Comparative functional expression of nAChR subtypes in rodent DRG neurons

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INTRODUCTION

Progress in investigating the mammalian nervous system has largely been achieved by molecular and systems neuroscientists at two very disparate levels. In between the molecular and systems levels, there is a forbidding complexity that arises from the many functionally divergent subclasses of neurons present at each anatomical locus. The complexity of cellular function is created, in part, by the differences in expression of many individual genes, across even closely related neuronal subclasses. Functional complexity is also created because individual gene products can combine to form various heteromeric receptor- and ion-channel subtypes in different neuronal subclasses. These factors create the functionally divergent neurons with distinct physiological roles found at any anatomical locus of the nervous system.

The molecular complexity is exemplified by nicotinic acetylcholine receptors (nAChRs), a family of ligand-gated ion channels. Functional nAChRs are pentameric, with 16 genes (in mammals) encoding different nAChR subunits. Because the subunits may be assembled into various combinations of heteromeric or (in some cases) homomeric combinations to form the functional pentameric ion channel, an enormous number of potential nAChR receptor subtypes are possible, with each combination that generates a functional receptor having a potentially distinct physiological function. The conventional approach to understand gene function, through targeted gene deletions (i.e., mutations in knockout mice or other model organisms), has limited value in this situation: ablation of a specific gene does not just result in the loss of function of one nAChR subtype, but of all functional nAChRs containing the subunit encoded by that gene. For example, if the α4 subunit is knocked out, then every functional nAChR that contains an α4 subunit (e.g., α4β4, α3β4, α6β4, α4β3, α4β2, etc.) would be functionally knocked out. This fact highlights the need for a pharmacological approach to selectively perturb the function of specific nAChR subtypes, in order to elucidate the physiological roles of each.

In this report, we have employed an approach to subdivide large, heterogeneous neuronal populations into subclasses by their functional expression of specific receptor- or ion-channel subtypes. Such neuronal subclasses (defined by expression of a single gene) could eventually be further subdivided into specific

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neuronal cell types (defined by a particular physiological function and expression of a cell-specific combination of genes), by cross-correlating the functional expression of several different genes across many individual neurons. The basic strategy is to monitor functional activity of specific receptor- and ion-channel subtypes in more than 100 individual cells simultaneously from a heterogeneous cell population, as we described previously (Teichert et al., 2012a,b). Using this experimental approach, we characterized dissociated rat and mouse lumbar dorsal-root ganglion (DRG) neurons that express functional nAChRs, and identified the particular nAChR subtypes expressed in different neuronal subclasses. In principle, this approach will allow us to investigate the various roles of different nAChR subtypes expressed in different somatosensory neurons. For example, we seek to understand the roles of α7 or α8A nAChRs in modulating different sensory modalities (e.g., sensations of pain, itch, temperature or touch) in the various neuronal subclasses responsible for transmitting these signals from the periphery to the central nervous system. This work represents a critical first step toward that goal.

MATERIALS AND METHODS

PREPARATION OF SOLUTIONS

The medium for culturing DRG neurons, “MEM + supplements,” was as follows: minimal essential media (MEM, from Invitrogen [Life Technologies]), was supplemented with 10% fetal bovine serum (FBS, from HyClone), penicillin (100 U/mL), streptomycin (100 μg/mL), 1× Glutamax (from Invitrogen), 10 mM HEPES, and 0.4% (wt/vol) glucose. The medium was adjusted to a pH of 7.4 with NaOH, filtered through a 0.22 μm filter under sterile conditions, and stored at 4°C until shortly before use, when it was allowed to warm to 37°C in a tissue-culture incubator with 5% CO2 atmosphere.

The “observation solution” (bath solution) for calcium-imaging experiments consisted of (in millimolar): 145 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 1 sodium citrate, 10 HEPES, and 10 glucose. A 1× stock of observation solution was prepared with penicillin-streptomycin at 100 U/mL and 100 μg/mL, respectively and stored at 4°C. No additional penicillin-streptomycin was added to the 1× observation solution. Sodium citrate and glucose were added to the 1× solution to yield their final concentrations given above. The 1× solution was adjusted to a pH of 7.4 with NaOH and stored at 4°C until used at room temperature.

