$–88 for each points) from cells incubated with 50 ng/ml 2.5S NGF for 48 h ($EC_{50} = 5.1$ μM). **c** N18D3 cultured with 50 ng/ml 2.5S NGF for 48 h showed the greatest intracellular Ca^{2+} elevation (left), which was prevented by co-incubation with 20 nM wortmannin (right)($n = 15$–144)

neuronal damages (Koh et al. 2004; Kim et al. 2005). It has also been studied as to whether these hybrid neurons can be a surrogate system for drug-induced peripheral neuropathy (Sanfeliu et al. 1999; Park et al. 2000; Park et al. 2008). However, no functional assessment of this line has been attempted regarding its sensibility despite it having a sensory neuronal origin. The present study focused on N18D3's sensory features by examining whether a repertoire of sensor molecules, the sensory TRP channels, endogenously operates in these hybrid cells. Our results show that, of sensory TRPs, TRPV4 substantially demonstrated its physiological and pharmacological functions upon TRPV4-specific stimuli (hypotonicity, warm temperatures, and 4αPDD) in N18D3 cells. The EC_{50} for the TRPV4 agonist of the N18D3 TRPV4 was close to that of native one. Therefore, TRPV4 in this line successfully mimic the native sensitivity.

Of six sensory TRPs tested, five are known to be expressed in mouse DRG and trigeminal neurons. TRPV1 is the archetypal one and four other TRPs (TRPV2, TRPV4, TRPM8, and TRPA1) were subsequently discovered (Dhaka et al. 2006; Hwang and Oh 2007). The sensory TRPs are able to sense distinct ranges of ambient temperature and different chemical substances. As well, heterogeneous subpopulations of sensory neurons express individual TRPs. In F-11 cells, it is both functionally and histologically confirmed that TRPV2 is

