In vitro culture of hiPSC-Derived Sensory Neurons: A viable human model to aid pain research and drug discovery

Key points

- Due to exciting research advances in stem cell biology, it is now possible to create physiologically-relevant human cell culture models using sensory neurons derived from human induced pluripotent stem cells (hiPSCs), to stimulate novel and translatable breakthroughs in pain research and drug discovery.
- hiPSC-Derived Sensory Neurons improve on animal cell culture models, which lack physiological relevance to humans, and can be misleading and non-translatable.
- Axol human iPSC-Derived Sensory Neurons express sensory neural markers that play a crucial role in nociception, and show expected changes in firing rates in response to chemical and thermal stimuli.
- This study shows that Axol hiPSC-Derived Sensory Neurons are a viable human cell culture model for pioneering research on pain disorders to enable the discovery of new therapeutic targets that could improve many patients' lives.

Aims and objectives

- This study investigated the possibility of developing an in vitro model using Axol hiPSC-Derived Sensory Neurons that can be used to recapitulate the sensation of human pain.
- Control rat-derived dorsal root ganglion (DRG) neurons and experimental hiPSC-Derived Sensory Neuron Progenitors (Axol Bioscience Ltd., UK) were cultured *in vitro*.
- Once mature, firing rate responses to thermal and chemical stimuli were measured on both control and experimental cultures using multi-electrode array (MEA) systems.
- In addition, the expression of relevant sodium ion channels and nociceptors in mature hiPSC-Derived Sensory Neurons was established using immunofluorescent imaging.

Background

Diseases that affect sensory systems, such as erythromelalgia and inflammatory autoimmune disease, can cause immense suffering and threaten millions of lives worldwide each year, posing serious public health and economic challenges. Understanding how sensory neurons work and how they respond to potential drug treatments are therefore crucial goals in the academic and healthcare domains, to uncover the unknown mechanisms of sensory disorders and discover new therapeutic targets.

Traditional methods have involved the in vitro culture of non-human mammalian neurons (usually derived from rats). However, the research community is now recognizing that these **animal cell culture models often lack suitable physiological relevance to make results translatable to humans**. This can cause downstream problems, such as false-positive drug screening results that mean drug candidates go on to fail in human clinical trials, ultimately causing unnecessary costs, risks and unpublishable results.

Yet, exciting research developments in stem cell biology over the last decade promise to generate physiologically-relevant human models. Specifically, human induced pluripotent stem cells (hiPSCs) can now be used to generate and culture human sensory neurons in vitro, giving researchers the crucial toolkit they need to make breakthrough research discoveries and identify life-changing therapeutic targets that are translatable to humans

Materials and Methods

Culture of rat neurons

Rat dorsal root ganglion (DRG) neurons obtained from male Wister rats (aged 10 weeks) were used as the control cell culture. These were cultured at 1.0 x 10^4 cells/cm^2 on 64-channel MEA chips (MED-R515A; Alpha Med Scientific Inc.) coated with Laminin 511 at 37°C in 5% CO_2/95% air atmosphere.

Culture of hiPSC-Derived Sensory Neuron Progenitors

Human iPSC-Derived Sensory Neuron Progenitors (ax0055) (Axol Bioscience Ltd., UK) were used as the experimental cell culture. The cells were accompanied by a cell culture system, including a Sensory Neuron Maintenance Medium (ax0060) and coating reagents SureBond+ReadySet (ax0052) for plating on glass, and SureBond-XF (ax0053) for plating on plastic (all Axol Bioscience Ltd., UK). A supplemental protocol guideline, 'Human iPSC-Derived Sensory Neuron Progenitors,' was followed to ensure best practices for cell thawing/plating, growth arrest of non-neuronal cells, and culture maintenance.

