Identification and Modulation of Voltage-gated Ca\textsuperscript{2+} currents in Zebrafish Rohon-Beard Neurons.

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ABSTRACT

Electrically excitable cells have voltage-dependent ion channels on the plasma membrane which regulate membrane permeability to specific ions. Voltage-gated Ca\(^{2+}\) channels (VGCCs) are especially important as Ca\(^{2+}\) serves as both a charge carrier and second messenger. Zebrafish (*Danio rerio*) are an important model vertebrate for studies of neuronal excitability, circuits, and behavior. However, electrophysiological properties of zebrafish VGCCs remain largely unexplored because a suitable preparation for whole-cell voltage-clamp studies is lacking. Rohon-Beard (R-B) sensory neurons represent an attractive candidate for this purpose because of their relatively large somata and functional homology to mammalian dorsal root ganglia (DRG) neurons. Transgenic zebrafish expressing green fluorescent protein in R-B neurons, (*Izl2b:EGFP*)\(^{ZC7}\), were used to identify dissociated neurons suitable for whole-cell patch-clamp experiments. Based on biophysical and pharmacological properties, zebrafish R-B neurons express both high- and low-voltage-gated Ca\(^{2+}\) current (HVA- and LVA-\(I_{Ca}\), respectively). Ni\(^{2+}\)-sensitive LVA-\(I_{Ca}\) occur in the minority of R-B neurons (30\%) and \(\omega\)-conotoxin GVIA-sensitive Cav2.2 (N-type) Ca\(^{2+}\) channels underlie the vast majority (90\%) of HVA-\(I_{Ca}\). To identify G-protein coupled receptors (GPCRs) that modulate HVA-\(I_{Ca}\), a panel of neurotransmitters was screened. Application of GABA/baclofen or serotonin produced a voltage-dependent inhibition while application of the mu-opioid agonist DAMGO resulted in a voltage-independent inhibition. Unlike in mammalian neurons, GPCR-mediated voltage-dependent modulation of \(I_{Ca}\) appears to be transduced primarily via a cholera toxin-sensitive G\(\alpha\) subunit. These results provide the basis for using the zebrafish...
model system to understanding $\text{Ca}^{2+}$ channel function, and in turn, how $\text{Ca}^{2+}$ channels contribute to mechanosensory function.

**KEY WORDS:**

Zebrasfish, Rohon-Beard neurons, Voltage-gated $\text{Ca}^{2+}$ channels, G-protein
INTRODUCTION

In excitable cells, such as neurons, Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels (VGCCs) contribute to electrical excitability, synaptic transmission, and signaling pathways involved with gene expression. Therefore, identification of VGCC subtypes, cellular distribution, and sensitivity to neuromodulators is fundamental for understanding neuronal physiology. VGCCs have been well characterized in neurons of mammalian and avian primary sensory ganglia (Scroggs and Fox 1992a). VGCCs on the readily accessible soma provide a model system for exploring the molecular underpinnings of G-protein coupled receptors (GPCRs) modulation and, by inference, presynaptic mechanisms underlying alteration of synaptic transmission at the less accessible presynaptic nerve terminal.

VGCCs are composed of α\(_1\) subunit and different auxiliary β and α\(_2\)δ subunits. Ten distinct genes encoding different α\(_1\) subunit have been identified and classified based on pharmacological and electrophysiological properties (Catterall et al. 2005). The Ca\(_{v}1\) (α\(_{1C}\), α\(_{1D}\), α\(_{1F}\), α\(_{1s}\)) subfamily encodes high voltage-activated (HVA) L-type channels which are sensitive to dihydropyridine antagonists (Bean 1984). The Ca\(_{v}2\) subfamily, comprised of the α\(_{1A}\), α\(_{1B}\), and α\(_{1E}\) genes products, correspond to HVA P/Q-, N-, and R-type channels, respectively, are selectively inhibited by ω-agatoxin IVA (Mintz and Bean 1993), ω-conotoxins GVIA or MVIIA (Mintz et al. 1991; Olivera et al. 1994), and SNX-482 (Newcomb et al. 1998). The Ca\(_{v}2\) class of VGCCs provide the main source of Ca\(^{2+}\) to support neurotransmitter release and are commonly modulated by heterotrimeric G-proteins acting downstream from GPCR activation (Dolphin 2003; Tedford and Zamponi...
Lastly, members of the CaV3 Ca$^{2+}$ channel family, $\alpha_{1G}$, $\alpha_{1H}$, $\alpha_{1I}$, correspond to low voltage-activated (LVA) T-type Ca$^{2+}$ channels (Catterall et al. 2005).

