FUNCTIONAL ENDPOINTS FROM HUMAN IPSC-DERIVED SENSORY NEURONS FOR PAIN DRUG DISCOVERY

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Abstract

Drug discovery workflows for neurological indications have traditionally relied on neuronal preparations from rodent tissues. However, the ability to test activity of compounds on human neurons is desirable, since rodent pharmacology data do not always predict effects in humans. Human iPSC-derived neurons are increasingly used to provide human *in vitro* pharmacology data, with the promise of improving decision-making for neuroscience drug discovery. In pain research, human iPSC derived sensory neurons have been used by recent studies to model primary sensory neurons of dorsal root ganglia. Although different types of human iPSC-derived neurons have been extensively characterized by gene expression, the most salient phenotype of neurons is electrical excitability, as neurons integrate synaptic inputs and respond by firing action potentials.
Patch-clamp electrophysiology assays are routinely used to characterize neuronal excitability and provide functional endpoints to assess effects of drug compounds. However, the basic electrophysiological properties of human iPSC-derived sensory neurons have not been well studied.
How well do iPSC-derived sensory neurons reflect the physiological state of native primary sensory neurons? How consistent is the phenotype?
How reproducible are the data?
To address these questions, we focused on human sensory neurons differentiated from iPSC-derived neural progenitors (Axol Bioscience, ax0055).
We conducted a thorough patch-clamp analysis, including: 1) current-clamp recordings to measure spontaneous and evoked action potential firing and 2) voltage-clamp recording to characterize tetrodotoxin-sensitive and tetrodotoxin-resistant Na currents (found in native neurons).
We also determined the concentration-response of tetracaine, a voltage- and frequency-dependent blocker of voltage gated Na currents. We recorded >500 neurons from twelve independent neural precursor differentiation experiments.
We conclude that human iPSC-derived sensory neurons reproduce several key electrophysiological properties expected from bona fide dorsal root ganglia sensory neurons and are useful for supporting drug discovery programs for pain.

Methods and study design for the characterization of Axol iPSC-derived sensory neurons

Cell culture
Frozen vials of human iPSC-derived sensory neuron progenitors (Axol Bioscience ax0055) were stored in the vapor phase of liquid nitrogen. Plating Media, Sensory Neuron Maintenance Media, neurotropic factors, and Maximizer supplement were also provided by Axol as part of the complete cell culture kit. Sensory neuron progenitors were thawed and plated at high density (113,000 cells/cm2) on poly-D-lysine (PDL) coated glass coverslips in Neural Plating Medium (Axol). Alternatively, cells were plated at low density (15,000 cells/cm2) on a confluent monolayer of rat cortical astrocytes growing on glass cover slips. The next day, the Neural Plating Medium was replaced with Sensory Neuron Maintenance Medium (Axol) supplemented with pen/strep, Maximizer, NGF (25 ng/ml), GDNF (25 ng/ml), BDNF (10 ng/ml), and NT-3 (10 ng/ml). On day 3, the cells were treated with mitomycin C (2.5 ug/ml) for 2 hours to arrest the growth and proliferation of dividing cells that did not differentiate into non-dividing neurons. After 2 hours, the medium was completely changed to remove the mitomycin C. On day 5, the medium was completely changed. Thereafter, the medium was refresh by ½ medium change three times per week.

Electrophysiology
Coverslips containing sensory neurons were transferred to an inverted microscope (Olympus IX73). Recordings were made using a Multiclamp 700B patch clamp amplifier (Axon) and signals digitized using a Digidata 1550B (Axon). PClamp 11 software was used for data acquisition (Clampex) and analysis (Clampfit). Cells were perfused by gravity with an external recording solution at room temperature at a flow rate of 1.5 ml/min. The external recording solution was comprised of (mM): 140 NaCl, 2.5 KCl, 2 CaCl2, 1.3 MgCl2, 10 glucose, 10 HEPES buffered to pH 7.3. Standard patch clamp methods were used for whole cell voltage clamp recordings. For voltage clamp recordings of Na currents, a Cs-based internal recording solution was used, comprised of (mM): 70 CsCl, 70 CsF, 3 MgCl2, 5 EGTA, 0.5 CaCl2, 10 HEPES buffered to pH 7.3.
For current clamp recordings of action potentials, patch pipettes were filled with a K-based internal recording solution comprised of (mM): 120 K-gluconate, 20 KCl, 3 MgCl2, 5 EGTA, 0.5 CaCl2, 10 HEPES buffered to pH 7.3.