Hanks’ balanced salt solution (HBSS), HEPES, and 2.5% trypsin were purchased from Invitrogen. DNase (II) type-I, collagenase-A, and acetylcysteine chloride (ACH) were purchased from Sigma Aldrich. α-Conotoxin GVIA, and α-conotoxin MVIIIC were purchased from Tocris Biosciences. All α-conotoxins were synthesized as previously described (Cartier et al., 1996). All stock solutions of pharmacological agents were diluted into observation solution to yield their final working concentrations described in Results and in the figures. Stock solutions were as follows: 1 M ACh in water; 200 μM α-conotoxin ATV [V11L/V16D] in observation solution; 50 μM α-conotoxin AuIB in observation solution; 10 μM α-conotoxin BuIB[TS5A, P6O] in observation solution; 200 μM α-conotoxin GVIA and MVIIIC in observation solution; 10 mM Nicardipine HCl in DMSO; Stock solutions of 100 mM PNU-120586 were prepared in DMSO and then completely diluted in one step into observation solution at 50°C while stirring to make 1 or 5 μM working concentrations. Fura-2-acetoxyethyl ester (Fura-2-AM, from Invitrogen) was dissolved in DMSO to produce a 1 mM stock solution, which was distributed into single-use aliquots and stored at −20°C. Fura-2-AM was used at 2.5 μM working concentration as described below.

PREPARATION OF 24-WELL PLATES

Silicone rings were cut with cork borers from 0.3 or 0.25 mm thick silicone sheets (Grace BioLabs). Each ring had an outer diameter of ∼14 mm and an inner diameter of ∼4 mm. The rings were washed sequentially with 70% ethanol, deionized filtered water, 100, and 70% ethanol. They were then autoclaved and dried. Each silicone ring was placed on the floor of a well of a poly-o-lysine coated 24-well tissue-culture plate (BD Biosciences) and sealed to the floor with gentle pressure applied with the tips of a pair of dull forceps. The exterior wells were not used for cultures; instead those wells (without silicone rings) and spaces between wells were half-filled with sterile distilled water to humidify the atmosphere above the plate.

For some experiments, the plate floor in the center of each silicone ring was coated with mouse laminin (BD Biosciences) by applying 30 μL of 10 μg/mL laminin, dissolved in Hank’s Balanced Saline Solution (HBSS). Laminin was not used for experiments that directly compared mouse and rat DRG cells. The plates were then placed in a 37°C tissue-culture incubator for ∼2 h to allow adequate time for laminin to coat the floor of each well. The laminin solution was replaced with MEM + supplements just prior to plating cells. This was done by aspirating the laminin solution and immediately adding 30 μL of MEM + supplements. Cells in suspension were added to the center of each silicone ring, onto the floor of the well, in the same manner (described in more detail below).

PREPARATION OF DRG CELLS

All procedures complied with the rules and regulations in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the institution’s Animal Care and Use Committee (IACUC) of the University of Utah Health Sciences Center. In all cases, rats were used were Sprague–Dawley and mice used were C57BL/6.

Several of the initial preparations of rat DRG cells were conducted as follows. After the rats were sacrificed with CO2, the lumbar dorsal root ganglia (L1-L6) were removed and placed in ice cold HBSS buffered with 10 mM HEPES, pH 7.2 (dissection buffer). The nerve roots were trimmed and the ganglia were then bisected or quartered, after which they were transferred to a 15 ml conical tube with 2 ml of dissection buffer containing 0.1% (wt/vol) collagenase-A and 0.25% (wt/vol) trypsin. The ganglia were incubated at 37°C for 60 min in the dissection solution containing enzymes, rinsed once with dissection solution, and then mechanically dissociated by trituration through a series of Pasteur pipettes of decreasing tip diameter (prepared by heating the
tip in a flame while rotating the pipette) in solution containing 5 mM MgCl₂ and 10 µg/mL DNase (II) type-I. The volume of the cell suspension was increased to 10 mL with dissection solution and then passed through a 70 µm cell strainer to remove large pieces of tissue. At this point, the cells were collected by centrifugation at 200 × g for 2 min, after which the supernatant was removed by aspiration and the cells were resuspended in MEM + supplements at a volume (typically ∼270 µL) and density suitable for plating cells in the previously prepared 24-well plates.

For experiments used to compare mouse and rat DRG cells, the following methods were applied consistently in preparing cell plates. First, the lumbar dorsal root ganglia were not coated with laminin, and no growth factors were added to MEM + supplements (without FBS) also containing 2.5 µM Fura-2-AM, which was freshly prepared by thawing the single-use stock aliquot of 1 mM Fura-2-AM in DMSO and adding it to MEM + supplements (without FBS), followed by vigorous vortexing for ∼20 s. The plate was placed in the 37°C incubator for 1 h and then at room temperature for 30 min to load the cells with Fura-2-AM dye, prior to calcium imaging. At this time, the media was replaced in each well with fresh MEM + supplements (at room temperature) without Fura-2. Just prior to imaging a particular well, MEM + supplements were replaced with observation solution (at room temperature) at least three times to completely remove free Fura-2-AM from the well.