Despite the wealth of information accumulated for mammalian and avian sensory neuron VGCCs, little is known about zebrafish (*Danio rerio*) VGCCs and modulation by GPCRs. Zebrafish represent an important model vertebrate for studies of neuronal excitability, circuits, and behavior as the cost of housing, ease of genetic manipulation, optical transparency, and ex vivo development are advantageous when compared with mammalian systems. However, VGCCs in zebrafish neurons remain largely unexplored due to the difficulty in isolating single identified neurons suitable for whole-cell voltage-clamp studies. Here, we utilized a transgenic zebrafish line expressing green fluorescent protein to develop a technique for isolating Rohon-Beard (R-B) neurons at 24 hrs post-fertilization (hpf). R-B neurons are primary sensory neurons that exist transiently during the early embryonic development of amniotes (Clarke et al. 1984; Lamborghini 1987; Soffe 1991; Williams et al. 2000) and are functionally replaced by dorsal root ganglion (DRG) neurons at later stages of development. Zebrafish embryos acquire a tactile response to mechanical stimulation during the initial phase of development that relies solely on R-B neuron function (Roberts 2000). Therefore, R-B neurons provide a simple, but functionally mature, sensory system at a time point when transient genetic manipulations such a morpholino antisense oligonucleotide-mediated suppression of protein translation are efficacious.

In this study, we show that enzymatically isolated R-B neurons are readily identified from GFP fluorescence and suitable for whole-cell voltage-clamp studies. Using this technique, we characterized LVA- and HVA-Ca$^{2+}$ currents ($I_{Ca}$). HVA-$I_{Ca}$
arose almost exclusively from CaV2.2 (N-type) channels as inferred from toxin-occlusion studies. Finally, we provide evidence that 5-HT, GABA, and DAMGO modulate HVA-Ca$^{2+}$ channels via different G-protein dependent mechanisms.

### MATERIALS AND METHODS

**Animals.** Zebrafish (*Danio rerio*) were housed at 28 °C in the fish facility on a 14/10 hr light/dark cycle. Embryos were maintained at 28 °C in embryo media consisting of 1 mM NaCl and 10$^{-4}$ % methylene blue. Staging was based on external morphology (Kimmel et al. 1995) and defined as hours or days post-fertilization (hpf or dpf, respectively). Wild-type embryos were obtained from AB, TL, or TAB type fish. The transgenic fish line, Tg(*Islet2b:EGFP*)$^{ZC7}$, was kindly provided by Dr. Chi-Bin Chien (Univ. of Utah Medical Center). The transgenic fish line Tg(*HuC:mCherry*) expressed the mCherry fluorescent protein driven by the *HuC* promoter. Adult fish were bred according to guidelines outlined in the Zebrafish Book (Westerfield 1995). After crossing, heterozygote embryos were selected using a fluorescence stereomicroscope. Maintenance of adult fish and experiments using embryos were performed following Institutional Animal Care and Use Committee guidelines of the NIH/NIAAA.

