Human Induced Pluripotent Stem Cell-Derived Nociceptors suitable for Automated Patch Clamp High Throughput Pain Drug Discovery

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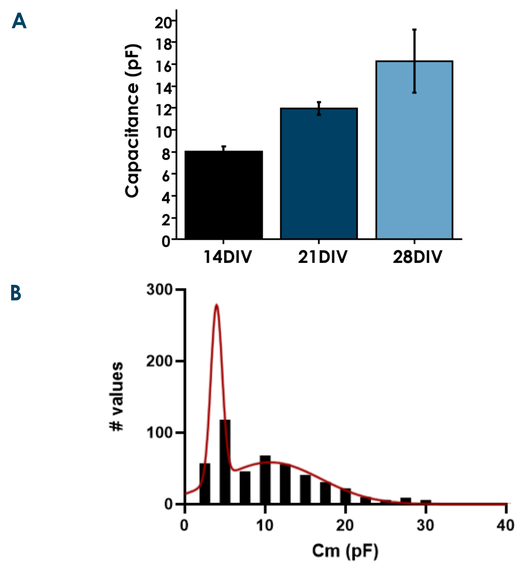
Introduction

There is an unmet need for novel non-addictive pain analgesics as the opioid epidemic continues. The ability to screen compounds on human sensory neuron nociceptors with high throughput would increase the efficiency and pace of preclinical pain drug discovery and improve translational success of new pain drug candidates. We have previously demonstrated that human nociceptors can be generated in an accelerated, scalable method from human induced pluripotent stem cells (hiPSCs), and that the hiPSC-derived nociceptors share similarities to human dorsal root ganglia based on whole-transcriptome profiling and expression of functional voltage- and ligand-gated channels important for nociception.

In this study, we utilise a novel dissociation method to enable automated patch clamp electrophysiological recordings of multiple ion channels in RealDRG™ cultures on the Patchliner and SyncroPatch 384 systems. The functional expression and biophysical and pharmacological properties of voltage-gated sodium (Nav) and potassium ion channels (Kv), and ligand-gated ionotropic GABA and P2X receptors were studied over 14, 21 and 28 days in culture, along with excitability properties and action potentials in current clamp.

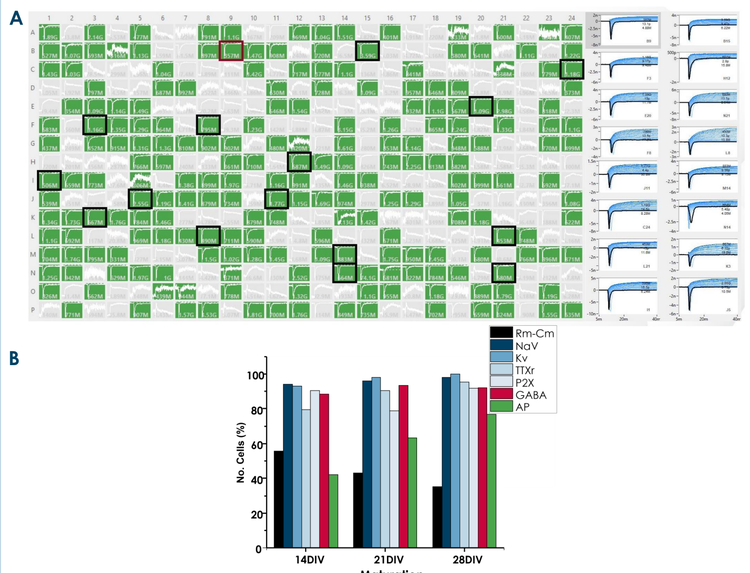
The percentage of cells with at least one evoked action potential increased from 42% to 77% over the course of maturation, with success rates decreasing from 56 to 35% as the cells matured. There was also an increase in voltage-gated sodium (Nav) and potassium (Kv) currents over time in culture, with success rates ranging from 94-98% and 93-100%, respectively. Most neurons had tetrodotoxin-resistant (TTXr) sodium currents, with a trend of increasing number of cells with Nav current and fraction of TTXr current per cell. A ligand puff protocol was developed to reproducibly evoke ligand-gated GABA-A and P2X receptor ion channels without desensitisation. These findings demonstrate the ability to functionally screen multiple analgesia drug targets in human iPSC nociceptors using high throughput automated patch clamp systems

Cell size reveals neuron maturation and diversity



Cell capacitance measurements of cell size reveals maturation and diversity of RealDRG™ sensory neuron phenotypes. A: Single neuron capacitance measurements are indicative of cell size, which increases as RealDRG™ sensory neurons mature during indicated days *in vitro* (DIV). **B:** Plot of cell capacitance from 3 NPC-384 chip recordings shows bimodal distribution typical of small and medium diameter sensory neurons, with a small tail of larger diameter cells.