**CELL CULTURE**

Typically, 30 µL of the cell suspension was then added to the center of the silicone ring in each well of a 24-well plate, which was previously prepared as described above. Each 24-well plate was then placed in the 37°C, 5% CO₂ tissue-culture incubator, and the cultures were resuspended at a volume (typically ∼270 µL) and density suitable for plating cells in the previously prepared 24-well plates.

**LOADING CELLS WITH FURA-2-AM**

After culturing the cells overnight, the 24-well plate was placed in a sterile tissue-culture hood. The 1 mL of MEM + supplements in each well was agitated by pipetting it up and down vigorously in the well to suspend all dead cells and dislodge any cells that were only loosely adherent. In general, the remaining adherent cells were viable DRG neurons and glia. The medium was replaced with 500 µL of fresh MEM + supplements (without FBS) also containing 2.5 µM Fura-2-AM, which was freshly prepared by thawing the single-use stock aliquot of 1 mM Fura-2-AM in DMSO and adding it to MEM + supplements (without FBS), followed by vigorous vortexing for ∼20 s. The plate was placed in the 37°C incubator for 1 h and then at room temperature for 30 min to load the cells with Fura-2-AM dye, prior to calcium imaging. At this time, the media was replaced in each well with fresh MEM + supplements (at room temperature) without Fura-2. Just prior to imaging a particular well, MEM + supplements were replaced with observation solution (at room temperature) at least three times to completely remove free Fura-2-AM from the well.

**VIDEO MICROSCOPY**

Images were obtained either with: (1) a 10×0.5 NA objective on an inverted Nikon Diaphot 200 microscope or (2) with a 10×0.4 NA objective on an inverted Olympus IX70 microscope with a reducing lens in front of the camera to image a larger field of view. With both microscopes, a Sutter Instruments Lambda LS light source (300-W Xenon arc lamp) fitted with a filter wheel and shutter (controlled by a Lambda 10-8 Smart Shutter, Sutter Instruments), was used as the source of excitation light at 380 and 430 nm. Images were acquired with a Nikon Digital Sight DS-Q1MC camera and controller and Nikon NIS elements software.

After loading cells with Fura-2-AM, a 24-well plate was fastened to the microscope stage. A brightfield image of a single field of view was captured and used to select regions of interest (ROIs) corresponding to a single cell, were monitored for changes in [Ca²⁺]ᵢ. Typically, ∼100 neurons were imaged for each experiment. The fluorescence emission was monitored at 510 nm for both 380 and 340 nm excitation. The exposure time for resting cytosolic calcium levels was adjusted for each experiment to a maximum ROI intensity of ∼3500 gray levels for 380 nm excitation and ∼1000 gray levels for 340 nm excitation. An image was captured at each excitation wavelength and the ratio of fluorescence intensities at excitation wavelengths of 340 and 380 nm was acquired either once per second or once per 2 s to monitor the relative changes in calcium concentration in each cell as a function of time.

**EXPERIMENTAL PROTOCOLS**

Calcium signals were elicited by a ∼15-µs application of 1 mM ACh (ACh pulse), in observation solution, as follows: the observation solution was aspirated from the well with a peristaltic pump controlled by a foot pedal, and observation solution containing 1 mM ACh was applied manually at the edge of the well from a pipette tip with a silicone tubing extension, the flexibility of the latter minimized any movement of the plate. After 15 s, the ACh solution was removed by aspiration and the cells were resuspended in 1 or 2 mL of MEM + supplements (without FBS), followed by vigorous vortexing for ∼20 s. The plate was placed in the 37°C incubator for 1 h and then at room temperature for 30 min to load the cells with Fura-2-AM dye, prior to calcium imaging. At this time, the media was replaced in each well with fresh MEM + supplements (at room temperature) without Fura-2. Just prior to imaging a particular well, MEM + supplements were replaced with observation solution (at room temperature) at least three times to completely remove free Fura-2-AM from the well.
was replaced completely with observation solution in the same manner. Typically, the observation solution was replaced three or four more times over the subsequent 45 s to remove any residual ACh from the well. This procedure was repeated as necessary, generally at intervals ranging from 5 to 8 min. In some cases, a high concentration of extracellular potassium (e.g., 30–50 mM [K+]o) was added to the bath at the beginning or end of each trial to help differentiate between neuronal and non-neuronal cells. Non-neuronal cells did not respond to depolarization by high [K+]o. Additionally, the soma of non-neuronal cells in these cultures are smaller in diameter than the somas of DRG neurons. A pulse of high [K+]o was applied to the bath in the same manner as an ACh pulse described above, with identical washing procedures. The ~15 s applications of ACh or [K+]o were represented in the figures by arrows at each respective time point, where the letter “X” represents ACh application and the letter “K” represents application of high [K+]o. Horizontal bars in each figure represent the application of other pharmacological agents to the bath solution for the duration of the bar (on the time scale of the X axis), as described in figures and figure legends. The experimental protocol shown at the bottom of each figure (or panel) corresponds to all calcium-imaging traces shown in that figure (or panel).