**Preparation of R-B neurons.** Larvae (<30 hpf) expressing EGFP were anesthetized in embryo media containing 0.02% tricaine (Sigma-Aldrich, St. Louis, MO). Dechorionated embryos were sacrificed by transection at a level caudal to the yolk sac (see Fig 2A). The trunk of the embryo was transferred to dissociation buffer containing (in mM) 0.6 EDTA, 5.5 Glucose, 5.4 KCl, 136.8 NaCl, 5.5 NaHCO$_3$, and 2 mg/ml trypsin TRL (Worthington Biochemical, Lakewood, NJ) and incubated for 30 min at
room temperature. After incubation, partially dissociated tissues were triturated with a Pasteur pipette in culture medium consisting of 60% L-15 (Invitrogen, Carlsbad, CA), 1 mM Na-HEPES (Sigma-Aldrich), 1% penicillin-streptomycin (Invitrogen), and 0.5% horse serum (Invitrogen). Dissociated R-B neurons were plated on poly-L-lysine (Sigma-Aldrich) coated tissue culture dishes and maintained overnight at room temperature prior to recording. In some experiments, neurons were incubated with either Bordetella pertussis (PTX; 500 ng/mL) or Vibrio cholerae (CTX; 500 ng/mL) toxin.

Electrophysiological recording. \( I_{Ca} \) was recorded using the conventional whole-cell patch-clamp configuration (Hamill et al. 1981). Patch electrodes were fabricated from borosilicate glass capillaries (1.5 mm outer diameter, 0.84 mm inner diameter; WPI Inc. Sarasota, FL) using a model P-97 micropipette puller (Sutter Instrument Company, Novato, CA). The patch electrodes were coated with Sylgard® 184 (Dow Corning, Midland, MI) and fire-polished to a final resistance of \(~8–9\ M\Omega\) when filled with the pipette solution described below. After rupturing the cell membrane, the mean access resistance was 18.5 ± 0.5 MΩ. The cell membrane capacitance was cancelled and series resistance was routinely compensated (>85% for both prediction and compensation; lag set to 10 \(\mu\)s) with an Axopatch 200B patch-clamp amplifier (Molecular Devices Inc., Sunnyvale, CA). The bath was grounded by an Ag/AgCl pellet connected via a 0.15 M NaCl/agar bridge. The liquid junction potential between the pipette and external solutions used for \( I_{Ca} \) isolation was approximately -1 mV and not corrected for. Voltage protocol generation and data acquisition were performed using custom-designed software (S5) on a Macintosh G4 computer (Apple Computer Inc., Cupertino, CA) equipped with an ITC-18 data acquisition interface (HEKA Instruments Inc., Bellmore, NY). Currents were
filtered at 2 kHz (-3 dB) using a four-pole low-pass Bessel filter, digitized at 10 kHz with a 16-bit analog-to digital converter in the ITC-18 data acquisition interface, and stored on the computer. All drug and bath solutions were applied to single neurons via a gravity-fed fused silica capillary tube connected to an array of seven polyethylene tubes. Drug application was started by switching the bath solution to a drug solution to avoid flow-induced artifacts. All recordings were performed at room temperature (21–24 °C).

Solutions and chemicals. To isolate $I_{Ca}$, patch electrodes were filled with a solution containing (in mM) 120 N-methyl-D-glucamine, 20 tetraethylammonium hydroxide (TEA-OH), 11 EGTA, 10 HEPES, 1 CaCl$_2$, 20 HCl, 4 MgATP, 0.1 Na$_2$GTP and 14 Tris creatine phosphate (pH 7.2, 295 mOsm/kg H$_2$O). The bath solution contained (in mM) 140 methanesulfonic acid, 145 TEA-OH, 10 HEPES, 10 glucose, 10 CaCl$_2$, and 0.0003 tetrodotoxin (pH 7.4, 320 mOsm/kg H$_2$O). Stock solutions were made for the following drugs: $\gamma$-aminobutyric acid (GABA), (RS)-baclofen, L-glutamic acid hydrochloride, oxotremorine M (all from TOCRIS Cookson Ltd., Ellisville, MO), nifedipine (EMD chemicals, Gibbstown, NJ), tetrodotoxin (TTX), $\omega$-agatoxin IVA, $\omega$-conotoxin GVIA, $\omega$-conotoxin MVIIA, SNX-482 (all from Alomone Labs, Jerusalem, Israel), Guanylyl 5‘-imidodiphosphate (Gpp(NH)p), $(\pm)$-norepinephrine, serotonin hydrochloride (5-hydroxytryptamine, 5-HT), NiCl$_2$, and CdCl$_2$ (all from Sigma-Aldrich).