High throughput success rate APC recordings



High quality, high throughput APC recordings from RealDRG sensory neurons. A: NPC-384 plate view of a voltage clamp Nav-Kv experiment, with green wells (cells) exceeding QC filters of >300 MΩ Rm and 2 pF Cm. Example current traces on *right* from cells highlighted by *black* boxes. **B:** Summary of experimental success rates for cell capture, cells with K_v and Na_v currents and P2X and GABA-A ligand-gated ionotropic receptor responses, and action potentials (AP), some of which increase with DIV maturation.

Methods

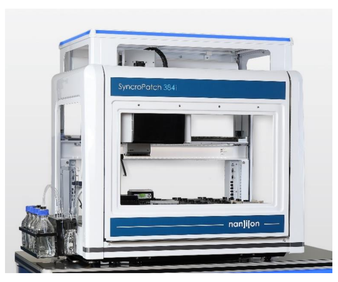
RealDRG™ sensory neurons:

Human stem cell-derived sensory neurons were rapidly differentiated and matured from human induced pluripotent stem cells (hiPSCs) using a validated protocol[1] that works across multiple hiPS donor cell lines[2]. Neural crest is induced from primary ectoderm and then differentiated using small molecules and growth factors, before sensory neurons are frozen and shipped to users for thawing and further maturation as desired. Of the key 173 evolutionarily-conserved human DRG-specific gene markers, over 90% are expressed in RealDRG sensory neurons; the remaining 10% of genes relate to myelination and immune cells and their absence reflects the fact that these iPSC-derived sensory neuron cultures are > 95% pure.

Automated patch clamp (APC) electrophysiology:

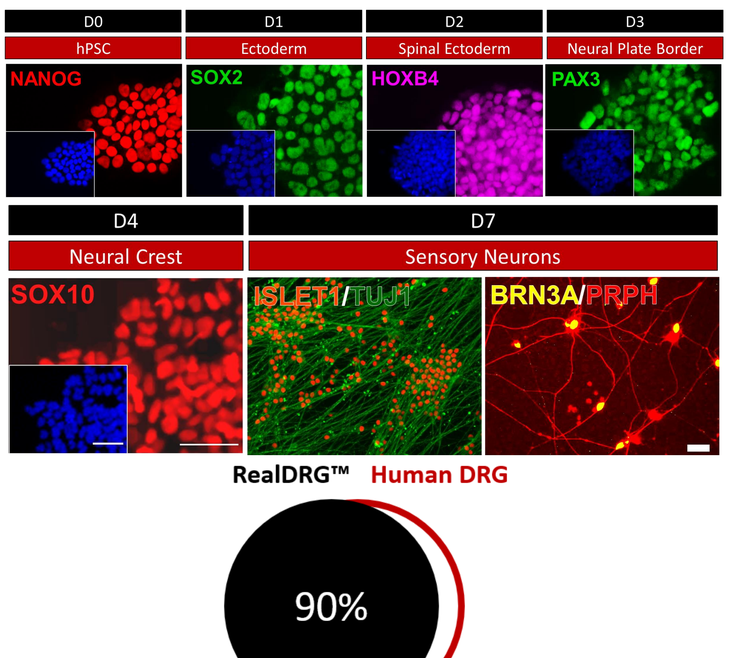
A novel dissociation method was used to prepare single cell suspensions from RealDRG™ cell cultures at 14, 21 and 28 days in culture suitable for APC electrophysiology on Nanion Patchliner and SyncroPatch384 platforms. Seal quality is excellent (>300 MΩ), enabling standard solutions and patch clamp protocols to be used to record voltage- and ligand-gated ion channel currents under voltage clamp, and record spontaneous and evoked action potentials under current clamp.