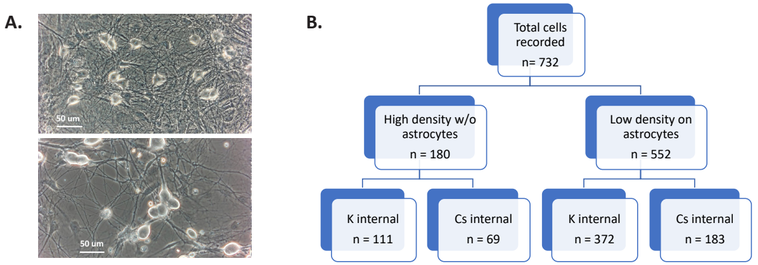


Figure 1. (A) Photomicrographs of Axol iPSC-derived sensory neurons (top) and adult rat dorsal root ganglion DRG neurons (bottom) at 7-day *in vitro*. **(B)** The electrophysiological properties were measured from 1-5 weeks as the hiPSC-derived neural progenitors matured into fully differentiated sensory neurons. Neurons plated at high density without astrocytes and at low density on astrocyte monolayers were compared.
732 cells from 15 independent batches were included in this study. The number of current clamp recordings of action potentials (K-internal) and voltage clamp recordings of Na currents (Cs-internal) are shown.

Development of passive membrane properties of human iPSC derived sensory neurons grown with and without astrocytes over 1 - 5 weeks *in vitro*

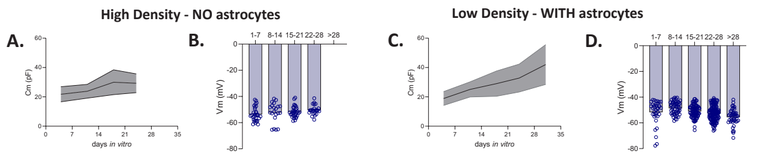


Figure 2. Cell capacitance **(A)** and resting membrane potential **(B)** of sensory neurons grown at high density without astrocytes as a function of differentiation time in culture.
Cell capacitance **(C)** and resting membrane potential **(D)** of sensory neurons grown at low density on astrocytes as a function of differentiation time in culture.
Shaded area represents 95% confidence interval.

Co-culture with astrocytes promotes the development of spontaneous activity

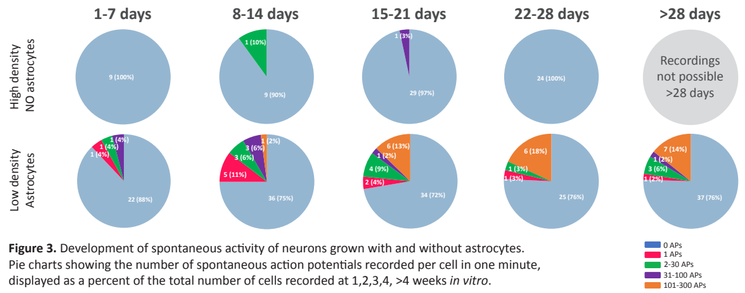


Figure 3. Development of spontaneous activity of neurons grown with and without astrocytes.
Pie charts showing the number of spontaneous action potentials recorded per cell in one minute, displayed as a percent of the total number of cells recorded at 1,2,3,4, >4 weeks *in vitro*.

Co-culture with astrocytes promotes the development of evoked activity

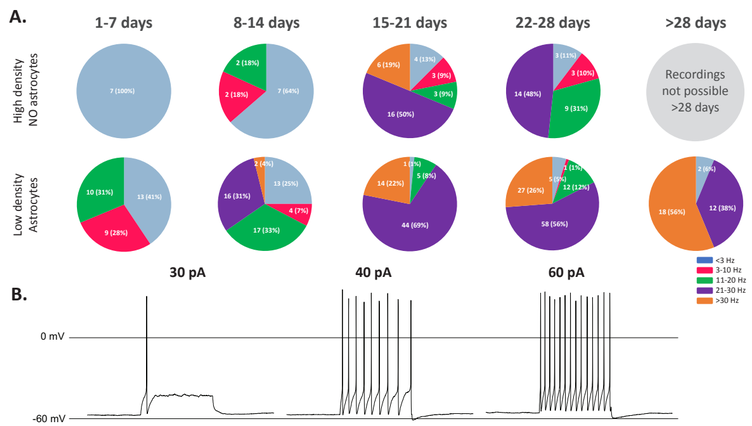


Figure 4. (A) Pie charts showing maximum number of action potentials in one second evoked by depolarizing current injection, displayed as a percent of the total number of cells recorded at 1,2,3,4, >4 weeks *in vitro*.

(B) Representative traces of action potentials evoked by one second depolarizing current injections of 30, 40, and 60 pA from a sensory neuron at 23 days *in vitro*.

(C) Number of action potentials evoked during one second depolarization of indicated current injection. Symbols indicate average of n = 48-64 cells ± sem.