**STATISTICAL DATA ANALYSIS**

The statistical data analysis of capsaicin sensitivity across DRG neuronal subclasses was performed as follows. We conducted 10 independent experimental trials for mouse DRG and 8 independent experimental trials for rat DRG. For each independent experimental trial, we executed the same experimental protocol in a different well containing a mixed population of DRG cells. Only neurons that responded to depolarization by 30 mM [K+]o were included in the data analysis. Neurons were parsed into different subclasses by functional nAChR expression as described in the Results. From each independent experimental trial, we calculated the percentage of capsaicin-sensitive DRG neurons within each neuronal subclass (sample means). These sample means were then compared to each other by single-factor Analysis of Variance (ANOVA) using Microsoft Excel. Significant differences were set at p-value < 0.05.

**RESULTS**

The experiments in this study were carried out with dissociated rat or mouse lumbar DRG neurons loaded with Fura-2 dye for calcium imaging. Several related experimental protocols were used to assess the functional expression of particular nAChR subtypes, as illustrated in the figures and as described in Materials and Methods. In general, pulses of 1 mM acetylcholine (ACh) were applied to a heterogeneous population of dissociated DRG neurons at regular time intervals to elicit transient increases in cytoplasmic-calcium concentration, [Ca2+]i, observed as peaks in the traces in each figure. Typically the responses of 50–150 neurons were imaged individually and simultaneously. ACh-elicited calcium signals observed prior to application of nAChR antagonists served as controls. ACh-elicited calcium signals observed following application of subtype-selective nAChR antagonists were used to identify the nAChR subtypes that were functionally expressed in each cell. The nAChR antagonists used in the experiments are summarized in Table 1. These are α-conotoxins with high selectivity for specific nAChR subtypes.

**IDENTIFICATION OF A RAT DRG NEURONAL SUBCLASS THAT FUNCTIONALLY EXPRESSES nAChRs CONTAINING α4 SUBUNITS**

Each trace shown in Figure 1 represents a single neuron's response. In Figure 1, calcium-imaging traces from three ACh-responsive DRG neurons are shown (bottom three traces), and from two non-ACh-responsive neurons (top two traces), all from a single experimental trial (one field of view from a single well). Following the application of 200 nM α-conotoxin ArIB[V11L;V16D] (hereafter ArIB[V11L;V16D]), an nAChR antagonist selective for the α7 subtype (Table 1; Whiteaker et al., 2007), there was no obvious effect on the ACh response. In contrast, the addition of 10 μM α-conotoxin BuIA (BuIA), a broad-spectrum antagonist of nAChRs (Table 1; Azam et al., 2003), resulted in complete inhibition of the ACh response (Figure 1). BuIA blocks most nAChR subtypes with rapid reversibility, but its inhibition of nAChRs that contain a β4 subunit is very slowly reversible (Azam et al., 2003). The apparently irreversible block (over the time interval shown in Figure 1) suggested that a β4-containing nAChR was likely to be the predominant nAChR subtype in a subset of cultured rat DRG neurons.

**UNMASKING A RAT DRG NEURONAL SUBCLASS THAT FUNCTIONALLY EXPRESSES α7 nAChRs**

Previous studies reported that α7 nAChRs are expressed in DRG neurons. This was demonstrated directly using standard electrophysiological techniques in a complementary study (Hone et al., 2012). However, using the protocol shown in Figure 1, there was no ACh response consistent with the α7 nAChR subtype. A possible reason for the failure to detect α7 nAChRs in that calcium transients elicited by the opening of α7 receptors are too small to be detectable using standard calcium-imaging methods, because of the rapid desensitization kinetics of α7 nAChRs. In

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**Table 1** | IC50 values for inhibition of nAChR subtypes by various α-conotoxins and their analogs (adapted from Hone et al., 2012).

