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Functional imaging in microfluidic chambers reveal sensory neuron sensitivity is differentially regulated between neuronal regions

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Abstract

Primary afferent sensory neurons are incredibly long cells, often traversing distances of over one metre in humans. Cutaneous sensory stimuli are transduced in the periphery by specialised end-organs or free nerve endings which code the stimulus into electrical action potentials that propagate towards the central nervous system.

Despite significant advances in our knowledge of sensory neuron physiology and ion channel expression, many commonly used techniques fail to accurately model the primary afferent neuron in its entirety. *In vitro* experiments often focus on the cell somata and neglect the fundamental processes of peripheral stimulus transduction and action potential propagation. Despite this, these experiments are commonly used as a model for cellular investigations of the receptive terminals.

We demonstrate that ratiometric calcium imaging performed in compartmentalised sensory neuron cultures can be used to directly and accurately compare the sensitivity and functional protein expression of isolated neuronal regions *in vitro*.

Using microfluidic chambers, we demonstrate that the nerve terminals of cultured DRG neurons can be depolarised to induce action potential propagation, which has both TTX-resistant and TTX-sensitive components. Furthermore, we show that there is a differential regulation of proton sensitivity between the sensory terminals and somata in cultured sensory neurons. We also demonstrate that capsaicin sensitivity is highly dependent on embryonic dissection age.

This approach enables a comprehensive method to study the excitability and regional sensitivity of cultured sensory neurons on a single cell level. Examination of the sensory terminals is crucial to further understand the properties and diversity of DRG sensory neurons.

Keywords: sodium channels, lidocaine, tetrodotoxin, capsaicin, development, TRPV1, ASIC, NGF, GDNF

Introduction

Afferent sensory neurons are the longest neurons in the body and signal stimuli from the periphery to the spinal cord. This process depends on the expression of proteins capable of transducing the stimuli and ion channels that generate action potentials. Traditional *in vitro* preparations of DRG neurons do not allow the processes to be studied in isolation, relying on the somal response for measurement. Microfluidic chambers now allow the fluidic separation of the soma from its peripheral neurites, allowing the peripheral regions to be assessed independently [32,39] and processes such as sensitisation and axotomy to be investigated [42].

Tissue damage and inflammation can increase the peripheral concentration of proton ions [36] exciting nociceptor terminals via activation of ASICs [44] and TRPV1 [7,41] and evoking pain [29]. Furthermore, sustained hyperexcitability in the periphery after nerve injury is a well-established

contributor to the onset of neuropathic pain, which involves rapid maladaptive changes in the injured axon. The accumulation of Nav1.7 and Nav1.8 in painful human neuromas is well documented [25] as is the peripheral upregulation of Nav1.3 after axotomy [6]. There are also additional changes in the trafficking and functional incorporation of TRPV1 [19,44] and P2X3 [10] to the axonal cell membrane after a peripheral insult. The independency from the soma is further confirmed by studies demonstrating that the axon has the ability to translate mRNA locally [18,23], rather than always relying on the transport of proteins synthesised in the soma.

The regional, subcellular variation in neuronal function is clear when one considers the physiology of a sensory neuron, with the soma located within the DRG and the peripheral terminals innervating structures often a considerable distance away. Previous studies have shown that sodium channels are functionally available in neurites at resting potential and contribute to electrogenesis in small-diameter afferent axons [43]. However, axons can rapidly adapt in response to peripheral insults, and may acquire electrophysiological properties distinct from the cell soma. We therefore sought to investigate whether the peripheral processes of cultured DRG neurons are fundamentally different to the cell soma in terms of functional excitability. We demonstrate that the axons are able to transduce algogenic stimuli and generate action potentials with a TTX-s and TTX-r component, providing an *in vitro* model of sensory neuron activation and action potential propagation. We show a remarkable difference in regional proton sensitivity between the soma and axons, with the periphery significantly more responsive to acid stimulation. Furthermore, we have exploited the fluidic isolation between soma and periphery to investigate capsaicin sensitivity. Again we show the periphery is more sensitive, and we can induce an action potential blockade using high concentration capsaicin only in TRPV1 expressing neurons. The functional expression of TRPV1 throughout embryogenesis has been investigated and we demonstrate that even after the onset of TRPV1 expression, a crucial developmental time point exists before which DRG neurons are unable to maintain TRPV1 expression *in vitro*.

Methods

Fabrication of microfluidic chambers using epoxy templates

Compartmentalised chambers were assembled by using a polydimethylsiloxane (PDMS) insert in which a pattern of channels and microgrooves were moulded, bonded to a glass bottom culture dish [32]. Our typical insert displays a pattern of approximately 100 microgrooves, 500 or 800 µm in length, 10 µm in width and 3 µm in height. Each microgroove is separated by a distance of 60 µm. Access to these microgrooves is secured by two channels, 1.25 to 2 cm in length, 1 mm in width and 100 or 150 µm in height (Fig.1). We took an additional step from the technique described in Park et al., 2006 by fabricating an epoxy resin mould from the original silicon master. This step was introduced due to the fragile nature of the silicon master used in the original publication [32] that are very susceptible to cracking; fabricating an epoxy resin mould from the silicon master resulted in a durable template that could be used many hundreds of times. All components of the Taab 812 epoxy resin (Taab) were mixed and the liquid mixture was kept under vacuum for at least 30 minutes to degas. This was poured onto a tin cup and a PDMS microfluidic chamber copied from the SU-8/silicon master was used as a mould. The PDMS device was set to float on the liquid epoxy resin with the relief pattern facing downwards. The epoxy resin was polymerised at 65°C overnight. The PDMS mould was then peeled from the hardened epoxy block.

PDMS replicas

PDMS inserts were replica moulded from the epoxy resin templates. A 10:1 ratio of PDMS prepolymer and curing agent (Sylgard 184, Dow Corning) was thoroughly mixed and degassed. The mixture is poured into the epoxy resin mould to approximately 4 mm thickness and allowed to cure for 45 minutes at 70˚C. Once solidified the PDMS piece was removed from the mould, the reservoir wells cut at either end of the channels using Harris Uni-Bore cutters (5-8 mm in diameter, Electron Microscopy Sciences) and the PDMS insert irreversibly adhered to the glass bottom of a WillCo culture dish (Intracell).