Drugs were prepared in distilled water, except for nifedipine, which was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was 0.1%, which did not affect $I_{Ca}$. For experiments with peptide toxins, bovine serum albumin (BSA) was added to all external solution at 0.5 mg/mL to minimize nonspecific binding. PTX and CTX holotoxins were purchased from List Biological Laboratories (Campbell, CA). All drugs
were diluted to the final concentrations from stock solutions on the day of the
experiment.

**Imaging.** Fluorescence images of zebrafish embryos were acquired with a
confocal microscope (Zeiss LSM510 META, Chester, VA) using a water immersion 25x
0.8 NA objective. For EGFP fluorescence, excitation was 488 nm and emission was
bandpass-filtered between 500–550 nm. The mCherry fluorescence was imaged using
565 nm excitation and emission was bandpass-filtered between 576–704 nm. Dissociated
cells were imaged on an inverted fluorescence microscope (Eclipse TE2000-U, Nikon,
Melville, NY). Screening for heterozygote transgenic embryos was performed on a
dissecting microscope with fluorescence attachment (MVX10, Olympus, Center Valley,
PA).

**Data analysis.** Current traces were analyzed using Igor Pro version 6
(WaveMetrics, Portland, OR) and statistical tests were performed with GraphPad Prism 5
for Mac OS X (GraphPad Software, La Jolla, CA). All data were expressed as mean ±
S.E.M. The percentage inhibition of $I_{Ca}$ (%) was determined using the equation $(I_{con} -
I_{drug})/I_{con} \times 100$, where $I_{con}$ and $I_{drug}$ are the $I_{Ca}$ amplitudes before and after drug
application, respectively. Statistical significance between two groups was determined
using the paired or unpaired Student’s t-tests, as appropriate. The modified Wald method
(http://www.graphpad.com/quickcalcs/ConfInterval1.cfm) was used to compute
confidence intervals of a proportion. Multiple comparisons were performed with a one-
way ANOVA followed by Dunnett’s post hoc test as indicated. $P < 0.05$ was considered
significant.
RESULTS

Preparation of R-B neurons using $Tg(Isl2b:EGFP)^{ZC7}$ zebrafish.

