IN VITRO CHARACTERIZATION OF HUMAN INDUCED PLURIPOTENT STEM CELL-DERIVED SENSORY NEURONS

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Discovery Stems From Here

INTRODUCTION

Human induced pluripotent stem cell-derived sensory neurons (hiPSC-sensory neurons) (Axol Bioscience) offer a physiologically relevant *in vitro* human model of pain perception.

The dorsal root ganglion (DRG) is the collection of sensory neuron cell bodies which project axons into the peripheral nervous system. These sensory neurons express key and unique nociceptors which are implicated in chronic pain conditions.

Here we present data on the characterization of hiPSC-sensory neurons assessing the expression and function of the DRG-specific voltage-gated sodium channels ($Na_v1.7$, $Na_v1.8$ and $Na_v1.9$) and transient receptor potential (TRP) ion channels, TRPV1, TRPA1 and TRPM8. Functional responses were evaluated against typical pain inducing molecules and chemotherapy drugs.

This data provides an in-depth characterization of Axol's hiPSC-sensory neurons demonstrating a viable human cell-culture model for pioneering research and drug discovery on both nociceptive and neuropathic pain disorders.

MATERIALS AND METHODS

Culture of hiPSC-Sensory Neuron Progenitors: Human iPSC-derived sensory neurons (Axol Bioscience ltd., UK) were cultured at $8.0x10^5$ cells/cm^2 on 384-channel 24-well multi-electrode array (MEA) chip and 64-channel MEA chips (Alpha Med Scientific) coated with Axol SureBond Coating Solution (Axol Bioscience ltd., UK) at 37°C in a 5% CO_2/95% air atmosphere.

Stimuli response experiments: Spontaneous extracellular field potentials were acquired at 37°C under a 5% CO_2 atmosphere using the high-throughput MEA system, here we simultaneously record extracellular potentials for 16 channels per well across 24-well plates (Presto, Alpha Med Scientific Inc.) and 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. Firing analyses and spike sorting were 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: Capsaicin (100nM), menthol (100µM) and wasabi (100µM) (Allyl isothiocyanate- AITC) were each applied to stimulate hiPSC-Sensory Neurons and AMG9810 (100nM), A967079 (300nM) and AMTB (50nM) were each applied to antagonise elicited responses in hiPSC-Sensory Neurons. Firing responses were measured 1 hour after administration in response to each of these chemical stimuli. Data provided by Dr Ikuro Suzuki (Tohoku Institute of Technology, Tokyo).

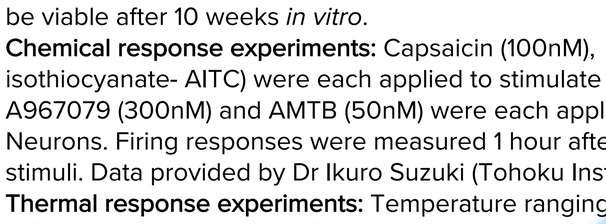
Thermal response experiments: Temperature ranging from 37°C to 46°C was applied to the culture using an MED64 thermal controller. The firing reaction of hiPSC-sensory neuron were measured in response to the different temperatures within this range, increasing by 1°C for each measurement.

Immunofluorescent imaging: Immunostains (TRPV1, TRPA1, TRPM8 $Na_v1.8$ and $Na_v1.7$ stains and β-tubulin III along with Hoechst and DAPI counterstains) were applied to mature hiPSC-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.

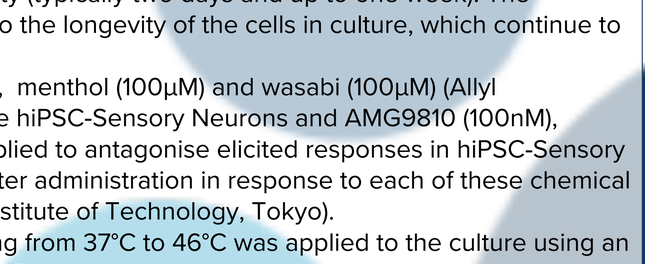
RNA Expression: cDNA from hiPSC-sensory neurons cultured for 8 weeks was compared to cDNA from human tissue from the dorsal root ganglion (DRG). PCR analysis (40 cycles; 55°C) confirmed the mRNA expression of *SCN9A* (82 bp, $hNa_v1.7$), *SCN10A* (149 bp, $hNa_v1.8$) and *SCN11A* (464 bp, $hNa_v1.9$) in Axol's hiPSC-sensory neurons. *SCN5a* (237 bp, $hNa_v1.5$) was included as a negative control. Data provided by Dr Edward Emery (University College London, London).