The dish and the PDMS insert (microgroove side up) were treated with two separate plasma treatments, separated by approximately 5 s and each lasting 25 s at <100W power in a plasma cleaner (Femto model, Diener Electronics). After venting the plasma cleaner the PDMS insert is placed microgroove side down in the centre of the glass bottom culture dish, ensuring it adheres evenly. Microfluidic chambers were then incubated at 65˚C for 5 minutes to complete the binding reaction. Sterile water is then added to flood all compartments and chambers are then stored in a sterile environment for up to 3 weeks.

Testing the fluidic isolation

Using a 2 compartment chamber, radioactive PBS was added to a single compartment and the accumulation of radioactivity measured in the adjacent compartment using phosphorimaging. One MBq of ^{125}Iodine (^{125}I, Perkin Elmer Life and Analytical Sciences) was added and the flow across the microgroove array was assessed over 5 days. ^{125}I was serially diluted from stock (3.7 GBq/ml) into PBS. The activity of each solution was measured using a PTW Curiementor 4 isotope calibrator (Freisburg). Accumulation of radioactivity was measured by exposing the chamber for 1 hour on an unmounted 20 x 25 cm phosphor screen (VWR international) at 0, 24, 48, 72 and 96 hours after addition of the solutions. The phosphor screens were subsequently scanned using a Typhoon 9410 Trio+ phosphorimager (GE Healthcare), with the scanning parameters set to 'best resolution'- 25 microns. Three volume conditions were investigated, in which ^{125}I solution was in a greater (200 µl vs 160 µl), equal (160 µl vs 160 µl) or lower (160 µl vs 200 µl) volume than PBS.

DRG neuron culture

Microfluidic chambers were prepared for culture 24 hours in advance by washing the compartments with 70 % ethanol for 30 minutes to reduce microbiological contamination. Ethanol was removed by washing extensively with sterile water. 40 µl of poly-L-lysine (PLL, 0.1 mg/ml in water, Biochrom AG) was added into each compartment allowing the entire channel to be filled. Chambers were stored at 5˚C overnight. The following day, PLL was removed and the chambers were washed with sterile water. Laminin (40 µg/ml in HBSS, Invitrogen) was subsequently added to each compartment. A minimum of 2 hours at 5˚C is required for laminin incubation, after which excess laminin was removed before the cells are plated into the chambers.

Time mated pregnant Sprague Dawley rats or C57BL/6 mice were killed by an ascending concentration of carbon dioxide and the embryos harvested. E15 or E16 embryos were used unless stated otherwise. DRGs from all cervical, thoracic and lumbar segments were dissected in calcium- and magnesium-free Hank's balanced salt solution (HBSS), and incubated in collagenase/dispase solution (10 and 12 mg/ml in HBSS, respectively; Sigma) at 37˚C for 60 minutes. The ganglia were triturated using fire-polished Pasteur pipettes until a single cell suspension was achieved, cells were then loaded onto a Percoll (Sigma) gradient and centrifuged for 8 minutes at 280 *x* g. After washing, cells were resuspended in complete Neurobasal medium (Invitrogen, Paisley, UK), containing 2 % B27 supplement (Invitrogen), 0.5 mM L-glutamine (Invitrogen), 1 % penicillin-streptomycin (Sigma) and 200 ng/ml recombinant human nerve growth factor (NGF, Genentech). Cells were subsequently plated in a volume of approximately 5 µl in one compartment only, using capillary action to draw the cell suspension along the entire compartment length. 10 µl of complete Neurobasal medium was placed in all other compartments. Cells were left to adhere for a minimum of 2 hours before flooding the channels with complete Neurobasal medium.

The microgroove array establishes a very high fluidic resistance between adjacent compartments. To maintain a fluidically isolated microenvironment a small volume difference between compartments is introduced. This creates a hydrostatic pressure difference, resulting in a slow but steady flow from the compartment with the greater volume, counterbalancing diffusion across the microgrooves. Therefore the compartment with the greater volume is fluidically isolated from the adjacent side. In order to encourage axonal growth through the microgrooves we exploit the fluidic resistance to create a uni-directional NGF gradient across the array. This was established and maintained according to Table 1, showing the two-compartment chemo-attraction protocol and Table 2, showing the three compartment chemo-attraction protocol.

The medium was changed on the indicated days by gently removing all fluid from the reservoirs, without removing the medium in the channels. Medium is slowly applied first to the somal compartment followed by adjacent compartments. 5 µM cytosine β-D-arabinofuranoside (Ara-C, Invitrogen) was added to the complete Neurobasal medium applied to the somal compartment between days 1 and 3 to inhibit DNA replication in dividing cells. Two days of Ara-C treatment resulted in a very pure neuronal culture.

Retrograde tracing in the compartmented chamber

In order to successfully identify neurons that projected from the somal compartment to adjacent compartments, 100 µl of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, Invitrogen, 40 µg/ml in complete Neurobasal medium) was applied to the terminal compartment, with 200 µl of complete medium in the somal compartment. Fluidic isolation of the axonal side containing the DiI results from the hydrostatic pressure imbalance corresponding to a 100 µl difference across the microgrooves. The somal compartment is always filled prior to adding the DiI to the axonal compartment.

Axon terminals were incubated with DiI for 1 hour at 37˚C, after which they were washed with extracellular fluid (ECF, 145 mM NaCl, 5 mM KCl, 10 mM HEPES, 10 mM D-Glucose, 2 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4). DiI labelled cell bodies were imaged with a 10 x FLUAR objective on a Zeiss Axiovert 200 inverse microscope and a DCLP 595 dichroic mirror with a BP 645/75 filter.

Immunohistochemistry

DRG cultures from rat litters dissected at E16 were prepared for immunocytochemistry at either 1 or 10 days in vitro. Cultures were washed with PBS, fixed with 1% PFA for 30 minutes and then blocked in 5% normal donkey serum for 2 hours at room temperature. Primary antibodies (Rabbit anti-PGP9.5, Ultraclone, 1:500; Biotin conjugated-IB4, Sigma, 1:100), were incubated at 4°C overnight. Cultures were washed and incubated with secondary antibodies for 2 hours at room temperatures before flooding with Vectashield mounting medium.