expressed (Bender et al. 2005). The sensitivity to an extremely noxious temperature and the current-voltage relationship of TRPV2 is readily reproduced in the F-11 lines and might become a valuable tool for future TRPV2 study. On the other hand, TRPV4 is mainly functional in N18D3. The different functional profiles of the two sensory neuronal cell lines suggest that each cell types may represent specific subpopulations. TRPV2 is known to be expressed in large-diameter sensory neurons (Bang et al. 2007b), and F-11 may represent the subpopulation. In the same manner, N18D3 might represent a TRPV4-positive subgroup. Indeed, different from TRPV2, a series of studies demonstrated that a group of small-diameter neurons show TRPV4-mediated responses and that these neurons play a role in mechanical and chemical nociception, suggesting that N18D3 hybrid cells may represent a nociceptive small-diameter subpopulation (Suzuki et al. 2003; Alessandri-Haber et al. 2005; Alessandri-Haber et al. 2006). But, it remains to be explored even in native DRGs, which subtypes among small-diameter neurons exclusively express TRPV4 or which specific neuronal markers are co-expressed. Rare accessibility due to a limited TRPV4-positive population is one of the reasons (Brierley et al. 2008; Ryu et al. 2010). Predominance of TRPV4 positivity in N18D3 cells emphasizes again the usefulness of this cell line in this context.
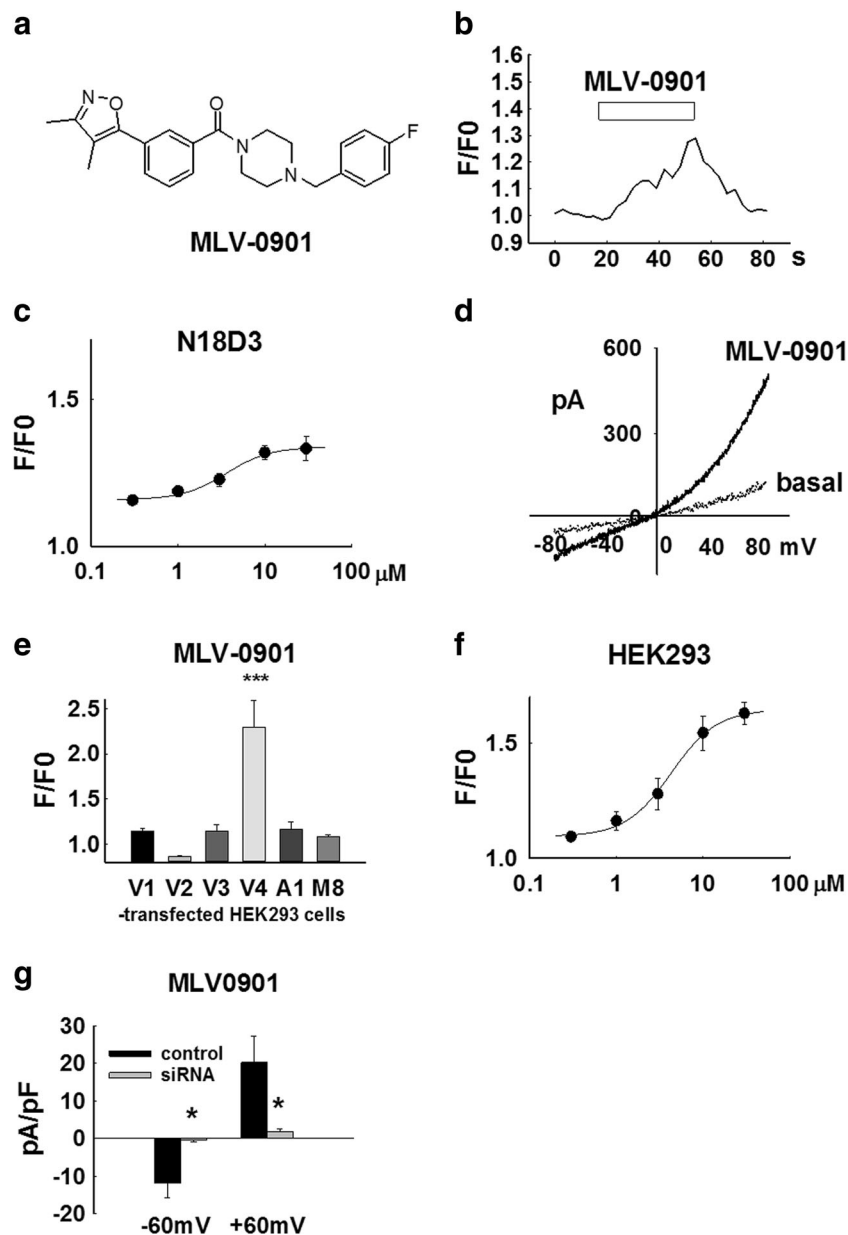
Fig. 4 A novel TRPV4 agonist is identified from experiments using the N18D3 cell line. **a** The chemical structure of the novel TRPV4 agonist, MLV-0901. **b** MLV-0901 (10 μM) elevated intracellular Ca^{2+} levels in N18D3 cells in the Fluo-3 Ca^{2+} imaging ($n = 18$). **c** Whole-cell current-voltage relationship of the current response of N18D3 to 10 μM MLV-0901. **d** Summary of the intracellular Ca^{2+} increases in the HEK cells transfected with TRPV4 or other five sensory TRP channels upon MLV-0901 treatments ($n = 25–88$ for each TRPs). The 10 μM MLV-0901 elicited Ca^{2+} influx only in the TRPV4-expressing HEK cells. The agonist sensitivities of each TRP-transfected cells were confirmed using their specific TRP agonist at concentrations given in Fig. 1b. **e** Concentration-response relationship of intracellular Ca^{2+} elevation in N18D3 cells in response to MLV-0901. Symbols represent the mean values of responses of Ca^{2+} influx ($n = 15–35$ for each points) from N18D3 cultured with 50 ng/ml NGF for 48 h ($EC_{50} = 3.8$ μM). **f** Concentration-response relationship of intracellular Ca^{2+} elevation in mTRPV4-transfected HEK293T cells in response to MLV-0901 ($n = 13–33$ for each points; $EC_{50} = 4.5$ μM). **g** Summary of N18D3 current responses upon 10 μM MLV-0901 at ±60 mV in whole-cell voltage clamp experiments using N18D3 cells transfected or untransfected with mTRPV4 siRNA (si3)

In F-11 cells, TRPV4 were also identified, but is not functionally active for unknown reasons (Bender et al. 2005). Interestingly, we found a similar discrepancy in that N18D3 cells contain TRPV1 mRNA and even express TRPA1 protein, but are without these TRP-mediated responses. Some post-transcriptional or post-translational determinants or subpopulation-specific co-factor molecules may likely be critical for such selective properties, but future studies are needed for their clarification.