| Subunit | α3β2 | α3β4 | α4β2 | α4β4 | α3β3/α2β3 | α3β4 |
|---------|------|------|------|------|------------|------|
| ArIB[V11L;V16D] | >20 μM | >20 μM | >20 μM | >20 μM | >20 μM | >20 μM |
| BuIA | 5.7 nM | 277 nM | >10 μM | 69.9 nM | 258 μM | 1.5 nM |
| BuIA[TS4-PHO] | >10 μM | 12 μM | >10 μM | >10 μM | >10 μM | 58.1 nM | >10 μM |
| AuIB | >100 μM | 750 nM | >100 μM | >100 μM | >100 μM | 73 μM | >10 μM |

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FIGURE 1 | Functional expression of nAChRs that contain β4 subunits in a subset of rat DRG neurons. One mM ACh was applied to dissociated rat DRG neurons at 8 min intervals as described in Section "Materials and Methods." This abbreviation applies to all subsequent figures. At the end of the experiment, 50 mM [K+]o (indicated by arrow above letter "K") was applied to the cells to elicit a calcium signal through a membrane depolarization. Cells that did not respond to 50 mM [K+]o were excluded from the analysis, because they were considered to be non-neuronal cells or non-viable neurons. The top two traces are from neurons that did not respond to ACh but did respond to 50 mM [K+]o. The bottom three traces are from neurons that responded to ACh and 50 mM [K+]o. At 10 min, after two ACh pulses, 200 nM ArIB[V1L;V16D], which selectively inhibits α7 nAChRs, was applied to the bath solution for the duration of the experiment, as indicated by a long horizontal bar. After the cells were exposed to ArIB[V1L;V16D] for 7 min, an ACh pulse was applied at 17 min. Very little, if any, block was observed after application of the α7-selective peptide. At 18 min, 10 μM BuIA was applied to the bath for 7 min as indicated by a short horizontal bar. This peptide is a broad-spectrum blocker of nAChRs. However, BuIA inhibition of most nAChRs is rapidly reversible; in contrast, it is very slowly reversible from nAChRs that contain β4 subunits. The apparently irreversible block of the ACh-responsive neurons by BuIA suggested that the predominant nAChR subtype in rat DRG neurons is a β4-containing nAChR.

In order to increase the magnitude of change in [Ca2+]i, we applied a positive allosteric modulator specific for α7 receptors, PNU-120596 (PNU). Experimental results in the presence of PNU are shown in Figure 2.

In Figure 2A, four cells (top four traces) responded to ACh directly, prior to the addition of PNU. Consistent with the experiment in Figure 1, their ACh responses were inhibited irreversibly over the time frame of the experiment by the addition of 10 μM BuIA. After 1 μM PNU was added, some neurons that previously did not respond to ACh began to respond robustly to ACh.
We employed the protocol shown in Figure 2A, bottom three traces). All ACh responses that were elicited in the presence of PNU (following application of BuIA) were blocked by 200 nM ArlB[VI14L;V16D]. As shown in Figure 2A, bottom three traces), a slow recovery of ACh responses was observed after washout of ArlB[VI14L;V16D].

These experiments suggested that DRGs contained two major subclasses of ACh-responsive neurons. The first subclass appeared to express predominantly β4-containing nAChRs. These responded directly to ACh in the absence of PNU and their ACh responses were blocked by BuIA irreversibly over the time course of the experiment. The second subclass of DRG neurons appeared to express predominantly α7 nAChRs. These responses to ACh only in the presence of PNU, and their ACh responses were blocked reversibly by ArlB[VI14L;V16D]. In most cases, neurons that responded to ACh prior to application of PNU did not respond to ACh in the presence of PNU after BuIA was applied. Thus, the data suggest that ACh-responsive DRG neurons express predominantly either α7 nAChRs or β4-containing nAChRs, or β4-containing nAChRs without substantial α7 expression (Table 2).

A DISTINCTIVE RAT DRG NEURONAL SUBCLASS THAT EXPRESS
BOTH α7 AND β4-CONTAINING nAChRs

We employed the protocol shown in Figure 2B multiple times in different trials, while simultaneously monitoring the responses from >50 individual DRG neurons in each trial. Notably, the experimental protocol in Figure 2B was done in the presence of a cocktail of voltage-gated calcium channel (VGCC) blockers, further supporting the evidence that the observed responses were mediated by nAChRs.