Calcium imaging and visualisation of response

For ratiometric calcium imaging, the somal compartment was incubated with complete Neurobasal medium containing 2 µM Fura-2 (Invitrogen) and 80 µM pluronic acid (Invitrogen) for 40 minutes at 37˚C. Compartments were washed with ECF and the culture chamber transferred to the recording stage mounted on a Zeiss Axiovert 200 inverted microscope. A 10 x FLUAR objective, DCLP 410 dichroic mirror and LP440 emitter filter were used for calcium imaging. Pairs of images at excitation wavelengths of 340 nm and 380 nm were captured every two seconds using a Till Photonics Polychrome IV monochromator at 200 - 400 ms exposure time. TILLvisION 4.0 software was used to analyse ratios of fluorescent images to give a time locked, pixel by pixel representation of intracellular calcium changes. Pseudocolour images represent the maximal response to a particular stimulus; these are created by capturing a single frame of emission fluorescence at pre-stimulus levels, and at peak fluorescent in response to a stimulus.

Within TILLvisION the baseline fluorescence is deducted from the peak fluorescence emission, leaving only a signal from cells which have responded. This image is transferred to Photoshop, converted to mono-colour and overlaid on a phase contrast image of the same neuronal field.

A gravity driven application system [12] was inserted into one the medium reservoirs of the compartment to be stimulated, with a suction device located in the connected reservoir so that a steady flow was established through the compartment of interest. Both applicator and suction device could be moved between compartments without changing the neuronal field that was imaged.

At all times during the imaging protocol, the compartments not receiving the stimuli contained a greater volume of ECF than the compartment receiving the stimuli, ensuring stimulant did not flow into adjacent compartments.

Statistics

For each set of results, the number of neurons and experiments are displayed in the text as ($n_{neurons}$= X, $n_{experiments}$= Y). $n_{neurons}$ refers to the total number of cells analysed. $n_{experiments}$ refers to the number of separate embryonic litters used. DRGs from one embryonic litter ($n_{experiments}$ = 1) were pooled and subsequently divided between chambers. All quantitative comparisons are presented as the arithmetic mean ± the standard error of the mean (SEM). Averages from one experiment were used to compute group means and measures of group variance. Statistical comparisons were always made on the basis of experimental number.

Results

Microgrooves create a fluidic barrier between compartments

In these experiments, devices with one or two microgroove arrays were used (Fig. 1A,B). In order to specifically stimulate the somata, axons or terminals of DRG neurons, the neurites were encouraged to grow through the microgroove array into a fluidically-isolated compartment. Fluidic isolation

occurs when there is a hydrostatic pressure imbalance, corresponding to a volume difference across compartments. We first investigated to what extent the microgroove array maintained a fluidic isolation between compartments using a sensitive radioactive method (Fig. 1C). When the radioactive solution was present at a lower volume (160 µl of ^{125}I in compartment A versus 200 µl of PBS in compartment B) no radioactivity could be detected in compartment B after 72 hours confirming that a complete fluidic isolation was maintained up to this point (Fig. 1D). At 96 hours, only 0.6 ± 0.2 % ($n_{experiments}$ = 4) of total radioactivity was detected in compartment B (Fig. 1D). When the volumes of ^{125}I in compartment A and PBS in compartment B were equal, fluidic isolation is maintained up to 24 hours and only 0.5 ± 0.1 % ($n_{experiments}$ = 4) of total activity is detected in compartment B at 48 hours (Fig. 1D). In control experiments, loading volumes were reversed and the radioactive solution was added in a greater volume. Without the hydrostatic pressure imbalance inhibiting its flow, radioactive solution was free to flow across the microgrooves resulting in 1.4 ± 0.2 % of total activity detected in compartment B at 24 hours, 2.1 ± 0.1 % at 48 hours, 3.1 ± 0.1 % at 72 hours and 4.2 ± 0.1 % at 96 hours (all time points, $n_{experiments}$ = 4) (Fig. 1D). These values were highly significant when compared to the reverse condition of ^{125}I in a lower volume ($p<0.001$ for every time point, 2-way ANOVA with Bonferroni post-hoc test). We conclude that fluidic isolation is maintained as long as there is a unidirectional hydrostatic pressure imbalance. Therefore, in all subsequent live cell imaging studies a hydrostatic pressure imbalance of at least 100 µl volume difference was maintained throughout the entire experiment, excluding that the functional response was therefore due to passive diffusion of chemical stimuli into the adjacent compartments.

It takes several days for neurons to grow neurites across the microgroove array and establish an axonal network in the adjacent compartment (Fig. 2). When immunohistochemistry is used to stain neurites and somata using equal amounts of antibody solution in either compartment it becomes apparent that the microgroove pattern is devoid of staining which further illustrates the fluidic isolation as antibodies did not penetrate significantly into the microgrooves when fluid levels were balanced..

Retrograde transport and functional development

To quantify this process we used 2 compartment chambers and applied the non-selective retrograde tracer, DiI in the terminal chamber (Fig. 3A) and quantified the number of neurons with fluorescently labelled somata at 3, 5, 7 and 10 days *in vitro* (DIV) (Fig. 3B, C). At 3 DIV axons are just beginning to emerge from the microgrooves. At this time point 31 ± 7% (n_{cells} = 762, $n_{experiments}$ = 8) were DiI labelled indicating the presence of a projecting neuron in the adjacent compartment (Fig. 3C,F). By 5 DIV the percentage of neurons with a projecting axon reached 55 ± 5% (n_{cells} = 510 , $n_{experiments}$ = 4) (Fig. C,F) and significantly increased to 76 ± 3% (n_{cells} = 898, $n_{experiments}$ = 7) by 7 DIV (Fig. 3C, F). Allowing the cells to remain in culture up to 10 DIV did not result in further accumulation of DiI (72 ± 11 %; n_{cells} = 318, $n_{experiments}$ = 5) (Fig. 3F), suggesting that after a week in culture the large majority of neurons grow neurites into the adjacent compartment.

To assess whether these neurites can transduce a stimulus and propagate an action potential we applied the non-selective depolarising agent KCl to the terminal compartment and studied the activation pattern at the somata using ratiometric calcium imaging with Fura-2 (Fig. 3A). Due to the complete fluidic isolation between compartments (Fig.1), a somal calcium influx in response to stimulation of the terminals cannot be attributed to stimulant diffusion and must rely on action potential propagation from the terminals to the soma. At 3 DIV only 60 ± 11% (n_{cells} = 762, $n_{experiments}$ = 8) of those with a projecting axon were activated by KCl (Fig. 3D-F), suggesting that the axons were capable of active transport, but expression of ion channels was not sufficient to propagate action potentials in a proportion of cells. However, during the 10 days in culture, the proportion of DiI labelled cells that could be activated by KCl applied to the terminals significantly increased compared to 3 DIV, with 82 ± 8% at 5 DIV (n_{cells} = 510, $n_{experiments}$ = 4), 90 ± 2% at 7 DIV (n_{cells} = 898, $n_{experiments}$ = 7) and 95 ± 2% at 10 DIV (n_{cells} = 318, $n_{experiments}$ = 5).