To obtain identified R-B neurons, a transgenic zebrafish line, $Tg(Isl2b:EGFP)^{ZC7}$ was utilized. $Tg(Isl2b:EGFP)^{ZC7}$, hereafter termed $Isl2b:EGFP$, is an $Isl2b$-promoter driven EGFP transgenic zebrafish line originally designed to label retinal ganglion cells that also facilitates identification of sensory neurons (Pittman et al. 2008). Previous studies revealed that the number of zebrafish R-B neurons peaks by 34 hpf and then gradually decreases as a result of programmed cell death (PCD), an event coincident with DRG development (Reyes et al. 2004; Svoboda et al. 2001; Williams et al. 2000). Therefore, we followed the development of sensory neurons in $Isl2b:EGFP$ embryos to determine the best stage for isolation of a pure R-B population. Figure 1A shows EGFP-labeled sensory neurons in the trunk of living $Isl2b:EGFP$ embryos imaged using confocal microscopy over a 24–31 hpf period. Prior to 33 hpf, R-B neurons were the sole EGFP-expressing cell type in the embryo trunk. However, beginning at 48 hpf, DRG neurons (arrow heads) became apparent distributed segmentally along the entire trunk ventral to the R-B neurons (Fig. 1). Contrary to previous studies (Patten et al. 2007; Svoboda et al. 2001; Williams et al. 2000), R-B neurons in $Isl2b:EGFP$ embryos were observed for up to two weeks post fertilization. During the latter part of this observation period, R-B neurons decreased in both size and number. To confirm the identity of the EGFP-labeled cells, we generated double-labeled zebrafish by crossing the $Isl2b:EGFP$ and $HuC:mCherry$ transgenic lines. The $Tg(HuC:mCherry)$ line expresses the fluorescent protein mCherry driven by the $HuC$ RNA-binding protein promoter (Park et al. 2000a; Park et al. 2000b) which serves a pan-neuronal marker (Kim et al. 1996; Park et al.
As illustrated in Figure 1B, R-B neurons could be recognized by several morphological features such as large soma size, dorsal position in the spinal cord, axonal trajectories, and double labeling with both EGFP and mCherry. The boundaries of the spinal cord in these images (Fig. 1B) are delineated by the mCherry fluorescence. Based on these results, we utilized embryos prior to 30 hpf for preparing R-B cells to avoid contamination with DRG neurons. In addition to DRG neurons, additional neurons expressing EGFP under the control of *Isl2b* promoter were observed in the head of *Isl2b:EGFP* embryos at 24 hpf (Fig 2A). These possibly included trigeminal, olfactory, retinal, and posterior lateral line ganglion neurons (Dambly-Chaudière et al. 2003). Therefore, the cranial portion of the embryo was removed prior to dissociation to assure a pure population of R-B neurons. Figure 2B shows fields of cells obtained following enzymatic and mechanical dissociation of the entire zebrafish trunk prior to 30 hpf. Although the phase contrast image shows a wide variety of cell types all displaying a similar appearance (Fig. 2B), individual dissociated single R-B neurons could be easily identified based on EGFP fluorescence (Fig. 2C). The acutely dissociated R-B neurons were spherical with a mean diameter of 10.4 ± 1.1 μm (n = 34) and devoid of neuronal processes; an ideal geometry for voltage-clamp studies. We confirmed the adequacy of space clamp in the isolated neurons by recording voltage-gated Na⁺ currents from the dissociated R-B neuron. Comparison of the Na⁺ current biophysical properties (Supplement data 1) with previous published results acquired using the nucleated patch-clamp technique (Pineda et al. 2005) were in good agreement.
Voltage-gated $I_{Ca}$ in R-B neurons.

$I_{Ca}$ from single dissociated R-B neurons were recorded using the whole-cell variant of the patch-clamp technique in solutions designed to isolate $I_{Ca}$ (see Materials & Methods). Recordings from R-B neurons of voltage-activated inward $I_{Ca}$ evoked with a 160 ms ramp command pulse from holding potential of -80 to +80 mV revealed two distinct subpopulations based on current trajectory. One group displayed only a high-threshold inward $I_{Ca}$ component based on the ramp current-voltage ($I$-$V$) curve which initiated near -40 mV and displayed a monotonic activation component (Fig. 3A). In contrast, the other group displayed a small but prominent hump at low-voltage potentials (-60 to -30 mV) in addition to a high-threshold current component (Fig. 3B). To further explore whether the low-voltage-activated $I_{Ca}$ component originated from T-type Ca$^{2+}$ channels, $I_{Ca}$ was evoked with a depolarizing step pulse to -30 mV from holding potential of -90 mV. As depicted in Figure 3B (bottom traces), a TTX-insensitive (300 nM) and Ni$^{2+}$-sensitive (100 µM) rapidly inactivating $I_{Ca}$ was recorded from R-B neurons with a hump in the ramp I-V; characteristics consistent with $I_{Ca}$ arising from T-type Ca$^{2+}$ channels. In contrast, only a minimally inactivating (over the 70 ms step pulse) $I_{Ca}$ was evoked from the population lacking an overt hump at low voltages (Fig 3A, bottom). The mean membrane capacitance ($C_m$) of R-B neurons with and without T-type Ca$^{2+}$ channels was similar, 3.0 ± 0.1 (n = 42) and 3.1 ± 0.1 (n = 98) pF, respectively, and not significantly different ($P = 0.51$, unpaired T-test). R-B neurons possessing both LVA- and HVA-$I_{Ca}$ comprised 30% (0.23–0.38, 95% CI, modified Wald method) of the total recorded R-B neuron population (Fig 3C, right).
Figure 4 depicts normalized (to maximal currents) $I_{Ca}$ $I-V$ relationships and superimposed current traces evoked by test pulses over the range -100 to +60 mV from a holding potential of -90 mV. In neurons lacking an LVA-$I_{Ca}$ component (Fig 4A), $I_{Ca}$ began to activate around -40 mV, reached a peak near 0 mV, and declined thereafter approaching a zero current asymptote near +60 mV. The $I_{Ca}$ trajectory was minimally inactivating at low-voltage potentials (Fig 4A, red traces) although greater inactivation (20–30% over 70 ms) was evident near voltages where the current was maximal. In neurons displaying an LVA-component, $I_{Ca}$ first appeared between -60 and -50 mV, again peaked near 0 mV, and declined thereafter (Fig 4B). The rapidly inactivating LVA-component was apparent at more hyperpolarized potentials than HVA-$I_{Ca}$ components (Fig. 4B, middle and right traces). The mean $I_{Ca}$ amplitudes for the LVA- (-30 mV) and HVA- (0 mV) components were $-0.02 \pm 0.002$ (n = 42) and $-0.24 \pm 0.02$ nA (n = 102), respectively (Fig 4 C). Mean current densities, determined with 10 mM Ca$^{2+}$ as the charge carrier, were $10.2 \pm 2.3$ and $78.7 \pm 5.3$ pA/pF for LVA- and HVA-current components, respectively.

