Characterization of Netrin-1 Receptor DCC in Human Pluripotent Stem Cell-Derived Sensory Neurons During Inflammatory Sensitization

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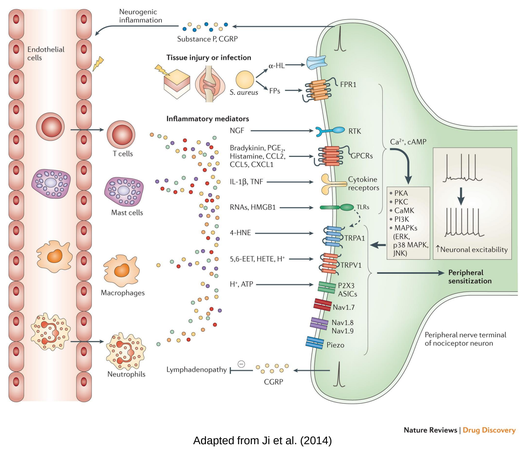
Abstract

Chronic pain remains a significant health issue that often diminishes one's quality of life and represents a large economic burden. In the dorsal root ganglia (DRG), specialized nociceptive sensory neurons are critical for the detection and transmission of noxious stimuli. Following injury or insult, nociceptors become sensitized, and exhibit increased cellular excitability, which represents a critical hallmark for the transition from acute to chronic pain[1]. Therefore, understanding the cellular and molecular mechanisms that regulate inflammatory sensitization is essential towards understanding the pathophysiology of chronic pain and developing more efficacious analgesics with limited adverse effects[2]. Using a recently developed human pluripotent stem cell-derived sensory neuron (hPSC-SN) differentiation protocol[2] and a cellular inflammation model comprised of a four-part cocktail of inflammatory mediators (inflammatory soup; hereby referred to as IS)[3], we show that our hPSC-SNs can be sensitized by 24 h treatment of using MEA recordings and through mass spectrometry (MS), identify specific proteins that were upregulated as a result of inflammation. Comparison of proteins identified by MS to be upregulated following 24 h IS with recently published pain-related databases such as the Priority Pain Genes Database, Human Pain Genetics Database, and the Dolorisk Group[4-6], we focused on the **DCC netrin 1 receptor (DCC)**, which has been previously shown to facilitate axonal elongation and sprouting, upregulated during inflammation, and play a role in neuropathic pain in rodent models[7,8].

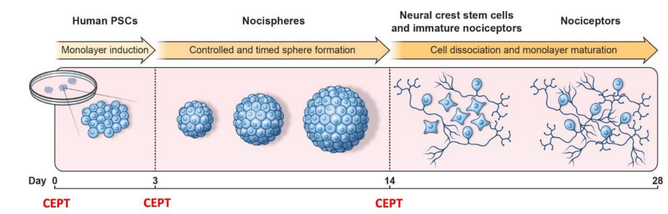


Adapted from Ji et al. (2014)

Objective: To characterize the subcellular expression and localization of DCC in hPSC-SNs in control and 24 h IS-treated groups using western blot and immunocytochemistry.