Therefore, whilst some cells which have a projecting axon are unable to transduce or propagate action potentials at early time points, by 7 and 10 DIV the neurites have matured and the vast majority now express the ion channels required for both stimulus transduction and action potential propagation.

Sensory neuron terminals can repeatedly respond

To test the reproducibility of the somal calcium response, we used a protocol with 8 consecutive KCl stimulations delivered to the terminals (Fig. 4) with an inter-stimulus (end to onset) period of 5 minutes. Repeated depolarisation of the sensory terminals (Fig. 4A) resulted in a very reproducible calcium response in the cell soma (Fig. 4B). The baseline between KCl stimulation was stable, with few random fluctuations and no gradual incline which would demonstrate calcium accumulation in the somata. After 4 stimulations 93 ± 2% (n_{cells} = 555, $n_{experiments}$ = 8) of neurons continued to exhibit a robust calcium transient and only 27 ± 4% (n_{cells} = 555, $n_{experiments}$ = 8) of neurons demonstrated tachyphalaxis after 8 stimuli (Fig.4E). The reproducibility of the calcium response meant these cultures were suitable for pharmacological investigations of action potential propagation.

Somal calcium transients induced by terminal stimulation require sodium channels

We next investigated the mechanisms responsible for the somal calcium transients after terminal stimulation. We tested the hypothesis that the sensory terminals transduce the depolarising stimuli and the axons propagate action potentials back to the cell soma, resulting in the measurable calcium influx. For this we used the 3 compartment chamber (Fig. 4A) and applied an excitatory stimulus to the most distal terminal compartment whilst applying drugs to the middle axonal compartment (Fig. 4A). Repeated stimulation of the terminals with KCl evoked reproducible calcium transients in the soma (Fig 4B) with little tachyphylaxis. Action potential propagation relies on voltage gated sodium channels (VGSC) that can be divided into tetrodotoxin (TTX)-sensitive (TTX-s) and TTX-resistant (TTX-r) channels [8].

When 1 µM TTX was applied to the axonal compartment, most neurons were no longer excitable (Fig. 4C) and only 18 ± 5 % (n_{cells} = 237, $n_{experiments}$ = 4, measured at the 6th consecutive stimulation) of neurons continued to discharge after prolonged application of TTX (Fig. 4D, E). This means that the majority of cultured DRG neurons require TTX-s sodium channels for action potential propagation. Between the 3rd and 4th, and 5th and 6th KCl stimulations we allowed the TTX to remain on the axon for prolonged periods of time. This was to ensure that increased exposure time did not result in blockade of all neurons. After this prolonged exposure the population of neurons with TTX-r properties remains constant, demonstrating that these neurons remain functional despite prolonged exposure to TTX at a concentration more than a 100-fold of the IC_{50} (Fig. 4E). Importantly, the TTX-resistant responses were immediately and completely abolished by application of the non-selective sodium channel blocker lidocaine (10 mM) (Fig. 4D, E). These experiments suggest that the calcium influx in the cell body of these neurons upon peripheral stimulation is entirely dependent on action potential propagation through VGSCs. We therefore provide a proof of concept that the pharmacology of action potential propagation in sensory neurons can be pharmacologically investigated *in vitro* using functional calcium imaging in compartmentalised microfluidic chambers.

We performed a TTX dose response using 6 concentrations of TTX ranging from 10 nM to 10 µM, and when analysing only the cells that are blocked by 1 µM TTX (presumably TTX-s neurons), the IC_{50} was determined as 55 nM. On the other hand, when analysing only the cells that remain active at 1 µM, the IC_{50} was found to be 25 µM. This near 500 fold difference in TTX sensitivity between these two populations is likely to reflect the relative ratio of TTX-s to TTX-r sodium channel expression.

Proton sensitivity is significantly different between the soma and sensory terminals

Acid induces a sharp, stinging pain in humans [29], and it has been shown that nociceptors are excited by protons through activation of cation channels [26]. TRPV1 and members of the ASIC family of ion channels have both been implicated in the transduction of acid stimuli [5,7,35]. To functionally study proton sensitivity across neuronal regions we used the 2 and 3 compartment

chambers. In the 2 compartment system, we applied a range of acid solutions in ascending proton concentration first to the terminal compartment then to the somal compartment, whilst imaging the same population of neurons. We quantified the percentage of KCl responsive neurons that were excited by acid when separately applied to the terminals and then to the soma. At every proton concentration tested, the terminals were significantly more sensitive than the soma (Fig. 5A-D). For example, application of pH 6.6 (n_{cells} = 600, $n_{experiments}$ = 9) to the terminals activated 74 ± 6 % of cells, compared to only 26 ± 7 % when the soma was stimulated. Similarly, pH 5.7 (n_{cells} = 781, $n_{experiments}$ = 12) depolarised 92 ± 2 % of cells when applied to the terminals, whereas somal stimulation activated only 49 ± 6 % of neurons (Fig. 5A).

Using the 3 compartment chambers, we then applied an acidic solution buffered to pH 5.1 first to the terminals, then to the axonal chamber which might also contain terminal processes and finally to the soma, whilst recording the calcium responses from the same population of cell somata. Of those cells that showed a response to KCl applied to the terminals (n_{cells} = 687, $n_{experiments}$ = 6), 91 ± 2% responded to protons applied to the terminals. Moreover, 95 ± 2 % of these cells also responded to pH 5.1 stimuli applied to the axon (Fig. 5E,F), indicating an almost complete overlap in responsiveness. This demonstrates that almost all neurons have proton sensitivity in the terminals and axon in this culture system. Intriguingly, in response to direct somal application of pH 5.1 in the same neuronal population, only 49 ± 12 % responded (Fig. 5B-F) revealing a differential regulation of proton sensitivity between neuronal regions. This shows that there can be a significant regional difference in the sensitivity to excitatory stimuli of a sensory neuron and that this regional diversity varies between different stimuli.

To understand the mechanisms governing the transduction of the acid stimuli in our culture system we applied the acid-sensing ion channel (ASIC) blocker amiloride [24] in combination with a pH 5.7 stimulus. Of the neurons that previously responded to a pH 5.7 stimulation in the absence of amiloride, only 21 ± 4 % responded in the presence of amiloride (Fig. 5E, ***p<0.001 paired t-test).