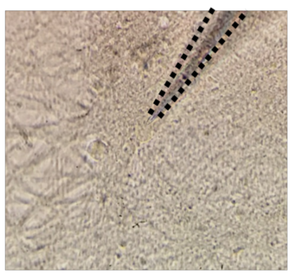
1. SODIUM CHANNEL EXPRESSION

A. $Na_v1.7$ and $Na_v1.8$ protein marker expression

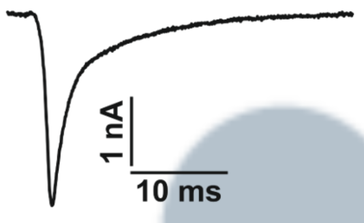


B. RNA expression analysis by cDNA PCR

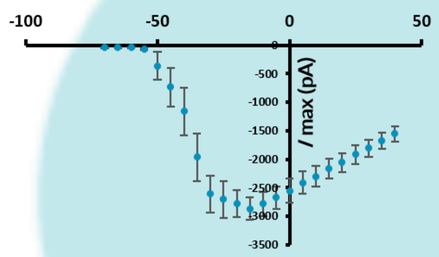




C. phase contrast



D. TTX-resistant sodium current



E. current-voltage plot

Figure 1: Molecular and Electrical Characterization of Axol's hiPSC-Sensory Neurons: voltage-gated sodium ion channels

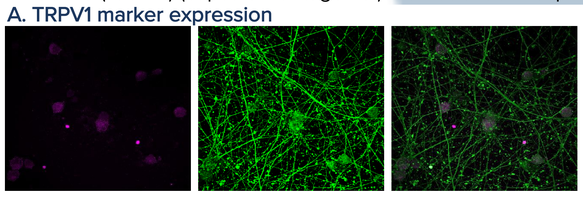
Nociceptive sensory neurons are unique in that they contain voltage-gated inward current sodium channels (Nav1.8 and Nav1.9) that are resistant to tetrodotoxin (TTX). The presence of these TTX-resistant ion channels was confirmed in Axol's hiPSC-sensory neurons. **A.** Immunocytochemistry data showed the expression of two key DRG specific ion channels $Na_v1.7$ and $Na_v1.8$. **B.** Axol's hiPSC-Sensory Neurons showed RNA expression of all three voltage-gated sodium ion channels, $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$. **C.** Phase contrast image of iPSC-sensory neurons 8 weeks in culture **D.** Example of a sodium-current elicited by a voltage step from -100 mV to -25 mV in the presence of TTX (0.5mM) **E.** Current-voltage plot of averaged Na-currents recorded from hiPSC-sensory neurons in the presence of TTX (n=9).

2.TRANSIENT RECEPTOR POTENTIAL EXPRESSION

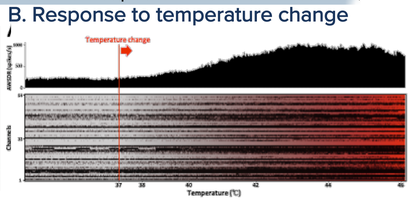
Figure 2: Molecular and Electrical Characterization of Axol's hiPSC-Sensory Neurons: potassium ion channels

TRPV1: Transient receptor cation channel TRPV1 is key in heat-induced pain with the nociception of heat greater than 43°C and is activated by chemicals such as capsaicin (chilli). **A.** immunostaining reveals expression of TRPV1 at 12 weeks *in vitro* (TRPV1 (purple), β-III tubulin (green)). Response of hiPSC-sensory neurons to temperature change; **B.** Raster plots at 64 electrodes from 37 to 46°C. Redline: starting time. **C.** Representative wave format 37 and 42°C. D. Rastor plot shows capsaicin evoked responses of hiPSC-sensory neurons before and after the application of 100nM capsaicin. hiPSC-sensory neurons treated with AMG9810 (100nM) (capsaicin antagonist) did not elicit a response when capsaicin was administered,

