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Supplementary Information for

An integrative approach to the facile functional classification of DRG neuronal subclasses

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Supplementary Information Text

SI MATERIALS AND METHODS

Constellation Pharmacology

Experiments were performed as described in detail previously [1-6] [7]. Briefly, lumbar DRG neurons from CGRP-GFP mice (in a CD1 genetic background) from ages P41-89 were dissociated by treating DRGs with trypsin followed by mechanical trituration. Cell suspension was filtered through 70 uM mesh, followed by 10-minute centrifugation at relative centrifugal force of 225g. The pellet obtained after centrifugation was resuspended in appropriate volume (~200uL) of culture media. Neurons were then plated into the center of a silicone ring that was previously attached to the floor of a 24-well poly-D-lysine coated plate. After allowing the cells to adhere to the floor of the plate for approximately one hour, 1 mL of culture media was added to each well. The culture media (MEM + supplements) consisted of minimal essential media (MEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 1x penicillin/streptomycin, 10 mM HEPES, and 0.4% (w/v) glucose, pH 7.4. The plated cells were placed in a 5% CO_2 incubator at 37 °C overnight. Cells were incubated with Fura2-AM dye for 1 hour at 37°C and 0.5 hour at room temperature. The fluorescence of 340nm/380nm excitation ratio (510 nm emission) was used as an indicator of the relative level of intracellular calcium in each cell while applying a set of pharmacological challenges over approximately an hour time course at room temperature. Exposure times ranges from 200-400 ms and each image was taken every 2 seconds for the duration of the experiment. Pharmacological challenges present in each experiment include allyl isothiocyanate (AITC) at 100 μM, menthol at 400 μM, capsaicin at 300 nM, K+ at 20mM and 40 mM, and RIIIJ at 1μM. Dendrotoxin K was used at 300 nM and 4-aminopyridine was used at 1 mM. Following the calcium imaging experiments, cells were incubated with Alexa-Flour 568 Isolectin B4 (IB4) at 2.5 μg/ml for 5 minutes at room temperature (~22 ^{o}C). Cells were then washed 3 times, incubated for 5 minutes with observation solution and washed 3 more times. Images were acquired using a Rhodamine filter set to identify IB4 positive non-peptidergic nociceptors.

Electrophysiological recordings from cultured DRG neurons

After performing calcium-imaging experiments on dissociated DRG neurons, cells of interest were chosen for current-clamp recordings in whole-cell mode. The intracellular pipette solution contained (in mM): 140 potassium aspartate, 13.5 NaCl, 1.8 $MgCl_2$, 0.09 EGTA, 9 HEPES, 14 creatine phosphate, 4 Mg-ATP and 0.3 Tris GTP. The pH of the solution was adjusted to 7.2 with KOH, and the osmolarity was adjusted to 290-300 mOsM with glucose. Frozen aliquots of this solution were thawed before use. The extracellular bath solution was the same as the DRG observation solution used for calcium imaging experiments and contained (in mM): 145 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1 Na-Citrate, 10 HEPES, and 10 Glucose. The pH of extracellular solution was adjusted to 7.4 with NaOH, and the osmolality was adjusted to 310-320 mOsM with glucose. The pipette resistance ranged from 3-5 MΩ. Cells with stable resting membrane potential below -40mV were used for recordings, which were made with a MultiClamp 700A amplifier and acquired with a DigiData 1440 digitizer. The amplifier and digitizer were under controlled by MultiClamp Commander and Clampex10.6 respectively (Molecular Devices, San Jose, CA).

Each DRG cell was stimulated by a series of 500-ms current injections from -100 pA to +200 pA in 25 pA steps, applied at a 0.2 Hz. The same current-pulse protocol was

performed in the presence of 1μM RIIIJ and upon peptide removal from the bath. The data was analyzed using clampfit and plotted in Graphpad Prism. Two-way ANOVA was used to test for the statistical significance using Graphpad Prism.

Simultaneous Ca^{2+} imaging and current clamp

Electrode Construction.

Patch pipettes were constructed from high-borate borosilicate capillary glass [WPI #1B150-4, 1.5-mm outer diameter (OD), 0.8-mm inner diameter (ID)] using a Flaming/Brown type puller (model P-97; Sutter Instruments). These pipettes had outside tip diameters of ~0.9–1.3 μm. Electrode tips are back-filled with a solution (pH = 7.4) consisting of (values in mM) 2 NaCl, 125 KCl, 2 $MgCl_2$-$6H_2O$, 10 HEPES and glucose at a concentration to bring the final osmolarity to approximately 290-300 mOsmol. These pipettes had resistances between 10 and 25 MΩ.