To confirm the reduction in responding neurons was not due to tachyphalaxis, we washed the amiloride out and reapplied pH 5.7 which resulted in activation of 91 ± 4 % of cells (Fig. 5E). Finally, to test the number of amiloride resistant neurons that expressed TRPV1, we carried out additional experiments where a capsaicin stimulus was included in the stimulation protocol. 72 ± 8 % of the amiloride resistant neurons were capsaicin-sensitive, meaning 5 % of the total pH 5.7 responsive population do not rely on TRPV1 or ASICs for proton transduction (Fig. 5E).

Regional variations in the sensitivity of neurons

To address whether the sensitivity differences as detected with proton stimulation extends to other nociceptive stimuli we carried out dose response experiments with capsaicin using 2 compartment microfluidic devices. Ascending concentrations of capsaicin were applied to the terminals whilst recording the somal calcium levels. The same capsaicin stimulation protocol was then separately applied to the same region of cell bodies that were previously recorded. Responding populations are quantified as a percentage of KCl responsive neurons. At all capsaicin concentrations a greater number of cells responded when the terminals are stimulated compared to stimulation of the soma (Fig. 6A). For example, with 0.3 µM capsaicin, 31 ± 8 % of cells were activated when the terminals were stimulated whilst only 18 ± 4 % responded upon somal stimulation. The overall sensitivity difference between the soma and terminals was significantly different ($F_{1, 42} = 4.9$, $p<0.05$, ANOVA).

We next asked whether high concentration capsaicin applied to the axonal compartment can induce a conduction block. Here we used the 3 compartment chamber, where we stimulated the terminals, applied the high capsaicin concentration to the axons, and imaged the cell bodies. Upon application of 100 µM capsaicin to the middle axonal compartment, 96 ± 3% ($n_{cells} = 474$, $n_{experiments} = 6$) of neurons expressing TRPV1 in the terminals responded to axonal stimulation with high concentration capsaicin (Fig. 6B), demonstrating that TRPV1 is functionally expressed along the entire length of the axon. We next tested whether high concentration capsaicin would affect action potential

propagation induced by stimulating the peripheral terminals with KCl. Using this paradigm, only 6 ± 3 % (n_{cells} = 474, $n_{experiments}$ = 6) of cells with capsaicin sensitive terminals were able to propagate action potentials through the high capsaicin concentration filled compartment (Fig. 6B). Conversely, of those neurons that display no response to capsaicin applied to the terminals (Fig. 6C), 94 ± 2 % (n_{cells} = 474, $n_{experiments}$ = 6) continue to propagate action potentials through the capsaicin filled compartment, resulting in a somal calcium influx (Fig. 6D, E). We therefore demonstrate that those neurons that can be functionally excited by capsaicin application to the terminals, also express TRPV1 along the length of the axon. High concentration capsaicin can be used as a specific blocker of action potential propagation in TRPV1-expressing neurons.

Developmental regulation of sensory neuron phenotypes

Sensory neurons acquire their functional properties in the second week of gestations in rodents and continue their maturation well into the postnatal period. This includes the maturation of the sensitivity to the TRP channel ligands capsaicin (TRPV1), menthol (TRPM8) and mustard oil (TRPA1). Moreover, all nociceptors initially express trkA, the high affinity receptor of nerve growth factor, and a pivotal diversification occurs in a subpopulation of nociceptors which down regulate trkA and develop into a lineage of nociceptors which can be identified by binding to the lectin B4 of Griffonia simplificolia. Figure 7 shows that IB4 binding is initially absent in neurons from E16.5 rats, but it is present in a subpopulation of neurons after 10 days in culture (Fig. 7A-G), suggesting the neurons are maturing and diversifying as they would in vivo.

*Capsaicin sensitivity is down-regulated in vitro when cultures are prepared from younger embryos*In order to assess the contribution of embryonic age to capsaicin sensitivity we prepared cultures from E15.5 and E16.5 rat embryos, and tested the functional expression of TRPV1 at the soma and terminals over 10 DIV. Assessment of the somal capsaicin sensitivity in mass cultures on the day of

dissection (day 0) revealed that 75 ± 1 % (n_{cells} = 485, $n_{experiments}$ = 5) of E16.5 DRG neurons were activated by capsaicin, whereas only 35 ± 8 % (n_{cells} = 316, $n_{experiments}$ = 4) of E15.5 rat DRG neurons were activated (Fig. 7H). Interestingly, E15.5 cultures almost entirely lost capsaicin sensitivity over 10 days in culture, with only 6 ± 3 % (n_{cells} = 366, $n_{experiments}$ = 4) activated at 7 DIV, whereas E16.5 cultures remained relatively constant at all time points tested, with 45 ± 5 % (n_{cells} = 563, $n_{experiments}$ = 7) activated at 7 DIV (Fig. 7I). There was an overall highly significant difference between E15.5 and E16.5 cultures ($F_{1, 61}$ = 242.4, p<0.001, ANOVA). Analysis of cells that successfully grew an axon through the microgrooves revealed a similar sensitivity pattern. We excluded any cells that could not be activated by KCl applied to the terminals, meaning we could not test early time points when the neurite has not yet grown through the microgroove array. However, when separately assessing capsaicin sensitivity at the soma (Fig. 7H) and terminals (Fig. 7I), we consistently observed a significantly greater capsaicin sensitivity in E16.5 cultures compared to the E15.5 cultures.

Similar experiments were performed in mouse DRG cultures prepared from E13.5, E14.5 and E15.5 embryos. In addition to capsaicin stimulation, we also stimulated the soma and terminals with the TRPM8 agonist – menthol. Similar to rat cultures, when DRG cultures were prepared from earlier embryonic ages (E13.5 and E14.5), the capsaicin sensitivity at day 0 was initially relatively high when tested at the soma (Fig.7J,K,L). For example, E13.5 cultures have 47 ± 4 % (n_{cells} = 209, $n_{experiments}$ = 3) capsaicin sensitive neurons, whilst in E14.5 cultures it is 55 ± 8 % (n_{cells} = 225, $n_{experiments}$ = 3). However, in cultures prepared from both embryonic ages, capsaicin sensitivity rapidly declined in the soma over 10 DIV (Fig.7J,K). However, capsaicin sensitivity in cultures prepared from E15.5 mouse embryos did not decline over time *in vitro*, and remained consistent at approximately 60 % throughout the 10 DIV (Fig. 7L). Whilst sensitivity of the terminals was generally slightly greater than that of the soma, as previously shown (Fig. 6A), it was always consistently lower in E13.5 and E14.5 cultures compared to E15.5.