Excitability is reduced by retigabine

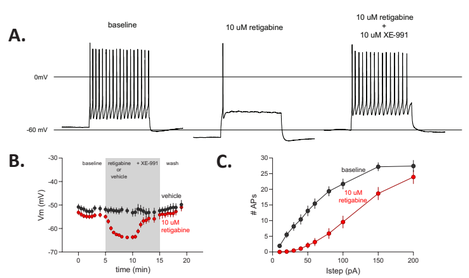
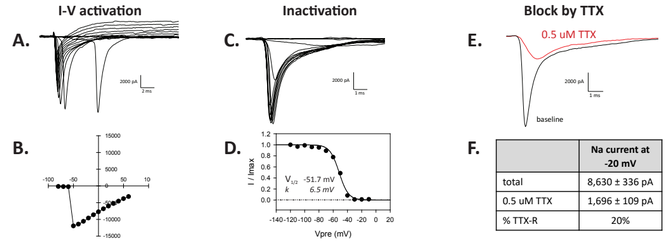


Figure 5. (A) A train of action potentials elicited by an 80 pA current injection.
10 uM retigabine (Kv7 channel opener) hyperpolarized the resting membrane potential and blocked spikes. Addition of the Kv7 antagonist XE-991 (10uM) reversed the effect of retigabine.

(B) Time course of the effects of retigabine and reversal by XE-991 on resting membrane potential.
Symbols represent mean ± sem of 21 hiPSC neurons.

(C) Retigabine reduced excitability by shifting the rheobase to the right.
Symbols represent mean ± sem of 17 hiPSC neurons.

Human iPSC sensory neurons exhibit TTX-sensitive and TTX-resistant Na currents



	Na current at -20 mV
total	8,630 ± 336 pA
0.5 uM TTX	1,696 ± 109 pA
% TTX-R	20%

Figure 6. (A) Inward Na currents in response to depolarizing voltage steps. **(B)** I-V relationship of activation. The "notch" indicates that the very large currents were not adequately controlled under voltage clamp.

(C) Inward Na current responses to a depolarizing step to -20 mV from a series of depolarizing pre-pulses. **(D)** I-V relationship of inactivation.

(E) Total Na current before and after addition of 500 nM TTX reveals the TTX-resistant Na current. **(F)** The total and TTX-resistant Na current amplitudes for n=104 cells presented as mean ± sem.

Tetracaine blocks Na currents in human iPSC sensory neurons

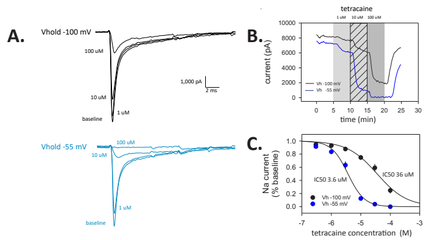


Figure 7. (A) Inward Na currents elicited from a holding potential of -100 mV (black) and -55 mV (blue) to partially inactivate the Na channels.

(B) Representative I-T plot showing the block of Na currents by the cumulative application of 1 uM → 10 uM → 100 uM tetracaine.

(C) Concentration-response of tetracaine shows that the block was voltage-dependent.
The IC_{50} for fully activated channels was 36 uM and the IC_{50} for partially inactivated channels was 3.6 uM.

Tetracaine blocks action potentials in human iPSC sensory neurons

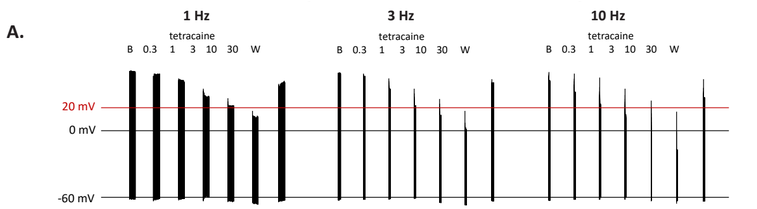
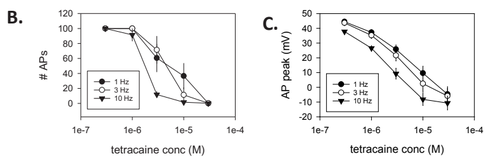


Figure 8. (A) Current clamp recordings showing trains of 100 action potentials ellicited at 1 Hz, 3 Hz, and 10 Hz. Cumulative application of increasing concentrations of tetracaine reduced action potentials at each frequency.



(B) Tetracaine reduced the number of action potentials in a concentration-dependent and frequency-dependent fashion.

(C) Tetracaine reduced the action potential amplitude in a concentration-dependent and frequency-dependent fashion.
Symbols represent average ± sem for n=10 neurons.

Conclusion

Human iPSC-derived sensory neuron progenitors developed several key electrophysiological properties expected from mature sensory neurons, making these cells useful for supporting drug discovery programs for pain.