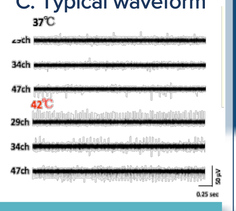
A. TRPV1 marker expression



B. Response to temperature change

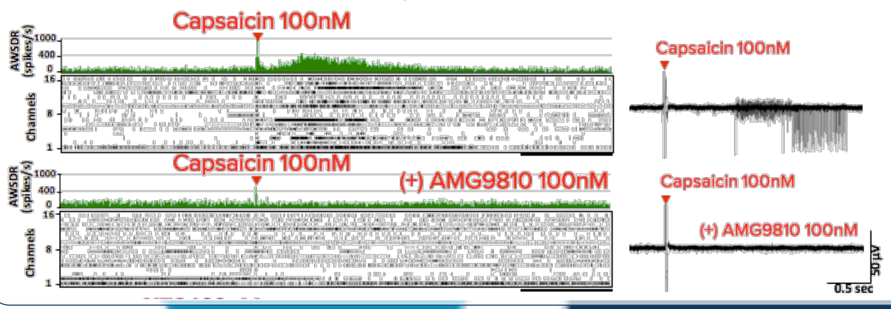


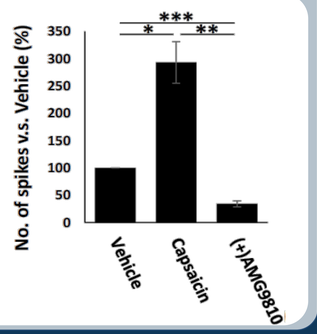
C. Typical waveform



Physiologically relevant firing peak at 43°C, representative of the activation temperature *in vivo* TRPV1 channels

D. Response to capsaicin & capsaicin antagonist

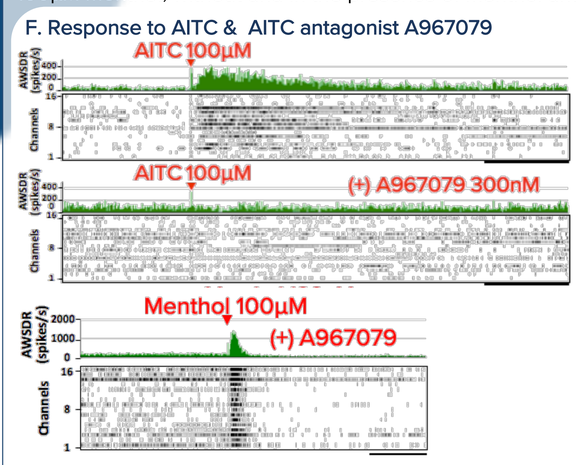


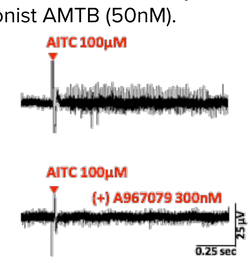


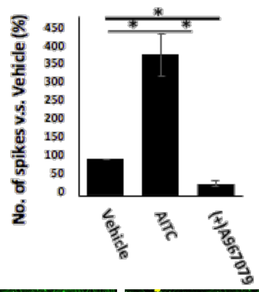
2.TRANSIENT RECEPTOR POTENTIAL EXPRESSION CONT.

Figure 2: Molecular and Electrical Characterization of Axol's hiPSC-Sensory Neurons: potassium ion channels **TRPA1:** Responds to pungent stimuli such as allyl isothiocyanate (AITC) (mustard oil/wasabi) and can also contribute to noxious cold sensitivity. **E.** Immunostaining reveals expression of TRPA1 at 12 weeks *in vitro* (TRPA1 (yellow), β-III tubulin (green)). **F.** Rastor plot shows the firing frequency of sensory neurons before and after the application of 100µM AITC without and in the presence of AITC antagonist A967079 (30nM), short burst firing was observed after administration of AITC which was blocked in the presence of the antagonist. **TRPM8:** Noxious cold stimuli (temperatures 10-15°C and below) and cooling compound menthol activates TRPM8. **G.** Immunostaining reveals expression of TRPM8 at 12 weeks in vitro (TRPM8 (blue), β-III tubulin (green)). **H.** Rastor plot shows menthol evoked responses of hiPSC-sensory neurons before and after the application of 100µM menthol, without and in the presence of menthol antagonist AMTB (50nM).