A minor fraction of the rat DRG neurons appeared to express both β4-containing nAChRs and α7 nAChRs (Figure 2B, top trace). Thus, the experiments establish four subclasses lumbar DRG neurons with respect to expression of nAChRs: (1) those

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### Table 2 Percentages of the total population of mouse and rat DRG neurons that exhibited functional expression of different nAChR subtypes.

|               | Mouse | Rat |
|---------------|-------|-----|
|               | P14   | P15–17 | P30–40 | P52 |
| % α7+ only    | 11    | 7     | 14      | 20   |
| % β4+ only    | 8     | 6     | 9       | 34   |
| % α7+ and β4+ | 3     | 2     | 5       | 15   |
| % ACh-unresponsive | 78 | 86   | 72      | 31   |
| % P14         | 45    | 46   | 48      | 47   |
| % P30–40      | 51    | 54   | 51      | 54   |
| % P52         | 51    | 54   | 51      | 54   |

*p* refers to the age of animals in postnatal days. For each age category of mouse or rat (column in the table), the percentages of DRG neurons were calculated from 5-9 independent experimental trials that encompassed >600 total neurons (cells responsive to depolarization by high extracellular potassium concentration) in each case (each column).

DRGs from mouse and rat include the same four neuronal subclasses with respect to AChR expression, but in substantially different proportions.

### Figure 3

Figure 3 demonstrates that mouse DRG neurons include those that express predominantly β4-containing nAChRs, (2) those that express predominantly α7 nAChRs, (3) those that express both α7 and β4-containing nAChRs, and (4) those that do not respond to ACh in the presence or absence of PNU (Table 2).

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**Species difference in capsaicin sensitivity among neuronal subclasses.**

After obtaining the data for Table 2, we then investigated the capsaicin sensitivity of each neuronal subclass defined by nAChR expression from mature mouse and rat DRG. Capsaicin activates the TRPV1 channel and may demarcate nociceptive neurons (Caterina et al., 1997; Caterina and Julius, 2001). In mouse DRG, we did not observe significant differences in the percentage of capsaicin-sensitive neurons across neuronal subclasses defined by nAChR expression (Table 3, ANOVA p-value = 0.65). However, in rat DRG, we did observe significant differences in...
ANOV A appears to be enriched for large-diameter neurons in both mouse and rat DRG (Table 3), it was evident that this neuronal subclass included both small- and large-diameter neurons (not shown).

**IDENTIFICATION OF α SUBUNITS PRESENT IN β4-CONTAINING nAChRs**

For the β4-expressing neurons, we wanted to identify the α subunits co-expressed with β4 to form functional ion channels. The experiment shown in Figure 4, using rat DRG neurons, was carried out with a-conotoxin AuIB (AuIB), which selectively inhibits α3β4 and α4β4 nACHRs over α3β4 and α4β2 nACHRs (Table 1; Luo et al., 1998). If the ACh-responsive cells were inhibited by 50 μM AuIB, this would suggest that the nACHr contained α3 or α6 subunits, but if they were not inhibited by AuIB, the α4 subtype might be present.

The experiment shown in Figure 4 demonstrates that the application of 50 μM AuIB did indeed inhibit the ACh responses in the β4-expressing subclass (top four traces). However, in contrast to the effect of BuIA, the inhibition was readily reversible upon washing, as expected for block of α3β4 and α4β4 nACHRs by AuIB. The reversible inhibition by AuIB, and the irreversible inhibition by BuIA, cumulatively suggest that the predominant β4-containing subtypes of nicotinic receptors present in DRG neurons are either α3β4 or α4β4 (or a combination of both subtypes). Notably, Figure 4 (top trace) also demonstrates that the subclass expressing both α3β4/α4β4 and α7 nACHRs can be detected with a different experimental protocol than the one employed in Figure 2B (top trace).

We then used 500 nM a-conotoxin BuAt[β4α3] (here-after BuAt[β4α3]) to selectively block α4β4 over α3β4 nACHRs (Table 3; Azam et al., 2010). BuAt[β4α3] partially blocked ACh-elicited responses from β4-expressing mouse DRG neurons (Figure 5). Similar results were obtained with rat DRG neurons, suggesting that α4β4 nACHRs mediate a portion of the ACh-elicited calcium signal in these neurons. However, the partial block by 500 nM BuAt[β4α3] also suggested that there is another nACHr subtype contributing to the ACh-elicited responses. The complete block of ACh-elicited responses by 50 μM AuIB (Figure 4) suggested that the second component is mediated by α3β4 nACHRs. Although there is no concentration of BuAt[β4α3] that is completely selective for α3β4 over α3β4 nACHRs (Table 1), it was apparent that the mix of α3β4 and α4β4 varied between cells (Figure 5). All ACh responses elicited in the absence of PNU were blocked in varying degrees by BuAt[β4α3]. Greater block suggested higher expression of α4β4 relative to α3β4.