**Pharmacology of $I_{Ca}$**

We next sought to identify the types of HVA-Ca$^{2+}$ channels functionally expressed in R-B neurons by sequentially applying pharmacological antagonists and toxins that occlude specific Ca$^{2+}$ channel types (Adams et al. 1993; Randall and Tsien 1995). $I_{Ca}$ was evoked every 10 s by a test pulse to 0 mV from a holding potential of -80 mV. Figure 5A and B illustrate the time-course of $I_{Ca}$ amplitude reduction following sequential cumulative application of different subtype-specific Ca$^{2+}$ channel blockers. Application of 10 $\mu$M nifedipine, a dihydropyridine L-type antagonist (Bean 1984), produced a slight
block (5.9%) of $I_{Ca}$. In the continued presence of nifedipine, addition of 0.5 μM ω-agatoxin IVA, a concentration sufficient to block both P- and Q-types of $I_{Ca}$ (Bernhardt et al. 1992; Mintz and Bean 1993), produced no additional overt reduction of $I_{Ca}$. Addition of ω-conotoxin GVIA to this mixture at a saturating concentration (3 μM), (Mintz et al. 1991; Olivera et al. 1994) resulted in a 90% block of total $I_{Ca}$ indicating that the large majority of $I_{Ca}$ arose from N-type (Ca,2.2) channels. The small residual current remaining following application of the three antagonists was abolished by application of the non-selective Ca$^{2+}$ channel blocker Cd$^{2+}$ (100 μM). To further characterize the toxin-resistant $I_{Ca}$ component, SNX-482 (300 nM), a selective antagonist of recombinant α1E channels, (Newcomb et al. 1998), was applied to R-B neurons but failed to produce overt block of $I_{Ca}$ (Fig 5B). Subsequent addition of ω-conotoxin MVIIA (3 μM), another N-type channel blocker (Olivera et al. 1994) resulted in an approximately 90% of reduction of total $I_{Ca}$ further substantiating the interpretation that $I_{Ca}$, under these recording conditions, was carried almost exclusively by N-type Ca$^{2+}$ channels. As summarized in Fig 5C, 98 ± 1% of total inward current was the Cd$^{2+}$-sensitive HVA-$I_{Ca}$, and 89 ± 1 and 87 ± 2% of inward currents were blocked by ω-conotoxin GVIA or ω-conotoxin MVIIA, respectively. There was no apparent difference in the spectrum of expressed HVA-Ca$^{2+}$ channel isoforms in R-B neurons displaying a LVA-$I_{Ca}$ component (89 ± 1 vs. 92 ± 2% for N-type; 3 ± 1 vs. 2 ± 1% for L-type; 4 ± 1 vs. 3 ± 1% for P/Q-type; 6 ± 1 vs. 2 ± 3% for R-type in R-B neurons with and without LVA-$I_{Ca}$ component, respectively). We conclude from these data that N-type Ca$^{2+}$ channels underlie the vast majority of VGCCs in R-B neurons, and that other Ca$^{2+}$ channel isoforms (L-, P/Q- and SNX-482 sensitive R-type) contributed less than 5% of the total $I_{Ca}$ (Fig 5D).
Endogenous G-protein modulation of N-type Ca\(^{2+}\) channels in R-B neurons.