Patchliner
4 or 8 recording channels



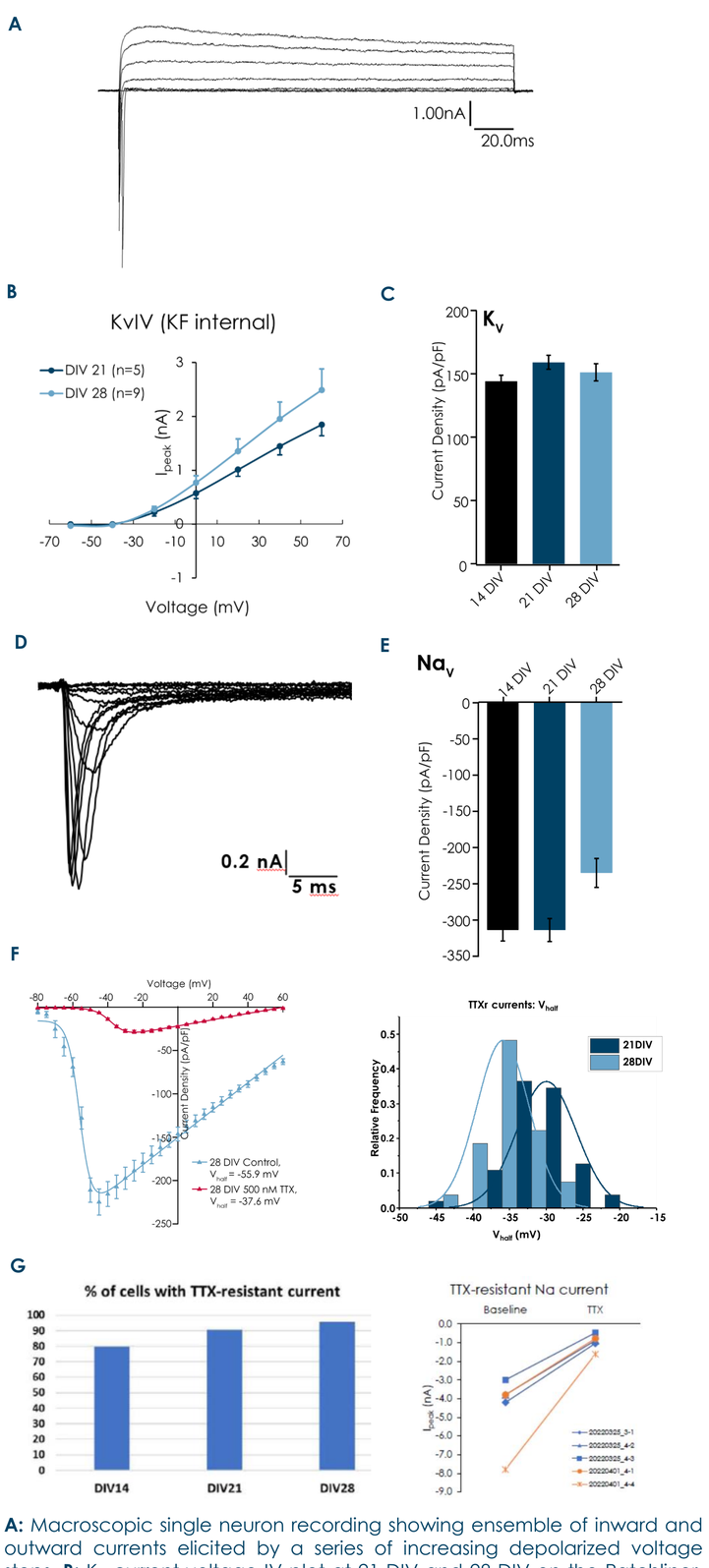
SyncroPatch 384
384 recording channels

Differentiation of RealDRG™ sensory neurons



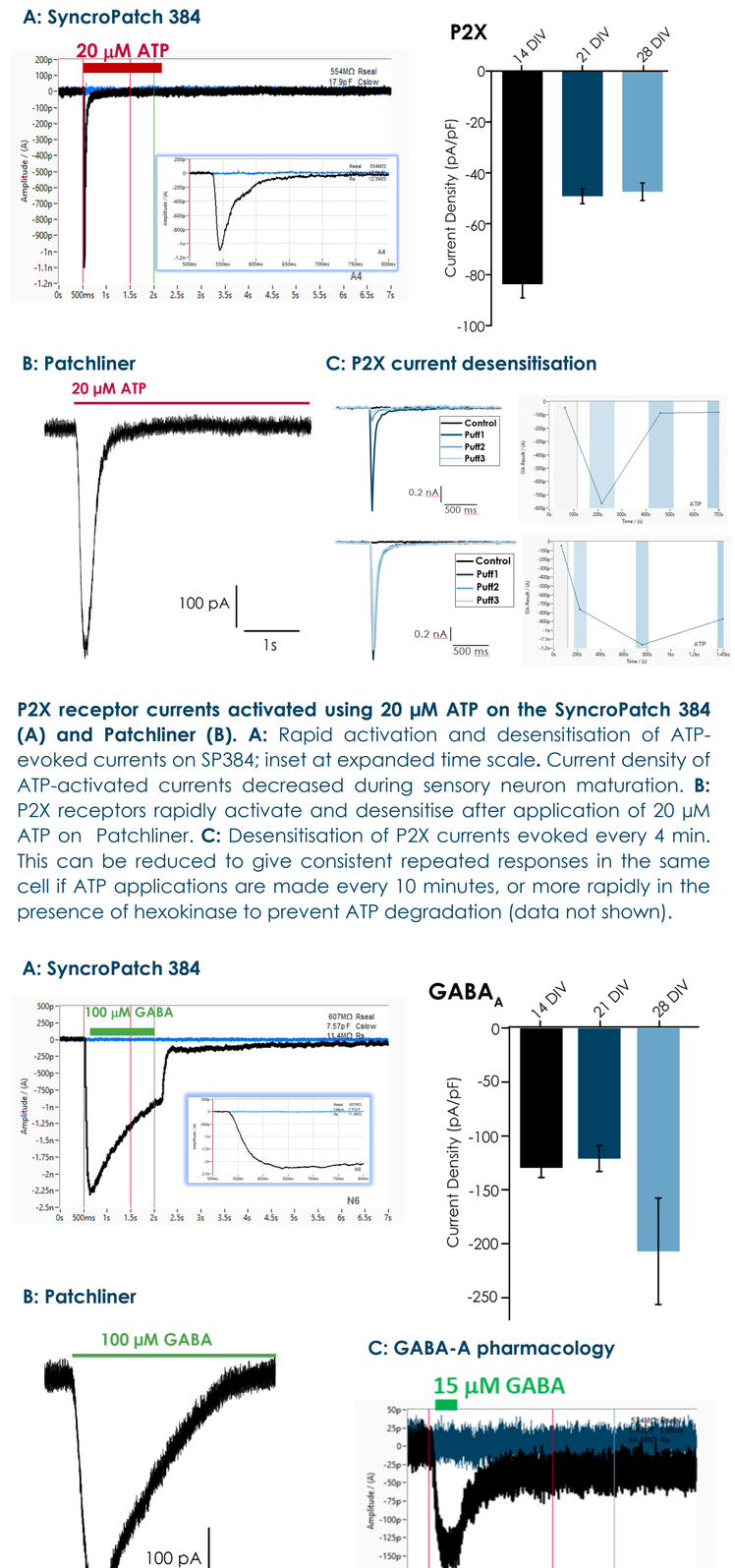
Differentiation of RealDRG™ from PSC progenitor through neuro-ectoderm and neural crest to sensory neuron revealed by key gene markers. Upper panels: Expression of classical markers for each cell type, culminating with sensory neuron markers TUJ1, ISLET1, Peripherin and BRN3A by day 7. **Lower:** RealDRG™ neurons express 90% of key sensory neuron genes that define the human DRG transcriptome[3,5,6], only lacking the 10% of genes related to myelination (Schwann cells) and immune cells (e.g. microglia).

Voltage-gated currents during maturation



A: Macroscopic single neuron recording showing ensemble of inward and outward currents elicited by a series of increasing depolarized voltage steps. **B:** K_V current-voltage IV plot at 21 DIV and 28 DIV on the Patchliner. **C:** Current density of K_V current does not change during maturation (data from SyncroPatch 384). **D:** A family of inward TTX-resistant Na_v current traces evoked by increasing amplitude of depolarizing voltage steps. **E:** Na_V current density decreases during maturation (total Na_V current). **F:** Na_v current IV plot at 28 DIV in the absence (light blue) and presence of TTX (red). TTXr currents activate at more positive potentials (typical of Nav1.8 channels[4]), and V_{half} of activation becomes more negative at later DIV. **G:** The % cells with TTXr current increases with DIV (SP384 data), and 10-20% of total current is carried by TTXr channels (Patchliner data).