Axol hiPSC-Derived Sensory Neuron Progenitors were cultured at 5.0 x 10^5 cells/cm^2 on 64-channel MEA chips (MED-R515A; Alpha Med Scientific Inc.) coated with Axol SureBond+ReadySet Coating Solution at 37°C in a 5% CO_2/95% air atmosphere. Guidelines on using the MED-R515A system to plate and culture Axol hiPSC-Derived Sensory Neuron Progenitors are available in a separate protocol document.

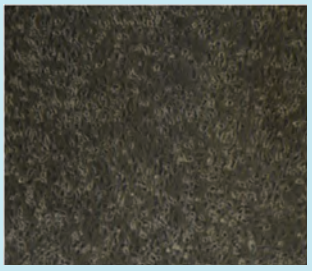
After two days *in vitro* (DIV), the culture medium was replaced with Sensory Neuron Maintenance Medium containing mitomycin C (Sigma-Aldrich Co. LLC) to remove the non-neuronal population. Non-neuronal death started to occur at five DIV and the full effects were apparent after seven DIV.

The cells were maintained in Sensory Neuron Maintenance Medium (supplemented with growth factors GDNF, NGF, BDNF, and NT-3) for a minimum of six weeks prior to performing the experiments. Half of the medium volume was replaced every three to four days. The identification of mature sensory neurons began after five weeks in culture.

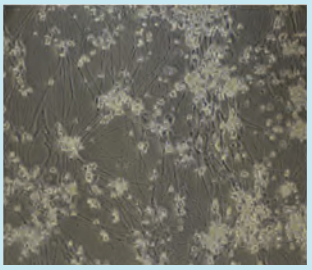
Five expert tips for the culture of hiPSC-Derived Sensory Neuron Progenitors:

Axol's scientists share their five top tips on culturing Axol hiPSC-Derived Sensory Neuron Progenitors:

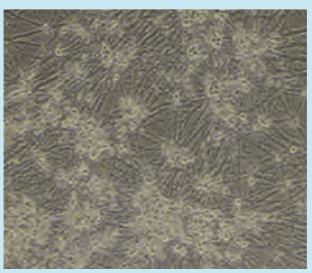
1. "About 24 hours after thawing vials of the Axol hiPSC-Derived Sensory Neuron Progenitors, you will see two types of cell under the microscope: fat, rounded neurons and darker, flatter cells (**see image A**). Sometimes you will see more of the flat cells, because the hiPSC-Derived Sensory Neuron Progenitors are embedded in these, but mitomycin C treatment will eliminate these flat cells."
2. "It is normal to observe significant cell death after mitomycin C treatment, which targets the flatter proliferating cells. Consequently, the population of hiPSC-Derived Sensory Neuron will be more homogeneous (i.e., almost 80-90% pure sensory neurons) post-mitomycin C treatment."
3. "For each addition of mitomycin C to the Sensory Neuron Maintenance Medium, always make fresh mitomycin C for best results."
4. "Under a phase contrast microscope, hiPSC-Derived Sensory Neuron appear slightly rounder, have larger somas, and are lighter in color than other neuronal subtypes (**see image B**); make sure you do not mistake them for dying cells!"
5. "After longer culture periods (approximately five to six weeks in vitro), neurites will become thicker and longer, and somas will become more spaced out (**see image C**)."



A) Pre-mitomycin C



B) Day 8 post-mitomycin C



C) Day 35 post-mitomycin C

Immunofluorescent imaging

Immunostains (TRPV1, TRPA1 and Nav1.7 stains and β-tubulin III and Hoechst counterstains) were applied to the mature hiPSC-Derived Sensory Neurons. Immunofluorescent imaging using confocal microscopy (Leica TCS SP8) was used to obtain images of the neurons to characterize their morphology and nociceptor expression.

Stimuli response experiments

Spontaneous and evoked extracellular field potentials were measured at 37°C under a 5% CO_2 atmosphere using a 64-channel MEA system (MED64-Basic; Alpha Med Scientific Inc.) at a sampling rate of 20 kHz/channel. Signals were low-pass filtered at 100 Hz and stored on a personal computer, with firing analyses and spike sortings performed using Mobius software (Alpha Med Scientific Inc.).