A number of neurotransmitters modulate N-type Ca\(^{2+}\) channels and the major modulation mechanism occurs via activation of heterotrimeric G-proteins (Herlitze et al. 1996; Hille 1994; Ikeda 1996). We thus examined the possible involvement of heterotrimeric G-proteins in modulating zebrafish N-type Ca\(^{2+}\) channels. \(I_{\text{Ca}}\) was evoked by a modified double-pulse voltage protocol (Elmslie et al. 1990) consisting of a test pulse to 0 mV (the “prepulse”), a strong depolarizing conditioning pulse to +80 mV, and second identical test pulse to 0 mV (the “postpulse”; Fig 6A). From the resulting \(I_{\text{Ca}}\), we determined the ratio of the postpulse to prepulse current amplitude measured isochronally at 10 ms after the start of the test pulse; a parameter known as the facilitation ratio (FR). The FR is an often-used metric for voltage-dependent Ca\(^{2+}\) channel inhibition mediated by G\(\beta\gamma\) subunits (Herlitze et al. 1996; Ikeda 1996). When determined in the absence of GPCR agonists, the basal FR is an indicator of tonic G-protein activation (Ikeda 1991b). The mean basal FR of dissociated R-B neurons was 0.87 ± 0.01 (n = 66); a result indicating little tonic G-protein activation. Previous studies have shown that Gpp(NH)p, a hydrolysis-resistant GTP analog, produces tonic inhibition of the \(I_{\text{Ca}}\) when introduced into mammalian sympathetic neurons, presumably by irreversibly binding to and activating G\(\alpha\) during basal nucleotide exchange (Ikeda 1996). To examine whether endogenous G-proteins modulate N-type Ca\(^{2+}\) channels in R-B neurons, 500 \(\mu\)M Gpp(NH)p was dialyzed into R-B neurons via the patch pipette solution. Prepulse amplitude (closed circles), postpulse amplitude (open circles), and the FR (open squares) were plotted as illustrated in Figure 6A and B together with superimposed current traces (right panel). Dialysis of normal pipette solution (which contain 0.1 mM GTP) into R-B
neurons produced no significant change of FR during the 250 s recording period (Fig 6A). In contrast, inclusion of 500 μM Gpp(NH)p in the pipette solution resulted, after a short delay, in a slowly increasing FR and $I_{Ca}$ traces with kinetic slowing during the prepulse, a hallmark of voltage-dependent modulation (Fig 6B). A comparison of FRs determined just following patch rupture (closed circles) and after 200 s of whole-cell recording (open circles) are summarized in Figure 6C. The mean FR increased from 0.89 ± 0.02 to 1.35 ± 0.04 (n = 8; $P < 0.001$, paired $t$-test) during dialysis with Gpp(NH)p compared with the control group which did not change (0.85 ± 0.02 to 0.85 ± 0.06, n = 6; $P = 0.74$) during an equivalent time period. Figure 6B bottom also shows that dialysis with Gpp(NH)p produced apparent inhibition of prepulse $I_{Ca}$ amplitude when compared with control group (57.3 ± 5 vs. 23.2 ± 4%, respectively). These results indicate that N-type Ca$^{2+}$ channels in zebrafish R-B neurons, isolated at an early stage of development, display all the properties of heterotrimeric G-protein modulation observed in adult mammalian peripheral and central neurons.