Interestingly, we observed an onset of menthol sensitivity across all embryonic ages and in both the soma and terminals, after being in culture for 3 to 7 days, depending on the dissection age , suggesting that unlike TRPV1, the functional expression of TRPM8 is not influenced by the embryonic age at which dissection takes place.

In an effort to understand the mechanisms leading to the downregulation of capsaicin sensitivity in DRG cultures from earlier embryos we prepared E14.5 mouse cultures. We then supplemented the medium of these cultures with additional neurotrophic factors to try and maintain the capsaicin sensitivity that is initially present at day 0. As expected, when only NGF is added, the capsaicin sensitivity declines to 4 ± 1 % at the soma and 20 ± 5 % at the terminals when tested at 7 DIV (Fig. 8A). Supplement of these cultures with NGF, NT3, GDNF and BDNF resulted in no further increase in capsaicin sensitivity (Fig. 8A). However, supplement with NGF, NT3, GDNF, BDNF and retinoic acid (RA) resulted in a significant increase in capsaicin sensitivity at 7 DIV to 37 ± 3 % ($p<0.01$) at the terminals and 24 ± 4 % at the soma ($p<0.01$) (Fig. 8A) (n_{cells} = 411, $n_{experiments}$ = 10). This suggests that RA is a critical factor for increasing the functional expression of TRPV1 *in vitro*, therefore we prepared E14.5 mouse cultures and cultured them only with NGF and RA. At 7 DIV, capsaicin sensitivity had increased from 12 ± 7 % and 6 ± 3 % (NGF alone) to 30 ± 2 % and 16 ± 2 % (NGF and RA) in the terminals and soma, respectively (Fig. 8B). We tested the effect of these different neurotrophin conditions on menthol sensitivity and found that there was no effect of promoting the sensitivity to the TRPM8 agonist (Fig. 8C,D). Therefore, we show that the functional expression of TRPV1 rapidly declines in 2 rodent species when dissected before a critical developmental time point. Supplement of the culture medium with RA can almost completely rescue the downregulation in E14.5 mouse cultures, but has no effect on menthol sensitivity.

Discussion

Here we describe the proof of concept that the combination of compartmentalised cultures and calcium imaging can be used to study the regional sensitivity of sensory neurons. This allows pharmacological examination of action potential propagation *in vitro*, which is not possible in conventional mass cultures. We demonstrate that the axons express membrane proteins involved in both action potential propagation (sodium channels) and stimulus transduction (TRPV1, ASICs) which are distinguishing features of a mature DRG neuron, and have found stark differences in the regional sensitivity to algogenic stimuli. We also show that there are key developmental time point after which the cells are capable of maintaining functional TRPV1 expression .

Stimulation of the neurites recapitulates the activation of a sensory neuron in its natural environment. Furthermore, the ability of the neuron to repetitively respond to this peripheral stimulation suggests the neurons possess electrophysiological characteristics akin to mature neurons. 1 µM TTX application to the peripheral branch of the sciatic nerve reduces the C-fibre compound action potential current by 89% [34]. Whilst not measuring compound action potential, but analysing individual cellular properties, we find 80 % of cells are blocked by 1 µM TTX, demonstrating the similarity to *in vivo* recordings where a large contribution of TTX-s VGSCs are also required for action potential propagation.

Expression of the capsaicin receptor, TRPV1, is often used to distinguish between sensory neuron subpopulation [9]. Capsaicin sensitivity has been demonstrated along the entire extent of sensory neurons [20]. When applied to the axon we observed a rapid calcium influx followed by complete conduction block, which has also been observed in both *in vivo* [33] and *in vitro* [37]. This was not observed in capsaicin insensitive neurons suggesting that TRPV1 activation is crucial to induce the blockade, and that the high concentration of capsaicin does not have off-target effects on TRPV1 negative cells.

We demonstrate that nearly all neurons are able to transduce proton stimuli when applied to the terminals. Similar to the capsaicin stimuli, we also show that the axon is also sensitive to acidic stimuli. A striking difference in the sensitivity to protons and capsaicin across neuronal regions was discovered, suggesting the expression of key transducing molecules in the terminals is separately regulated to the cell soma. Anterograde transport of the TRPV1 receptor in a form capable of ligand binding has been reported [38]. Furthermore, during inflammation the axonal transport of TRPV1 mRNA is increased, suggesting local translation in the axon may contribute to the hypersensitivity of primary afferents [40]. ASIC2 and ASIC3 are also predominantly transported in an anterograde direction [2,14,27]. Our experiments demonstrate that despite being grown in artificial culture conditions, the neuron ensures the peripheral neurites are capable of responding to potentially noxious stimuli, and that this sensitivity is remarkably greater than at the cell soma. TRPV1 has also been shown to be predominantly localised at the neuronal growth cone and filopodial structures, acting as a key regulator for axonal guidance [17]. The presence of TRPV1 in these peripheral structures correlates well with chemo-sensation, and possibly explains the differential capsaicin sensitivity we observe.

Using microfluidic chambers we have shown that neurons replicate many of the developmental milestones which can be identified *in vivo*.

DRG neurogenesis in the rodent occurs in two developmental waves mediated by neurogenins and neurogenin-1 acting between E11 and E13 is thought to predominantly gives rise to TrkA expressing nociceptors [30]. Functional expression of TRPV1 is soon detected after these neurogenesis waves, beginning at E12.5 in a small proportion and increasing dramatically to approximately 64% of all DRG neurons at E14.5 [19]. Other sequential waves of TRP channel expression occur e.g. TRPM8 occur later in embryonic life. The onset of TRPV1 sensitivity at E12.5 relates well with our findings of acutely dissociated neurons from E13.5, E14.5 and E15.5 mouse embryos. However, E13.5 and E14.5 mice cultures loose capsaicin sensitivity over time in culture, whereas E15.5 mice maintain this

sensitivity over 10 DIV. We observe no progressive neuronal death over the culture period, suggesting the functional expression of TRPV1 is downregulated in cultures obtained from younger embryos rather than a progressive neuronal loss. In addition to TRPV1, we observe that TRPM8 expression develops after several days *in vitro* in cultures from E13.5, E14.5 and E15.5, at a time point that correlates closely to E18.5 (Figure 7) when it emerges *in vivo*. It is the same developmental age when there is a transition in growth factor dependency [31] and we also observe develop the emergence of non-peptidergic neurons which stain with isolectin B4 of Griffonia simplificifolia (Figure 7).