Using N18D3, we found a novel synthetic TRPV4 agonist. Pharmacological profiles of this compound obtained from N18D3 and from transfected HEK cells were similar, indicating that the utility of N18D3 for pharmacological identification of novel TRPV4 ligands may be promising. This cell line may have such advantages: different from heterologous cell systems, no additional genetic manipulation including transfection is required; stability and similarity in the expression levels analogous to those of native neurons may avoid possible aberrations in responsiveness and healthiness stemming from ectopic expression, and the cells may conserve authentic sensory neuronal machinery and a cytosolic microenvironment that possibly modulates TRPV4's activity or ligand sensitivity in a natural fashion. MLV-0901 has a chemically distinct structure compared to a well-known agonist, 4αPDD. The aliphatic chain moieties (~10 carbons), which lack in the MLV-0901 structure, seem necessary for the potency of 4αPDD for TRPV4 activation (Vriens et al. 2007). On the other hand, RN-1747, a latest compound reported to activate TRPV4, shares the benzyl piperazine backbone with MLV-0901 (Vincent et al. 2009). Furthermore, these two compounds exhibited similar EC_{50}s (~5 μM). It is possible that these two novel compounds bind to a similar region of the channel protein. Collectively, MLV-0901 and RN-1747 may provide a novel backbone towards reproducibly designing of synthetic TRPV4 ligands, as well as an agonistic standard useful for TRPV4 functional studies.

Taken together, the results from the present study suggest that N18D3 is a novel cell line system that resembles TRPV4-expressing sensory neurons. This cell line may enable better exploration of the roles for TRPV4 in sensory neuronal function and modulation. Further, pharmacological modulators for TRPV4 can be more readily identified by this convenient cell line, possibly contributing to devising an effective control strategy for TRPV4-mediating diseases not limited to pain but including neuropathies and skeletal dysplasias (Nilius and Voets 2013). In this context, MLV-0901, a novel TRPV4 agonist, may help drug design for developing therapeutics by offering useful chemical information.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Alessandri-Haber N, Joseph E, Dina OA, Liedtke W, Levine JD (2005) TRPV4 mediates pain-related behavior induced by mild hypertonic stimuli in the presence of inflammatory mediator. Pain 118(1–2):70–79

Alessandri-Haber N, Dina OA, Joseph EK, Reichling D, Levine JD (2006) A transient receptor potential vanilloid 4-dependent mechanism of hyperalgesia is engaged by concerted action of inflammatory mediators. J Neurosci 26(14):3864–3874

Alexander R, Kerby A, Aubdool AA, Power AR, Grover S, Gentry C, Grant AD (2013) 4α-phorbol 12,13-didecanoate activates cultured mouse dorsal root ganglia neurons independently of TRPV4. Br J Pharmacol 168:761–772

Bang S, Hwang SW (2009) Polymodal ligand sensitivity of TRPA1 and its modes of interactions. J Gen Physiol 133(3):257–262

Bang S, Kim KY, Yoo S, Kim YG, Hwang SW (2007a) Transient receptor potential A1 mediates acetaldehyde-evoked pain sensation. Eur J Neurosci 26(9):2516–2523

Bang S, Kim KY, Yoo S, Lee SH, Hwang SW (2007b) Transient receptor potential V2 expressed in sensory neurons is activated by probenecid. Neurosci Lett 425(2):120–125

Bang S, Yoo S, Yang TJ, Cho H, Kim YG, Hwang (2010a) Resolvin D1 attenuates activation of sensory transient receptor potential channels leading to multiple anti-nociception. Br J Pharmacol 161(3):707–720

Bang S, Yoo S, Yang TJ, Cho H, Hwang SW (2010b) Farnesyl pyrophosphate is a novel pain-producing molecule via specific activation of TRPV3. J Biol Chem 285(25):19,362–19,371