Whole-Cell Recording

The DRG neurons plated on a coverslip with polylysine-D coating were approached by a patch-electrode using a three-axis Microdrive (MPC-200) while maintaining positive pressure and applying -1nA square-wave pulses(2Hz) via Multiclamp 700 A (Axon Instruments) to monitor resistance. Cell contact was monitored both under the microscope in brightfield and via a small increase in the voltage change; upon contact, negative pressure was applied to the pipette to increase the resistance of the seal to giga-ohm levels (~1.5GΩ)[8]. Once the seal resistance has increased, we started measuring calcium influx using the ratio of bound to unbound fura-2. Either a sinewave current of ±1nA or negative pressure was used to rupture the patch and gain electrical access to the intracellular membrane potential. Digidata 1440A (Axon CNS) was used to acquire membrane potential and injected current in separate channels at a sampling rate of 10000Hz each. The resistance and capacitance of neuronal membrane were measured by injecting an occasional current pulse of -0.1nA and fitting a double exponential to differentiate between potential drop across pipette and cell[9].

Transgenic Mice

Two strains of transgenic reporter mice were used for identification of specific somatosensory neuronal subclasses: CGRP-GFP mice and PV-ires-Cre; Ai14 reporter mice. CGRP-GFP mice (strain name: STOCK Tg(Calca-EGFP)FG104Gsat/Mmucd) were created by the Gensat project as previously described [10]. In this mouse strain, GFP expression is driven by the gene-regulatory elements of calcitonin gene-related peptide (CGRP), which primarily labels peptidergic nociceptors in the somatosensory neuronal cell population. We crossed PV-IRES-Cre-ER driver mice (Jackson lab stock # 008069) with Ai14 reporter mice (Jackson lab stock #007908) to drive the expression of tdTomato in proprioceptive neurons in the progeny. All these mice were graciously provided by David Ginty lab.

Single Cell Transcriptomics

After constellation pharmacology experiments were performed, individual cells were picked using fire polished glass pipettes with optimized diameter. Cells were lysed and mRNA was reverse transcribed to generate cDNA, which then underwent whole transcriptome amplification, all using the QIAseq FX Single Cell RNA library kit

according to the manufacturer's standard protocol (Qiagen Sciences, 19300 Germantown Rd., Germanton, MD 20874). The amplified cDNA was used to construct a sequencing library for the Illumina NGS platform, also using the QIAseq FX Single Cell RNA library kit. The amplified cDNA was fragmented to 300 bp in size, treated for end repair and A-addition, followed by adapter ligation and then cleanup with Agencourt AMPure XP magnetic beads (Beckman Coulter Life Sciences, 350 Lakeview Parkway S. Drive, Indianapolis, IN 46268. The cDNA library was submitted to the HCI high-throughput genomics core facility (High Throughput Genomics Shared Resource, Huntsman Cancer Institute Room 1753, 2000 Circle of Hope, University of Utah, Salt Lake City, UT 84112) for library quality control and sequencing.

Methods from the sequencing core

Sequencing libraries (25 pM) were chemically denatured and applied to an Illumina HiSeq v4 paired end flow cell using an Illumina cBot. Hybridized molecules were clonally amplified and annealed to sequencing primers with reagents from an Illumina HiSeq PE Cluster Kit v4-cBot (PE-401-4001). Following transfer of the flowcell to an Illumina HiSeq 2500 instrument (HCS v2.2.38 and RTA v1.18.61), a 125 cycle paired-end sequence run was performed using HiSeq SBS Kit v4 sequencing reagents (FC-401-4003).

Bioinformatics Methods (Alignment and QC)

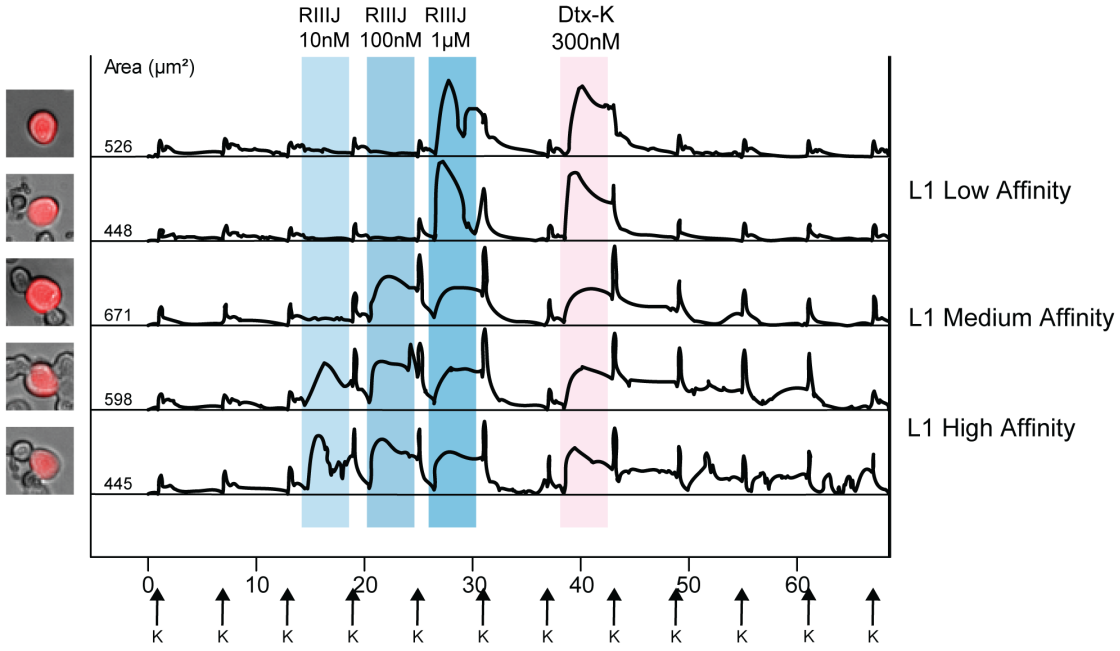
Illumina adapters were trimmed from the paired-end (mate length 125-bp) reads by cutadapt (v1.16)[11]. Reads and adapters were required to overlap a minimum of 6 bp for trimming to take place and reads shorter than 20 bp were discarded. Adapter trimming was followed by initial sample quality control metrics were generated with FASTQC (v0.11.5)[12]. Trimmed reads were aligned with STAR (v 2.5.4a)[13] using the ENCODE parameterization. Additional alignment metrics were generated with Picard's 'CollectRNASeqMetrics' utility (v2.9.0)[14] and QoRTs' (v1.3.0)[15]. Finally, the QC metrics were collated with multiqc (v1.6)[16].