Pre- and perinatally, the discharge properties of embryonic sensory neurons also undergoes of maturation. At E11.5 66% of neurons exhibit immature action potentials, but none at E15.5 at E11.5, with none detected at E15.5. The presence of a hump on the falling phase of the action potential, indicative of nociceptive neurons arises at E12.5 [28]. Interestingly, at E16.5 current injection was unable to elicit more than one action potential but with increasing age the percentage increased with the majority of neurons at birth firing trains. [28]. However, at E16 well defined receptive fields that can respond to chemical, heat and mechanical stimulation are already found [15, 16]. As we have not combined our current calcium imaging experiments with electrophysiological recordings, we are unable to discern how many action potentials are evoked by a given stimulus. Using fura calcium imaging of porcine DRG cultures, it was shown that a single electrical pulse in the soma induced a small but significant fluorescent signal in the neurite [22] and this can also be shown for genetically encoded calcium indicators [13] (and see below)

The nociceptive-specific sodium currents Nav1.8 and Nav1.9 first emerge at E15 and E17, respectively, and undergo a period of upregulation during late embryonic and early post-natal ages [4]. The TTX-s Nav1.3 begins to be downregulated at E17 [45]. The electrophysiological properties of cultured neurons are therefore likely to be modified depending on time of tissue harvest, duration of culture and culture conditions.

NGF has a clear role in maintaining capsaicin sensitivity in cultured, adult DRG neurons, as its removal leads to a rapid reduction in capsaicin sensitivity without neuronal death [44]. NGF alone however, is insufficient to maintain capsaicin sensitivity in early embryonic cultures and retinoic acid can significantly increase the functional expression of TRPV1 in E14.5 mouse cultures at 7 DIV. Retinoic acid-induced neuronal differentiation of TRPV1-transfected SHSY5Y neuroblastoma cells resulted in a 6 fold increase in TRPV1 protein [1]. Hundreds of genes are positively upregulated by retinoids, including those encoding $p75^{NTR}$, TrkA, TrkB, TrkC and Ret [3]. Studies in Xenopus oocytes have shown heterologously expressed TrkA and TRPV1 can associate with PLC-γ to form a protein complex which results in a 30-fold increase in proton evoked currents through TRPV1 [11], suggesting retinoid induced overexpression of neurotrophin receptors may interact to functionally increase capsaicin induced responses.

A recent exciting technical advance has been the use of genetically encoded sensitive calcium indicator GCaMP which is a protein engineered from green fluorescent protein, the calcium-binding protein calmodulin and a calmodulin-interacting peptid This has demonstrated that single electrical impulses can evoke a small, but discernible, calcium transient in sensory neurons *in vitro* [13]. Trains of electrical stimulation gave rise to stronger responses which fused to a single calcium transient. A direct comparison of fura- and GCaMP –mediated calcium imaging has not been made in microfluidic chambers to allow for a head to head comparison of the relative sensitivity and specificity.

GCaMP imaging has also been used to study the somal calcium response of DRG neurons in response *in vivo* and electrical stimulation of the neuronal peripheral terminals evoking as little as 3 to 4 action potentials elicited robust calcium transients of the soma [13]. This preparation holds great promise for future simultaneous investigations of groups of neurons and may circumvent issues

associated with the study of sensory neurons *in vitro* such as axotomy- or culture-induced gene expression.

In conclusion the use of the soma as a model of sensory neuron sensitivity has its limitations, as there may be differences in the functional expression between subcellular regions, especially in neurons that span the longest distances in the body such as DRG neurons. Indeed, we have demonstrated a remarkable difference in the sensitivity to protons, when comparing peripheral neurites to the cell somata, verifying the importance of this approach for sensory neuron investigations. We further illustrate that this method is highly amenable to site-specific pharmacological interventions, as stimuli and inhibitors can be applied specifically to terminals, axons or soma, which enabled us to investigate the properties of action potential propagation *in vitro*.

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Conflict of Interest Statement

The authors declare no competing financial interests

Supplemental video content

Video content associated with this article can be found at http://links.lww.com/PAIN/A539

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Figure legends

Fig 1. The microgroove array creates a high fluidic resistance barrier between compartments. ***A, B,*** Microfluidic chambers can be fabricated either as a 2 or 3 compartment system. Individual compartments are illustrated with the addition of a coloured dye. Channels (i) are connected to the medium reservoirs (ii) and are separated by a microgroove array. The medium reservoirs provide access to the channels enabling medium exchange. ***C,*** Schematic explanation of radioactive flow experiment, showing the radioactive ^{125}I solution is loaded in one compartment, whereas PBS is loaded in the adjacent compartment. ^{125}I solution is loaded in either a lower, equal or greater volume than the PBS in the adjacent compartment. ***D,*** Quantification of radioactivity in compartment B. Fluid isolation of compartment B is maintained up to 72 hours, when a hydrostatic pressure imbalance is established by loading a lower volume of ^{125}I solution. ***p<0.001 (lower volume vs. greater volume).

Fig 2. DRG sensory neurons can be cultured in microfluidic chambers and their axons encouraged to grow though the microgroove array. ***A,*** Photomicrograph of three microgrooves opening into a channel loaded with a blue dye. ***B,*** Brightfield image of DRG sensory neurites emerging from the microgrooves and branching into the terminals compartment. ***C,*** Immunocytochemical image of the entire microfluidic culture. DRG sensory neurons are stained with PGP9.5 and project neurites from the soma compartment through the microgroove array into the terminals compartment. ***D,*** Magnification of the selected area in C, showing the PGP9.5 labelled neurites emerging from the microgrooves. ***E,*** Magnification of the selected area in C, showing PGP9.5 labelled somata and their neurites entering the microgrooves.

Fig 3. Action potential propagation and axonal transport in neurons cultured in microfluidic chambers. ***A,*** Representation of the chambers showing neurons growing through the microgrooves. DiI application and KCl stimulation is applied to the isolated terminal compartment. Somal accumulation of DiI and live calcium responses are recorded from fura-2 loaded cell somata. ***B,*** Brightfield image of DRG cell soma at 3 and 7 DIV. Pseudocolour image of ***C,*** DiI labelled cell somata, ***D,*** cells depolarised in response to stimulation of the terminals. ***E,*** Merged image of DiI labelled cells and those with a functional response. Yellow cells display both a functional response and DiI labelling. ***F,*** Quantification of cells that are DiI labelled and the percentage of these that can be activated by potassium stimulation applied to the terminals. *p<0.05, ***p<0.001. Scale bar - 100µm.