Bang S, Yoo S, Yang T, Cho H, Hwang S (2011a) 17(R)-resolvin D1 specifically inhibits TRPV3 leading to peripheral antinociception. Br J Pharmacol 165(3):683–692

Bang S, Yoo S, Yang TJ, Cho H, Hwang SW (2011b) Isopentenyl pyrophosphate is a novel antinociceptive substance that inhibits TRPV3 and TRPA1 ion channels. Pain 152(5):1156–1164

Bang S, Yoo S, Yang TJ, Cho H, Hwang SW (2012) Nociceptive and pro-inflammatory effects of dimethylallyl pyrophosphate via TRPV4 activation. Br J Pharmacol 166(4):1433–1443

Bender FL, Mederos YSM, Li Y, Ji A, Weihe E, Gudermann T, Schafer MK (2005) The temperature-sensitive ion channel TRPV2 is endogenously expressed and functional in the primary sensory cell line F-11. Cell Physiol Biochem 15(1–4):183–194

Brierley SM, Page AJ, Hughes PA, Adam B, Liebregts T, Cooper NJ, Holtmann G, Liedtke W, Blackshaw LA (2008) Selective role for TRPV4 ion channels in visceral sensory pathways. Gastroenterology 134(7):2059–2069

Chen W, Mi R, Haughey N, Oz M, Hoke A (2007) Immortalization and characterization of a nociceptive dorsal root ganglion sensory neuronal line. J Peripher Nerv Syst 12(2):121–130

Dhaka A, Viswanath V, Patapoutian A (2006) Trp ion channels and temperature sensation. Annu Rev Neurosci 29:135–161

Guler AD, Lee H, Iida T, Shimizu I, Tominaga M, Caterina M (2002) Heat-evoked activation of the ion channel, TRPV4. J Neurosci 22(15):6408–6414

Hwang SW, Oh U (2007) Current concepts of nociception: nociceptive molecular sensors in sensory neurons. Curr Opin Anaesthesiol 20(5):427–434

Jahnel R, Bender O, Munter LM, Dreger M, Gillen C, Hucho F (2003) Dual expression of mouse and rat VRL-1 in the dorsal root ganglion derived cell line F-11 and biochemical analysis of VRL-1 after heterologous expression. Eur J Biochem 270(21):4264–4271

Kim JG, Koh SH, Lee YJ, Lee KY, Kim Y, Kim S, Lee MK, Kim SH (2005) Differential effects of diallyl disulfide on neuronal cells depend on its concentration. Toxicology 211(1–2):86–96

Kim KY, Bang S, Han S, Nguyen YH, Kang TM, Kang KW, Hwang SW (2008) TRP-independent inhibition of the phospholipase C pathway by natural sensory ligands. Biochem Biophys Res Commun 370(2): 295–300

Koh SH, Kim SH, Kwon H, Kim JG, Kim JH, Yang KH, Kim J, Kim SU, HJ Y, Do BR, Kim KS, Jung HK (2004) Phosphatidylinositol-3 kinase/Akt and GSK-3 mediated cytoprotective effect of epigallocatechin gallate on oxidative stress-injured neuronal-differentiated N18D3 cells. Neurotoxicology 25(5):793–802

Liedtke W, Choe Y, Marti-Renom MA, Bell AM, Denis CS, Sali A, Hudspeth AJ, Friedman JM, Heller S (2000) Vanilloid receptor-related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor. Cell 103(3):525–535

Nilius B, Voets T (2013) The puzzle of TRPV4 channelopathies. EMBO Rep 14(2):152–163

Park SA, Choi KS, Bang JH, Huh K, Kim SU (2000) Cisplatin-induced apoptotic cell death in mouse hybrid neurons is blocked by antioxidants through suppression of cisplatin-mediated accumulation of p53 but not of Fas/Fas ligand. J Neurochem 75(3):946–953

Park IH, Kim MK, Kim SU (2008) Ursodeoxycholic acid prevents apoptosis of mouse sensory neurons induced by cisplatin by reducing P53 accumulation. Biochem Biophys Res Commun 377(4):1025–1030