The responses of the culture to chemical and thermal stimuli were first measured after seven DIV. These measurements were repeated multiple times, with the interval between trials depending on the recovery and replication of the cells' spontaneous firing activity (typically two days and up to one week). The experiments could be repeated multiple times due to the longevity of the cells in culture, which continue to be viable after 10 weeks *in vitro*.

Chemical response experiments: 100µM of capsaicin, menthol and wasabi (Allyl isothiocyanate- AITC) were each applied to the mature rat DRG neurons and Axol hiPSC-Derived Sensory Neurons using the MED64 probe. Firing responses of both culture types were measured in response to each of these chemical stimuli.

Thermal response experiments: Temperature ranging from 37°C to 47°C was applied to the culture using an MED64 thermal controller. Mature DRG and hiPSC-Derived Sensory Neuron cell firing responses were measured in response to the different temperatures within this range, increasing by 1°C for each measurement.

Results

After 33 DIV on the 64-channel MEA chips, spontaneous firing activities were typically observed in the hiPSC-Derived Sensory Neurons, mimicking their typical *in vivo* behavior. In addition, immunofluorescent imaging revealed that sensory neural markers relevant to human nociception (TRPV1, TRPA1 and Nav 1.7) were all expressed in the hiPSC-Derived Sensory Neurons after five weeks in culture (**Figure 1**).

After 14 DIV, the hiPSC-Derived Sensory Neurons showed a significant increase in firing rates in response to elevated temperatures, and showed the highest increase at 43°C (**Figure 2**). In addition, they showed concentration-dependent responses and changes in firing rates in response to all three chemical stimuli: capsaicin, menthol and wasabi (Allyl-isothiocyanate- AITC) (**Figure 3**).

Lastly, the hiPSC-Derived Sensory Neurons could be classified into 27 distinct types based on their physiological responses to the three chemical compounds: capsaicin, menthol and wasabi (AITC) (**Figure 4**).

Figure 1: After 5 weeks in culture, Axol hiPSC-Derived Sensory Neurons express TRPV1, TRPA1 and Nav 1.7.

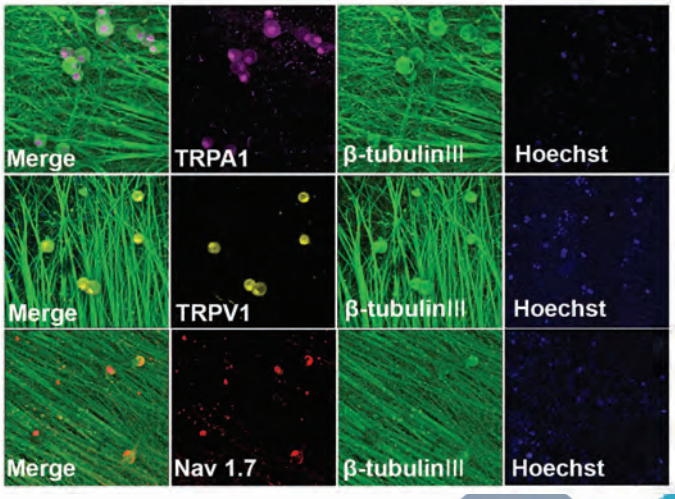


Figure 2: Number of spikes (%) in response to increasing temperature after 14 DIV and after 21 DIV

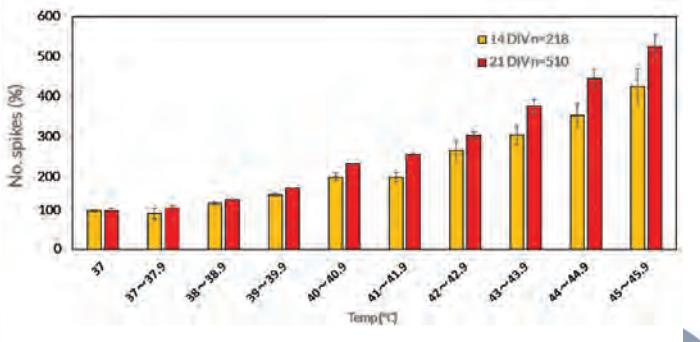


Figure 3: Neuronal firing responses of hiPSC-derived sensory neurons to chemical stimuli: capsaicin, menthol and wasabi (AITC).