In house bioinformatics

Custom R[17] scripts are used to test for transcriptional differences in gene and isoform counts using a variety of statistical techniques including but not limited to: DSEQ2[18], logistic regression, random forest[19], rpart[20], t-test. Resampling techniques (bootstrap and permutations) are used to control for false positives. Significant differences between cell categories for specific genes/isoforms are then tested in validation assays.

<insert page break then Fig. S1 here>

Fig. S1. Heterogeneity in RIIIJ response within L1 large diameter DRG neurons.



L1 responded to RIIIJ with varying affinity. Those with low affinity for RIIIJ responded only to concentration of RIIIJ ≥ 1 uM with a smooth DE. L1 neurons with medium RIIIJ affinity responded with a smooth DE only at concentrations ≥ 100 nM. Subclass one neurons with high affinity for RIIIJ responded with a smooth DE at all concentrations. In all cases Dtx-K replicated the smooth DE seen in the presence of 1uM but in the neurons with high RIIIJ affinity the $[Ca^{2+}]_i$ was much slower to return to baseline.

Table S1. Variability in Subclass Distribution from Individual DRG Cultures

Cell culture:	Age (days)/Sex	Total Neurons	# Neurons Assigned to Subclass L1	L2	L3	L4	L5	L6
1	43/m	772	12	5	10	11	19	11
2	47/f	919	8	20	10	8	1	10
3	53/f	1114	11	12	9	21	9	22
4	56/m	1140	14	9	24	11	15	24
5	66/f	1427	12	13	3	14	7	17
6	89/m	1788	14	7	12	29	10	26
Total, all cultures		27,216	174	166	198	285	144	340

Calcium-imaging experiments were conducted on various DRG cell cultures? . Selected results are summarized here to illustrate the variability in Subclass distributions from different DRG cell cultures, which also indicates that neuronal L1-6 were identified in all of our cell cultures prepared for this study. The results were analyzed and summarized as presented in the main text, Figure 2 and here. Cultures from mice of different ages were chosen to show the full age range of animals analyzed. The six Subclasses defined in this work were characterized using large-diameter DRG neurons, with cross-sectional cell area >500 μm^2 as the arbitrary lower size limit. The striking phenotypes elicited by LI and L2 allowed us to identity putative L1 and L2 DRG neurons with cell somas smaller than the designated size threshold, which are otherwise indistinguishable in their phenotypes from the DRG neurons >500 μm^2 used to define these Subclasses. This analysis suggests that 44% of L1 neurons and 22% of L2 neurons were smaller than the 500 μm^2 threshold; in the case of the L1 neurons, the frequency of small proprioceptors increased with the age of the mice in the sample analyzed (if only cultures from mice under 57 days of age were included in the analysis, the frequency of L1 neurons below 500 μm^2 fell to 32%). Thus, the true number of neurons that belong in each Subclass defined in Fig. 2 and summarized here are probably an underestimate in every case, since there are neurons in each class with cell somas smaller than the lower size threshold of 500 μm^2<insert page break here>

Table S2. **Summary of labeled proprioceptor experiments**

Cell culture:	Age (days)/Sex	Total Neurons	tdTomato+	L1
1	167/f	963	6	5
2	167/f	1272	30	29
3	60/f	714	12	10
4	60/f	1001	15	13
5	52/m	832	23	22
6	52/m	1248	21	15
7	40/f	488	32	22
Total, all cultures		6518	139	116

Neurons from 7 wells of td-Tomato parvalbumin labeled mice were analyzed by calcium imaging. Parvalbumin is an indicator of proprioceptive neurons in the DRG. A small portion of total neurons from each well expressed tdTomato. All neurons identified as L1 by the RIIIJ smooth DE expressed the tdTomato. 83% of the neurons expressing tdTomato also responded to the presence of RIIIJ with a smooth DE.

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