Raymon HK, Thode S, Zhou J, Friedman GC, Pardinas JR, Barrere C, Johnson RM, Sah DW (1999) Immortalized human dorsal root ganglion cells differentiate into neurons with nociceptive properties. J Neurosci 19(13):5420–5428

Rimmerman N, Bradshaw HB, Hughes HV, Chen JS, SS H, McHugh D, Vefring E, Jahnsen JA, Thompson EL, Masuda K, Cravatt BF, Burstein S, Vasko MR, Prieto AL, O'Dell DK, Walker JM (2008) N-palmitoyl glycine, a novel endogenous lipid that acts as a modulator of calcium influx and nitric oxide production in sensory neurons. Mol Pharmacol 74(1):213–224

Ruan B, Pong K, Jow F, Bowlby M, Crozier RA, Liu D, Liang S, Chen Y, Mercado ML, Feng X, Bennett F, von Schack D, McDonald L, Zaleska MM, Wood A, Reinhart PH, Magolda RL, Skotnicki J, Pangalos MN, Koehn FE, Carter GT, Abou-Gharbia M, Graziani EI (2008) Binding of rapamycin analogs to calcium channels and FKBP52 contributes to their neuroprotective activities. Proc Natl Acad Sci U S A 105(1):33–38

Ryu JJ, Yoo S, Kim KY, Park JS, Bang S, Lee SH, Yang TJ, Cho H, Hwang SW (2010) Laser modulation of heat and capsaicin receptor TRPV1 leads to thermal antinociception. J Dent Res 89(12):1455–1460

Sanfeliu C, Wright JM, Kim SU (1999) Neurotoxicity of isoniazid and its metabolites in cultures of mouse dorsal root ganglion neurons and hybrid neuronal cell line. Neurotoxicology 20(6):935–944

Stein AT, Ufret-Vincenty CA, Hua L, Santana LF, Gordon SE (2006) Phosphoinositide 3-kinase binds to TRPV1 and mediates NGF-stimulated TRPV1 trafficking to the plasma membrane. J Gen Physiol 128:509–522

Story GM, Peier AM, Reeve AJ, Eid SR, Mosbacher J, Hricik TR, Earley TJ, Hergarden AC, Andersson DA, Hwang SW, McIntyre P, Jegla T, Bevan S, Patapoutian A (2003) ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. Cell 112(6):819–829

Strotmann R, Harteneck C, Nunnenmacher K, Schultz G, Plant TD (2000) OTRPC4, a nonselective cation channel that confers sensitivity to extracellular osmolarity. Nat Cell Biol 2(10):695–702

Suzuki M, Mizuno A, Kodaira K, Imai M (2003) Impaired pressure sensation in mice lacking TRPV4. J Biol Chem 278(25):22,664–22,668

Vincent F, Acevedo A, Nguyen MT, Dourado M, DeFalco J, Gustafson A, Spiro P, Emerling DE, Kelly MG, Duncton MA (2009) Identification and characterization of novel TRPV4 modulators. Biochem Biophys Res Commun 389(3):490–494

Vriens J, Owsianik G, Janssens A, Voets T, Nilius B (2007) Determinants of 4 alpha-phorbol sensitivity in transmembrane domains 3 and 4 of the cation channel TRPV4. J Biol Chem 282(17):12,796–12,803

Watanabe H, Vriens J, Suh SH, Benham CD, Droogmans G, Nilius B (2002) Heat-evoked activation of TRPV4 channels in a HEK293 cell expression system and in native mouse aorta endothelial cells. J Biol Chem 277(49):47,044–47,051

Wood JN, Bevan SJ, Coote PR, Dunn PM, Harmar A, Hogan P, Latchman DS, Morrison C, Rougon G, Theveniau M et al (1990) Novel cell lines display properties of nociceptive sensory neurons. Proc Biol Sci 241(1302):187–194

Zimmermann K, Hein A, Hager U, Kaczmarek JS, Turnquist BP, Clapham DE, Reeh PW (2009) Phenotyping sensory nerve endings in vitro in the mouse. Nat Protoc 4(2):174–196