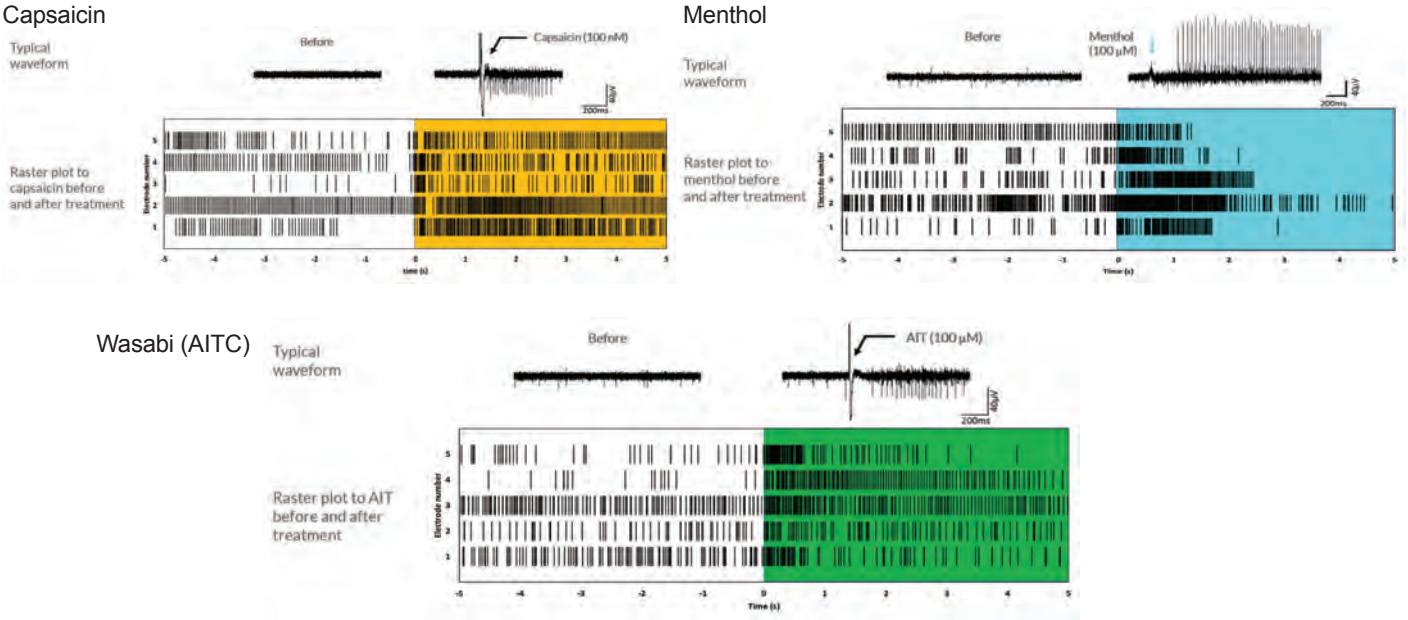
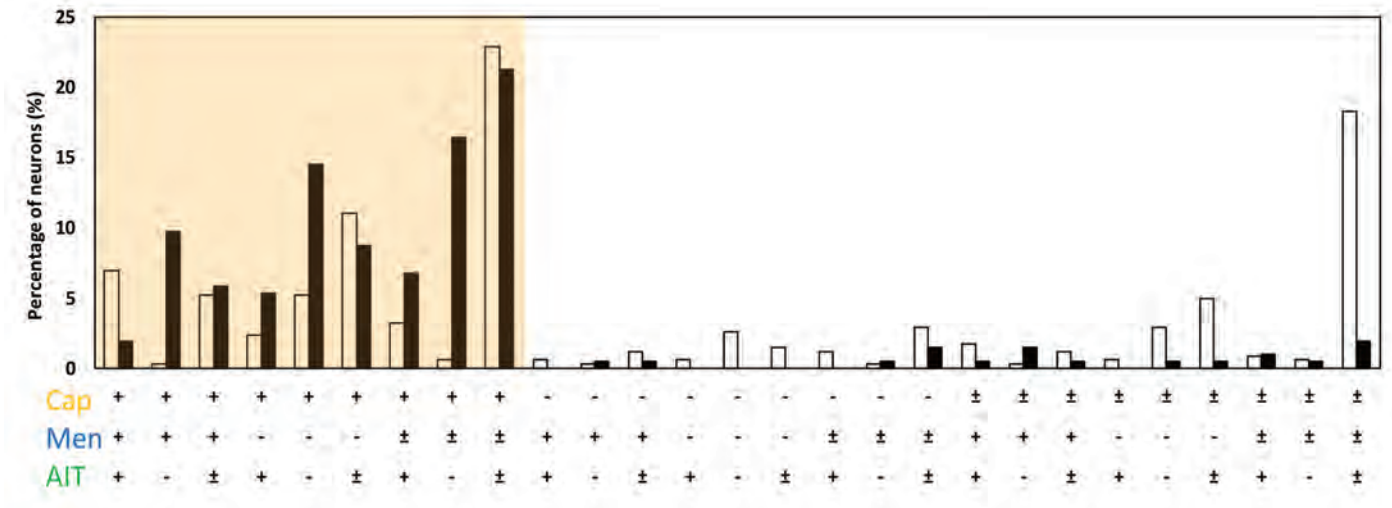