**SHOULDS OF CALCIUM TRANSIENTS MEDIATED BY VOLTAGE-GATED CALCIUM CHANNELS AND MITOCHONDRIAL Na+/Ca2+ EXCHANGE**

In the experiment shown in Figure 2A, considerable variability was observed in the decay kinetics of the ACh-elicited calcium signals in different neurons. Following a pulse of ACh, in some neurons [Ca2+]i returned to baseline relatively rapidly (sharp peaks, e.g., top trace), while in other neurons [Ca2+]i returned to baseline slowly (broad peaks, e.g., fourth trace from top), often with characteristic shoulders observed on the peaks. As shown in the top three traces of Figure 2B, the peaks were notably sharpened in the caspase sensitivity of these neuronal subclasses (Table 3, ANOVA p-value < 0.001). These data suggest that in rat DRG the neuronal subclass that expresses only α7 nACHRs is significantly enriched for TRPV1 expression (94% of these neurons were caspase sensitive), whereas the neuronal subclass that expresses only β4-containing nACHRs included a significantly lower percentage of TRPV1-positive neurons (36%) than the other three neuronal subclasses (Table 3). Although the neuronal subclass that expresses both α7 and β4-containing nACHRs appears to be enriched for large-diameter neurons in both mouse...
cells that responded to ACh prior to application of PNU, when a cocktail of VGCC inhibitors was present in the bath solution. The variation in the shape of the peaks apparently was caused (in the absence of PNU) by a membrane depolarization through nAChR activation that was sufficient to trigger the activation of VGCCs to variable extents.

In some cases, the broad shoulders of the peaks mediated by α7 nAChR activation (with PNU) persisted in the presence of VGCC blockers (Figures 2 and 4). In these cases, the response profile hypothetically may be due to the release of calcium from internal calcium stores, triggered by the opening of the α7 nAChR. With VGCC blockers in the bath solution, such ACh-response profiles were only observed in the α7-nAChR-expressing neurons in the presence of PNU. In DRG neurons, shoulders of calcium transients elicited by membrane depolarization were previously shown to be mediated by a mitochondrial Na\(^+\)/Ca\(^{2+}\) exchanger (NCX; Baron and Thayer, 1997; Castaldo et al., 2009). Upon strong depolarization, the increase in cytoplasmic Ca\(^{2+}\) is translocated into the mitochondria via a mitoch

### Table 3 | Average percentages of neurons responsive to 300 nM capsaicin and their average cross-sectional cell areas (cell size) within different mature DRG neuronal subclasses defined by functional nAChR expression.

| DRG neuronal subclass | Mouse (P40) | Rat (P47) |
|-----------------------|-------------|-----------|
|                       | Average % capsaicin sensitive | Average cell size (μm\(^2\)) | Average % capsaicin sensitive | Average cell size (μm\(^2\)) |
| a7+ only              | 44%         | 350       | 94%         | 396       |
| p4+ only              | 56%         | 334       | 36%         | 410       |
| a7+ & p4+             | 45%         | 392       | 79%         | 527       |
| ACh-unresponsive      | 43%         | 244       | 77%         | 380       |

*P* refers to the age of animals in postnatal days. Mouse data was compiled from 10 independent experimental trials that encompassed >700 total neurons (cells responsive to depolarization by high extracellular potassium concentration). Rat data was compiled from eight independent experimental trials that encompassed >650 total neurons.

#### DISCUSSION

In this study, we characterized the spectrum of rat and mouse DRG neurons with regard to their nAChR-expression profiles, using a cellular neuropharmacological platform that we established previously (Teichert et al., 2012a,b). Using this approach, we can directly compare individual cellular responses from greater than 100 DRG neurons simultaneously in a single experimental trial (one field of view from a single well). From the data compiled, four broad categories of rodent DRG neurons could be defined with respect to expression of different nAChR subtypes: (1) neurons that express predominantly β4-containing nAChRs with α3 and α5 subunits; (2) neurons that express predominantly α7 nAChRs; (3) neurons that express a combination of α3β4α6β4 and α7 nAChRs; and (4) neurons that do not express nAChRs. However, in rat, each of the first three neuronal subclasses (that express nAChRs) encompasses a higher percentage of the total DRG cell population than in mouse (Table 2). Approximately 70–80% of rat DRG neurons expressed functional nAChRs, whereas only ~15–30% of mouse DRG neurons expressed functional nAChRs, at all developmental time points tested in our study (Table 2).