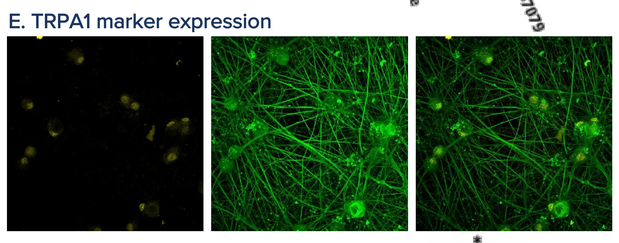
F. Response to AITC & AITC antagonist A967079



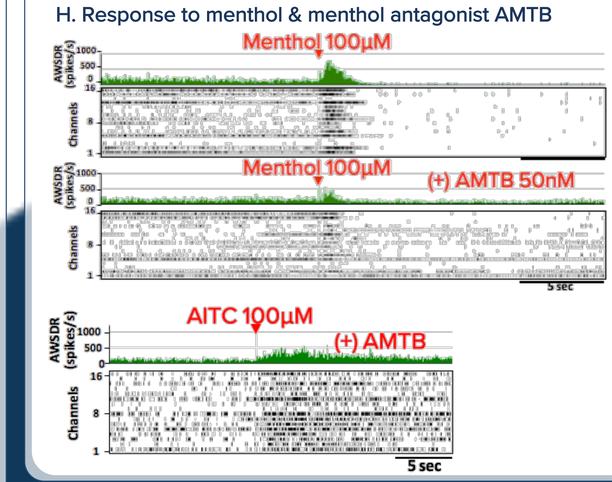


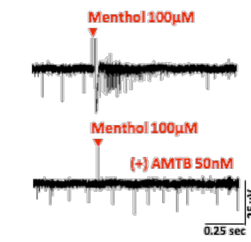


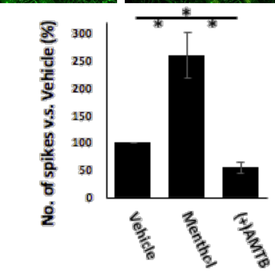
E. TRPA1 marker expression



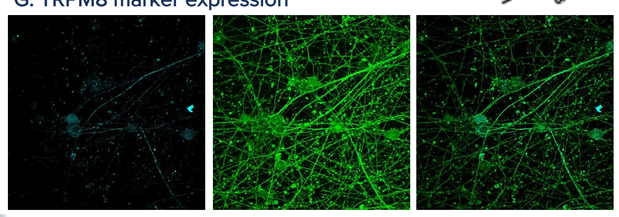
H. Response to menthol & menthol antagonist AMTB







G. TRPM8 marker expression



3. CHEMOTHERAPY-INDUCED NEUROPATHIC PAIN

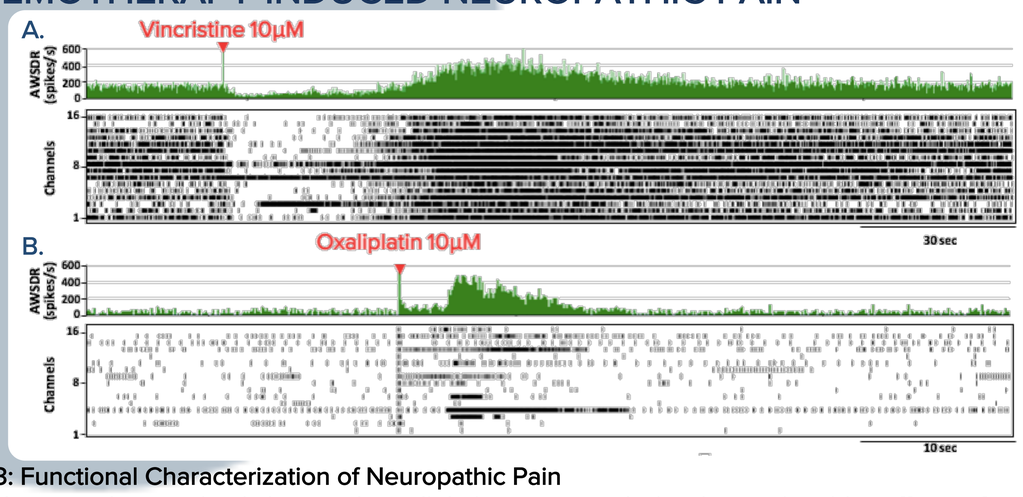


Figure 3: Functional Characterization of Neuropathic Pain

Chemotherapy drugs, vincristine and oxaliplatin, can result in the toxic side effect of peripheral neuropathy. The application of both of these chemotherapy drugs to hiPSC-sensory neurons resulted in an acute increase in firing rate, the time taken for the hiPSC-sensory neurons to respond was slow in comparison to the application of capsaicin, menthol and AITC. Figure 3: Rastor plot and array-wide spike detection rate (AWSDR) before and after administration of A. vincristine 10µM and **B.** oxaliplatin 10µM.