Materials and Methods

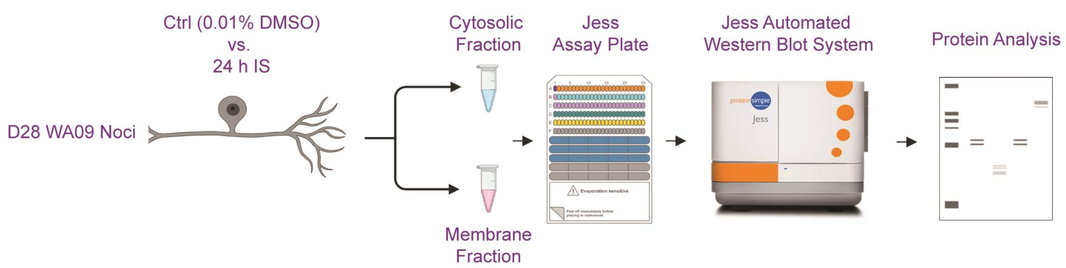
A hPSC-SN Differentiation



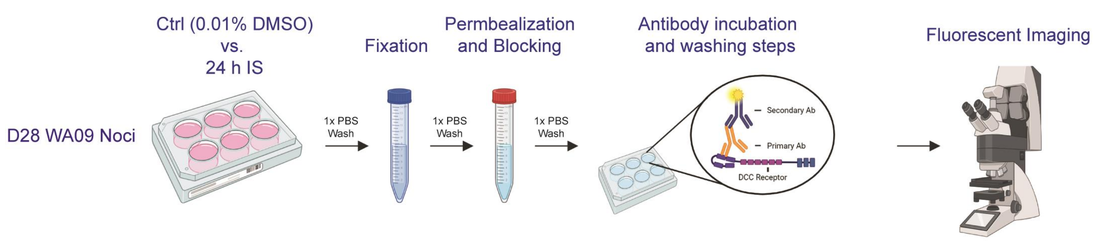
E6 medium	E6 medium	DMEM/F12 medium
0.2 μM CHIR98014	0.5 μM CHIR98014	N2 supplement
2 μM A 83-01	2 μM A 83-01	B27 supplement (w/o vitamin A)
	1 μM DBZ	BDNF/GDNF/NGF/NT-3 (25 ng/ml each)
	25 nM PD173074	1 μM PD0332991

Fig 1. Schematic Overview of hPSC-SN Differentiation and Experimental Methods. (**A**) hPSC-SNs are differentiated via monolayer induction (D0-D3), followed by formation and maturation of nocispheres (D3-D14), dissociation at D14, and further maturation in monolayer culture (D14-28). Adapted from Deng et al. (2023). (**B**) Flowchart depicting procedure for Western blotting experiments. (**C**) Flowchart depicting procedure for immunocytochemistry experiments.

B Western Blot



C Immunocytochemistry



Results

Mass Spectrometry Analysis of Sensitized hPSC-SNs

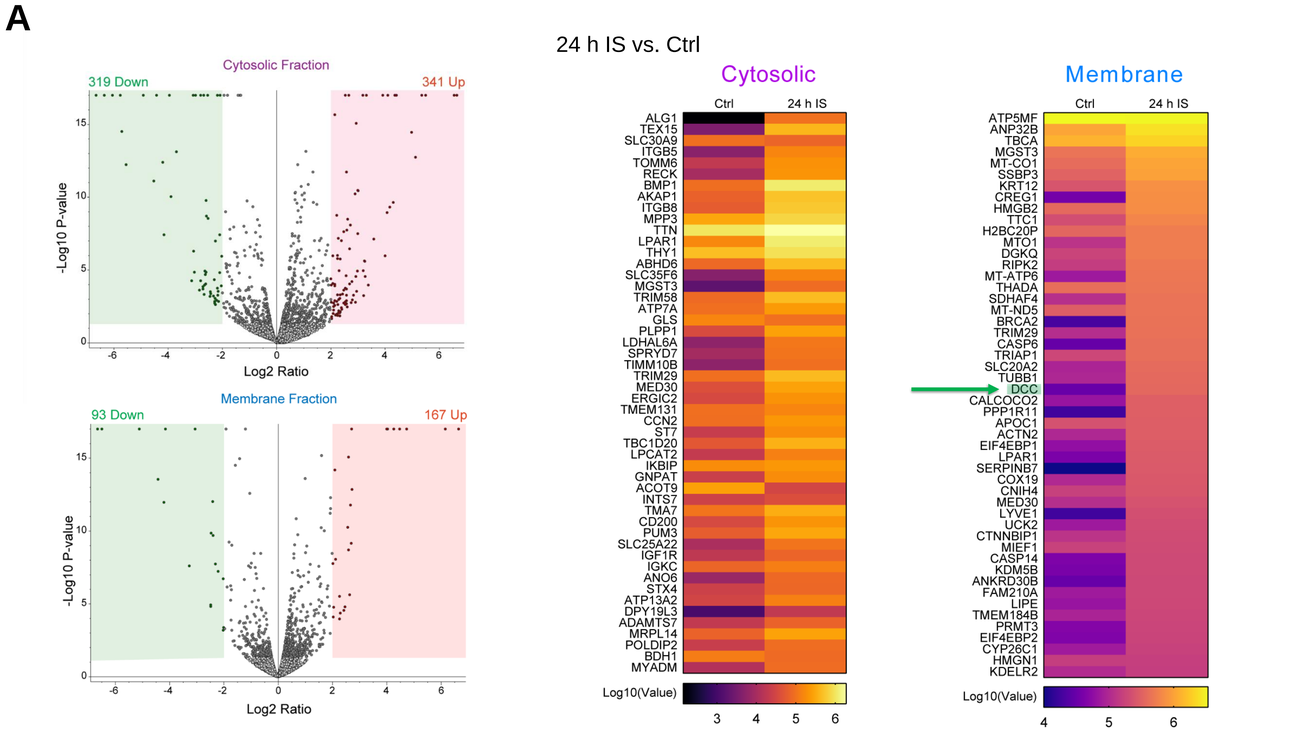


Fig 2. Proteomic Analysis Reveals Netrin-1 Receptor DCC is Upregulated after 24 h IS Treatment. (**A**) Volcano plots depicting significantly upregulated and down-regulated proteins in cytosolic and membrane subcellular fractionations from Ctrl and 24 h IS hPSC-SNs. (**B**) Heatmap of the top 50 upregulated proteins in cytosolic and membrane fractions following 24 h IS. DCC was found to be significantly upregulated in membrane compartments.