Ligand-gated ionotropic receptor currents



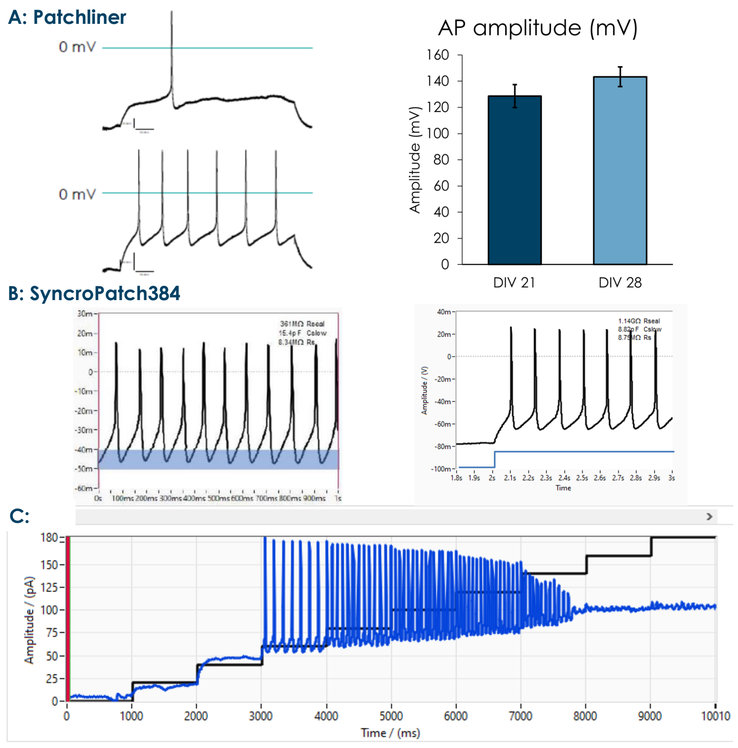
P2X receptor currents activated using 20 µM ATP on the SyncroPatch 384 (A) and Patchliner (B). A: Rapid activation and desensitisation of ATP-evoked currents on SP384; inset at expanded time scale. Current density of ATP-activated currents decreased during sensory neuron maturation. **B:** P2X receptors rapidly activate and desensitise after application of 20 µM ATP on Patchliner. **C:** Desensitisation of P2X currents evoked every 4 min. This can be reduced to give consistent repeated responses in the same cell if ATP applications are made every 10 minutes, or more rapidly in the presence of hexokinase to prevent ATP degradation (data not shown).

$GABA_A$ receptors activated by fast application of GABA on SyncroPatch 384 (A) and Patchliner (B). There was a variable degree of desensitisation between sensory neurons and platforms. *Inset* shows time course of activation on an expanded time scale. GABA current density increased at 28 DIV compared with 21 or 14 DIV. **C:** GABA currents were inhibited by the competitive $GABA_A$ antagonist Bicuculline (10 µM, *blue trace*).

Ion channel expression in RealDRG™

Key nociceptor ion channels are expressed in RealDRG™. A: Phase contrast morphology of RealDRG™ neurons, and IHC labelling with sensory neuron markers TUJ1, Peripherin, Nav1.7 and Nav1.8. **B:** RNA sequencing data reveals rapid and sustained expression of genes SCN9A, SCN10A and SCN11A (encoding $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$, respectively), and TRPV1 during 4 weeks of *in vitro* maturation. **C:** Comparison of expression of ion channels in RealDRG™ sensory neurons with native adult human DRG[3,5,6].

Action potential recordings on APC platforms



Action potentials recorded on Patchliner (A) and SyncroPatch 384 (B). A: Single and repetitive action potentials evoked by a 500 msec current step on Patchliner; AP amplitude increases with DIV maturation. **B:** Spontaneous (left) and evoked APs (right) on SyncroPatch 384. **C:** Membrane excitability assessed with a series of increasing current steps on SP384.

Summary

- **RealDRG™** sensory neurons recapitulate the differentiation and maturation of human DRG and express most of the characteristic markers of A and C-fibre nociceptors and mechanoreceptors.
- **RealDRG™** sensory neurons are well suited to automated patch clamp as they produce large, smooth soma membranes with simple dendritic processes for high quality sealing and good voltage control.
- **RealDRG™** allow high success rate recordings on **SyncroPatch 384** and **Patchliner automated patch clamp** devices.
- **A TTX-sensitive Na_V** current was reliably recorded in **almost all cells** at 14, 21 and 28 DIV.
- **A TTX-resistant current** was present in many cells, around 10-20% of total inward current amplitude. Pharmacological dissection is ongoing.
- RealDRG sensory neurons exhibit low rates of **spontaneous action potential activity**, typical of native rodent and human DRG neurons.
- **Action potentials** can be evoked with single or incrementing current injections, for assessment of sensory neuron membrane excitability.
- The occurrence of **action potentials increases with RealDRG maturation**.
- **Flexible liquid application capabilities** allow study of fast ligand-gated ionotropic receptor responses typically found in sensory neurons.
- **P2X-mediated currents** were reliably recorded on SP384 after desensitisation and ATP degradation were reduced. Responses increased during maturation, but current density decreased.
- A **GABA-activated current** was recorded on SyncroPatch 384 and Patchliner, which was blocked by the GABA-A antagonist Bicuculline. The % of cells with responses was maintained over 14, 21 and 28 DIV, and GABA-A current density increased as sensory neurons matured.
- **RealDRG™** sensory neurons represent an affordable and scalable solution for more predictive human translational analgesia studies.

References

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