4. OXALIPLATIN-INDUCED COLD HYPERSENSITIVITY

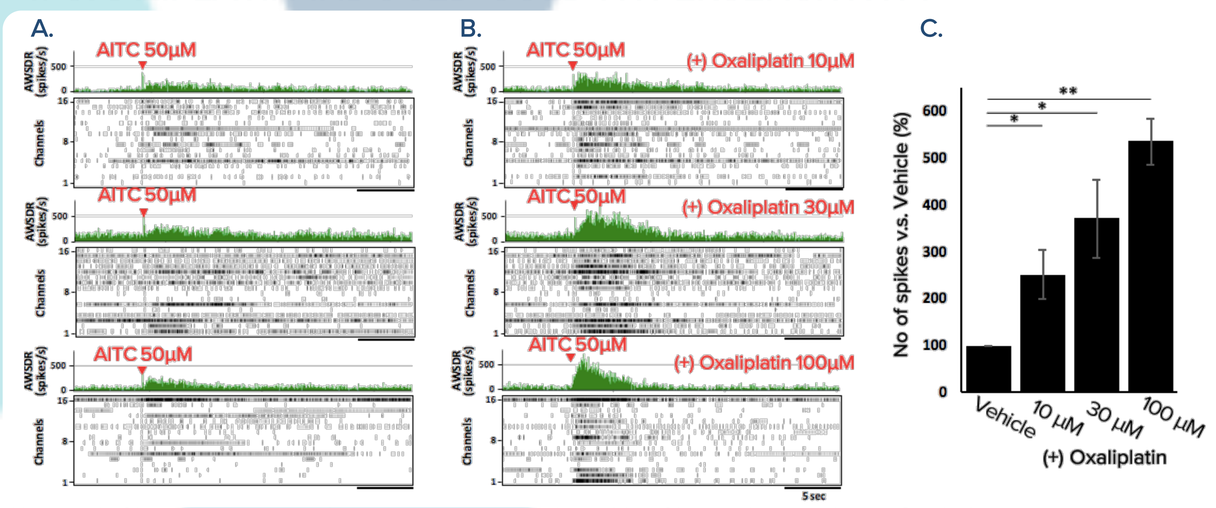


Figure 4: Characterization of Hypersensitivity Induced by Chemotherapy Drug Oxaliplatin

Oxaliplatin has further been implicated in the exacerbation of cold sensation. Here Oxaliplatin was shown to increase the firing rate of hiPSC-sensory neurons in response to AITC in a dose-dependent manner. Oxaliplatin results in a hypersensitivity to cold stimuli in hiPSC-sensory neurons. **A.** The response of hiPSC-sensory neuron to the application of AITC 50µM (control) **B.** hiPSC-sensory neuron treated with varying concentrations of oxaliplatin (10, 30 and 100µM), after 2 hours AITC 50µM was administered and the response was measured. **C.** Percentage increase of the number of firing spikes compared to the vehicle control. Treatment of oxaliplatin resulted in a dose-dependent increase in firing rate. (n=6, *p < 0.05, **p < 0.0005).

CONCLUSION

Extensive profiling of nociceptive ion channel expression and function determined that Axol's hiPSC-sensory neurons:

- Express key voltage-gated sodium channels, Nav1.7 and the DRG-specific, TTX-resistant channels, Nav1.8 and Nav1.9.
- Express nociceptive TRP channels TRPV1, TRPA1 and TRPM8.
- Functionally respond to the noxious stimuli heat, capsaicin, AITC and menthol.
- Show evoked responses to capsaicin, menthol and AITC were TPRV1, TRPM8, and TRPA1 channel dependent responses.
- Produce a pain response after administration of chemotherapy drugs.
- Exhibit hypersensitivity to cold stimuli, AITC, when treated with oxaliplatin.

Axol's hiPSC-Sensory Neurons offer a well characterized, translatable in vitro model to identify pain responses to toxic compounds and development of analgesic drugs to combat the painful side effects of chemotherapy treatment.

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