Membrane Fraction

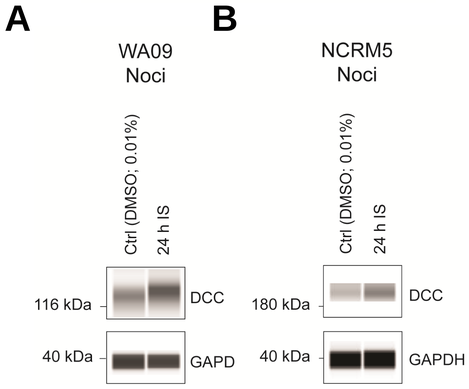


Fig 3. Western blot images showing upregulation of DCC after 24 h IS in WA09 and NCRM Noci. D28 WA09 (**A**) and NCRM5 (**B**) hPSC-SNs showing increased DCC expression in membrane subcellular fractions after 24 h IS treatment. Difference in DCC MW suggests different proteolytic cleaving processes or receptor isoforms between human embryonic and induced pluripotent stem-cell derived sensory neurons.

Immunocytochemistry of DCC

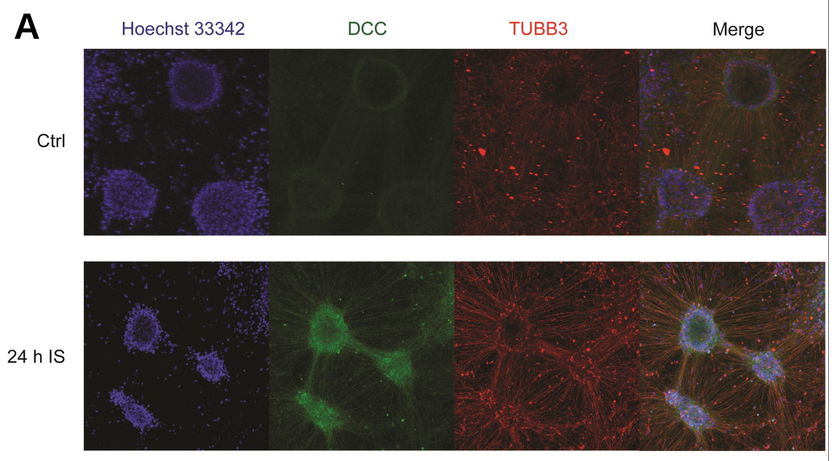


Fig 4. Immunocytochemistry of hPSC-SNs after 24h IS demonstrating increased DCC in WA09 Noci. (**A**) Ctrl and 24 h IS-treated WA09 D28 Noci were co-stained for Hoechst 33342 (blue), DCC (green), and TUBB3. DCC puncta appears to be more strongly expressed following 24 h IS treatment. TUBB3 staining of axons appears to exhibit stronger expression after 24 h IS treatment compared to Ctrl.

Summary and Future Directions

Here, we demonstrate that the Netrin-1 receptor, DCC, as previously shown from our mass spectrometry proteomic analysis, appears to be upregulated in hPSC-SNs following 24 h IS treatment as demonstrated by our western blot and immunocytochemistry experiments. Interestingly, though 24 h IS treatment appears to increase DCC expression in both WA09 and NCRM5 hPSC-SNs, we observed differences in DCC's molecular weight, suggesting distinct isoform expression between human embryonic and induced pluripotent stem-cell derived sensory neurons. In addition, DCC expression appears to be stronger after 24 h IS treatment in both somal and axonal regions of hPSC-SNs, and 24 h IS treatment also appears to facilitate axonal sprouting, as previously shown[7,8]. Though our initial findings suggest a putative role of DCC in regulating inflammatory sensitization, future replicates will be needed to confirm and quantify these findings. Overall, our work demonstrates DCC as a potential regulator of inflammatory sensitization and chronic pain. Future directions will be aimed at characterizing downstream signaling pathways of DCC, as well as high-throughput functional and drug screening assays to determine whether altering DCC receptor activity modulates sensory neuron activity and inflammatory sensitization, and can serve as a potential drug target for mitigating chronic pain.

References

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