Figure 4: The 27 types of hiPSC-derived sensory neurons and rat DRG neurons defined by their firing response rates to capsaicin (Cap), menthol (Men) and wasabi (AIT). + = increase in firings; - = decrease in firings; and ± = no change.



Conclusions

This study showed that cultured Axol human iPSC-Derived Sensory Neurons display the typical characteristics and firing responses of human sensory neurons, indicating that they are a viable human model for studying the mechanisms of pain disorders and for identifying therapeutic targets. In addition, electrophysiological measurements in cultured hiPSC-Derived Sensory Neurons using multi-electrode arrays (MEA) systems were found to be a suitable toxicological assay, which could be applied to the drug screening of peripheral nerves.

Instead of relying on conventional animal-derived cell models that are limited in their translation to humans, this study shows that researchers can now access an effective, physiologically-relevant human model. This will enable them to produce and disseminate breakthrough discoveries and new translatable therapeutics, which will potentially reduce the risks and costs in follow-up human clinical trials. Adopting this state-of-the-art approach promises to generate groundbreaking research and the discovery of novel disease therapeutics to improve the quality of many patients' lives.

Highlighted products used in this application note and where to find them

Product Name	Product Code	Supplier
Human iPSC-Derived Sensory Neuron Progenitors (Male)	ax0055	Axol Bioscience
Sensory Neuron Maintenance Medium	ax0060	Axol Bioscience
SureBond+ReadySet	ax0052	Axol Bioscience
SureBond-XF	ax0053	Axol Bioscience
64-channel MEA probe for MED64-Basic	MED-R515A	Alpha MED Scientific Inc
Mitomycin C	M4287	Sigma
Glial-Derived Neurotrophic Factor (GDNF)	450-10	Peprotech
Nerve Growth Factor (NGF)	450-01	Peprotech
Brain-Derived Neurotrophic Factor (BDNF)	450-02	Peprotech
Neurotrophin-3 (NT-3)	450-03	Peprotech

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