In rat DRG, there were significant differences in the percentages of capsaicin-sensitive (TRPV1 expressing) neurons between nAChR-defined neuronal subclasses. Within the neuronal subclass that expresses only α7 nAChRs, 94% of the neurons were capsaicin sensitive, whereas only 36% of the neurons were capsaicin sensitive in the subclass that expresses only β4-containing nAChRs (Table 3). In contrast to rat DRG, there were no significant differences in the percentages of capsaicin-sensitive neurons among the mouse DRG neuronal subclasses. Presently, we do not know why these apparent differences exist between rat and mouse DRG neurons, but the results suggest...
that the α7 subclass may be enriched for nociceptors in rat DRG, whereas the β4-containing subclass may be comprised largely of non-nociceptive neurons. It is notable that a higher percentage of rat DRG neurons expressed α3β4/α6β4 nAChRs, α7 nAChRs, and TRPV1 channels than mouse DRG neurons (Table 2 and 3). Although this TRPV1 difference (capsaicin sensitivity) between mouse and rat has been observed in other studies (Caterina et al., 2000; Taggert et al., 2001; Haberberger et al., 2004; Hjerling-Leffler et al., 2007), the generally broader expression pattern of multiple ion channels among rat DRG neuronal subclasses requires further investigation in future comparative studies. We should also consider the possibility that other nAChR subtypes may not be expressed in the soma of DRG neurons used for calcium imaging in this study, but may be present in nerve endings, axons, or synaptic terminals. These possibilities may also be investigated in future studies.
In this study, all of the ACh responses observed in DRG neurons from mouse and rat were mediated by nAChRs and not nMaChRs. This was demonstrated by blocking of ACh-elicited responses by atropine, and by the lack of block by BuIA.

We observed some differences in pharmacology between mouse and rat; mouse DRG neurons required a higher concentration of PNU (5 μM in mouse vs. 1 μM in rat) to amplify responses mediated by the α7 nAChR. Furthermore, although the ACh responses in the presence of PNU were specifically inhibited by ArIB[V11L;V16D] in both mouse and rat, the affinity of the peptide for the mouse α7 nAChR was apparently lower than for rat (incomplete block in mouse), and more rapidly reversible (Figures 2B and 3). These relatively minor pharmacological differences do not compromise the conclusion that the nAChR subtypes expressed in DRG neurons are the same in both species.

Within the four broad categories of neurons defined by this study, diverse response phenotypes were observed in each. For example, following an ACh pulse (prior to application of PNU), some neurons responded with relatively rapid calcium decay kinetics (sharp peaks; e.g., Figure 2A, top two traces), while other cells demonstrated relatively slow calcium decay kinetics (broad peaks) with distinctive shoulders on each peak (e.g., Figure 2A, third and fourth traces from top). When a combination of different VGCC antagonists was applied to the bath, only the sharp ACh-elicited peaks in the presence of PNU were seen to be functionally coupled to L-type VGCCs (Dajas-Bailador et al., 2002, 2005). Various nAChR subtypes, VGCC subtypes and mitochondrial Ca2+ transport may be functionally coupled in specific DRG neuronal subclades to regulate cytoplasmic Ca2+ concentration, which is a common endpoint of almost all signaling in the nervous system, regulating diverse processes from neurotransmitter release to gene-expression changes (Hille, 2001; Shen and Yakel, 2009). Among our future directions is a plan to explore the functional coupling between nAChRs and other signaling components within specific DRG neuronal subclasses in more detail.

There are a plethora of generalizations about the mammalian nervous system based on studies using mice and rats as model systems. This study provides a molecular assessment of divergent cell types at a population level between these two species, demonstrating clear similarities, but also important species differences. Clearly some caution needs to be exercised with respect to the standard practice of using mouse as a molecular genetic system, and rat for physiological studies. An isolated study from either species could easily be over-interpreted in its breadth of applicability across mammalian species or in its translational value.

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AUTHOR CONTRIBUTIONS

Arik J. Hone, Baldomero M. Olivera, and Russell W. Teichert designed research. Nathan J. Smith, Tosifa Memon, Simon Bossi, Thomas E. Smith, and Russell W. Teichert conducted research. Nathan J. Smith, Arik J. Hone, Tosifa Memon, Simon Bossi, Thomas E. Smith, Baldomero M. Olivera, and Russell W. Teichert analyzed data. J. Michael McIntosh, Baldomero M. Olivera, and Russell W. Teichert wrote the paper.

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