Fig 4. Axonal conduction can be studied in microfluidic cultures. ***A,*** Schematic of a 3 compartment chamber, showing neurons growing through 2 microgroove arrays. KCl is applied in 5 minute intervals to the terminals whilst TTX and lidocaine are applied to the axonal compartment. Live calcium imaging is performed on the fura-2 loaded cell somata. ***B,*** Representative calcium transients of a single cell. The evoked calcium response is highly reproducible. Calcium transients from two cells blocked (***C),*** or not blocked (***D)*** by 1µM TTX. TTX resistant response are always blocked by addition of the non-selective sodium channel blocker lidocaine. ***E,*** The percentage of neurons responding to each consecutive stimulation and different drug treatments. ***p<0.001

Fig 5. Proton sensitivity is differentially regulated between neuronal regions in DRG neurons. ***A,*** Percentage of responding neurons that are depolarised by various acid stimuli applied to the terminals and separately to the soma. ***B,*** Representative pseudocolour images showing peak calcium response from pH 5.1 stimuli applied to the terminals, axons and soma, in a 3 compartment system. ***C,*** Representative calcium transient showing a cell that is depolarised by acid when applied to the terminals and axon, but is insensitive at the cell soma (asterisk in B). ***D,*** Calcium transient of a cell

depolarised by acid applied to terminals, axon and soma. (arrowhead in B). ***E,*** Quantification of responding cells showing the majority of pH 5.7 responsive cells are blocked by amiloride. ***F,*** Overlap of capsaicin sensitive and amiloride resistant cells showing a small percentage (5%) that do not rely on TRPV1 or ASICs for pH detection. Scale bar – 50 μm. ***p<0.001

Fig 6. The terminals display a greater sensitivity to capsaicin than the soma, and a high capsaicin concentration induces a conduction block in TRPV1 expressing neurons. ***A,*** Dose response curve of capsaicin-induced activation when applied to the terminals and soma. Responding populations are expressed as a percentage of cells that respond to 100 mM KCl when applied to the terminals. ***B,*** Example calcium transient and schematic of a cell that is excited by capsaicin when applied to the terminals and axon. Cells that are capsaicin sensitive in the terminals are unable to propagate KCl evoked action potentials through the high concentration capsaicin compartment. ***C,*** Representative calcium transient and schematic of a cell that is insensitive to capsaicin when applied to the terminals and axon. The schematic demonstrates that the high concentration capsaicin stimulation applied to the axon does not block KCl evoked action potential propagation. ***D,*** Quantification of neurons that continue to propagate action potentials through the high concentration capsaicin, showing that the vast majority of capsaicin sensitive neurons are blocked. ***E,*** Quantification of neurons that continue to propagate showing capsaicin insensitive neurons are unaffected by high concentration capsaicin.

Fig 7. IB4 binding develops *in vitro* and capsaicin sensitivity is down-regulated in cultures prepared from early rat and mouse embryos. ***A, B, C,*** Immunocytochemical staining for IB4 and PGP9.5 in E16.5 rat cultures, after 1 day *in vitro.* No IB4 positive neurons are detected. ***D, E, F,*** Immunocytochemical staining for IB4 and PGP9.5 in E16.5 rat cultures, after 10 days *in vitro.* IB4

binding is abundantly detected. ***G, H,*** At high magnification IB4 binding can be detected both internally and on the membrane in neurons cultured for 10 days. ***I, J,*** Quantification of capsaicin sensitive cells in mass cultures over 10 DIV prepared from E15.5 and E16.5 rat embryos. At all-time points, E15.5 cultures have significantly fewer cells that functionally express TRPV1. The terminals display significantly reduced capsaicin sensitivity in E15.5 cultures compared to E16.5 cultures. ***K, L, M*** Embryonic mouse cultures from E13.5, E14.5 and E15.5 mouse embryos. ***K,*** E13.5 mouse cultures, capsaicin sensitivity rapidly declines over time *in vitro* in the cell soma, and remains low in the terminals. The onset of menthol sensitivity occurs after 5 DIV at the cell soma. ***L,*** E14.5 mouse cultures, capsaicin sensitivity similarly declines over the first 3 DIV when tested at the somata, and is also relatively low at the terminals. Menthol sensitivity is first detected at 4 DIV at the cell somata. ***M,*** E15.5 mouse cultures, capsaicin sensitivity is maintained at all-time points tested in both the somata and terminals. Menthol sensitivity is first detected at 3 DIV in the somata.

Fig 8. Retinoic acid upregulates capsaicin sensitivity in E14.5 mouse DRG cultures***. A,*** Percentage of cells that respond to capsaicin stimulation of the soma and terminals after 7 DIV in NGF-containing medium is not increased by the addition of BDNF NT3 and GDNF, but by RA ***B,*** NGF and RA alone are capable of increasing capsaicin sensitivity at 7 DIV. ***C, D,*** Supplementation of NGF-containing culture medium with additional neurotrophins or RA has no significant effect on menthol sensitivity measured at 7 DIV.

Table 1: Chemo-attraction protocol for two compartment chambers

	Somal compartment	Terminal compartment
Day 0	200 ng/ml NGF in 200 µl	200 ng/ml NGF in 200 µl
Day 1	20 ng/ml NGF in 160 µl	200 ng/ml NGF in 200 µl
Day 3	0 ng/ml NGF in 160 µl	200 ng/ml NGF in 200 µl
Days 5, 7 and 9	0 ng/ml NGF in 200 µl	200 ng/ml NGF in 200 µl

Table 2: Chemo-attraction protocol for three compartment chambers

	Somal compartment	Axonal compartment	Terminal compartment
Day 0	200 ng/ml NGF in 100 µl	200 ng/ml NGF in 100 µl	200 ng/ml NGF in 100 µl
Day 1	20 ng/ml NGF in 100 µl	200 ng/ml NGF in 120 µl	200 ng/ml NGF in 120 µl
Day 2	0 ng/ml NGF in 80 µl	20 ng/ml NGF in 100 µl	200 ng/ml NGF in 120 µl
Day 3	0 ng/ml NGF in 100 µl	0 ng/ml NGF in 100 µl	200 ng/ml NGF in 120 µl
Day 5, 7 and 9	0 ng/ml NGF in 100 µl	0 ng/ml NGF in 100 µl	200 ng/ml NGF in 120 µl