**Coupling of GPCRs to N-type Ca$^{2+}$ channels in R-B neurons**

To identify GPCRs that modulate Ca$^{2+}$ channels in R-B neurons, we screened agonists that activate GABAb (GABA or baclofen, 100 μM), μ-opioid (DAMGO, 1 μM), adrenergic (norepinephrine, 10 μM), muscarinic ACh (oxotremorine-M, 10 μM), metabotropic glutamate (L-glutamate, 100 μM), or serotonin (5-HT, 10 μM) receptors. Of these agonists, robust $I_{Ca}$ modulation was observed following application of GABA/baclofen, 5-HT, or DAMGO. Figure 7A illustrates representative time courses of prepulse amplitude (closed circles), postpulse amplitude (open circles), and FR (open squares) prior to, during (black line), and after agonist washout. Extracellular perfusion
of either GABA (100 μM) or the specific GABAbR agonist baclofen (100 μM) resulted in $I_{Ca}$ inhibition via a voltage-dependent mechanism. The onset and washout of agonist effect was rapid occurring with the 10 s interval between test pulses. The $I_{Ca}$ modulation displayed all of the hallmarks of voltage-dependent Gβγ-mediated inhibition, namely slowing of activation during the prepulse, relief of inhibition during the postpulse, reversal of kinetic slowing in the postpulse, and increased FR (Ikeda 1991b; 1996).

Similar phenomenology was seen following application of 5-HT. In contrast, application of DAMGO produced a predominately voltage-independent inhibition of $I_{Ca}$ based on the absence of overt kinetic slowing in the prepulse trace or increase in the FR during agonist-mediated inhibition (Fig. 7B). The effects of neurotransmitter application on R-B neuron $I_{Ca}$ are summarized in Figure 7C and D. GABA/baclofen and 5-HT produced a mean $I_{Ca}$ inhibition of between 40-45% that was relatively consistent; inhibition was > 20% in all neurons tested and the coefficient of variation (CV) was 15–22%. DAMGO produced a more variable inhibition with a mean inhibition of 28% and a CV of 87%.

Given the sample size (n = 15), it was unclear whether this variation arose from a multimodal distribution. NE and Oxo-M produced small and variable inhibitions, 11.5 ± 3.3 (n = 9) and 7.7 ± 2.8% (n = 6), respectively, while a small sampling of L-glutamate treated neurons failed to display an overt inhibition (1.3 ± 1.2%, n = 3).

Mean FRs during agonist application are shown in Fig. 7D. Activation of either GABAbR (via baclofen) or 5-HT receptors resulted in mean FRs of 1.12 ± 0.04 (n = 17) and 1.12 ± 0.03 (n = 6), respectively. The basal FR (prior to drug application) for these conditions, 0.84 ± 0.03 and 0.83 ± 0.02, respectively, were comparable to the basal FR reported in Fig. 6. Although the increases in FR during agonist application were not as
large as those reported for comparable conditions in mammalian neurons (e.g., often ≥ 2), the results support the idea that activation of GABAbR or metabotropic 5-HTRs result in $I_{Ca}$ modulation with a voltage-dependent component. Conversely, application of DAMGO produced a small decrease in mean FR (mean difference of -0.06, paired $t$-test, $P = 0.014$, n = 15) when controlled with pre-drug values. Hence, DAMGO-mediated $I_{Ca}$ inhibition was voltage-independent suggesting a discrete signaling mechanism when compared with the GABAbR or 5-HTR responses.

**Effects of Pertussis and Cholera toxin pretreatment**

The class of heterotrimeric G-protein involved in GABAbR and 5-HTR modulation of $I_{Ca}$ was examined by pre-incubating dissociated R-B neurons in either PTX (500 ng/mL) or CTX (500 ng/mL) for 15–17 hours prior to recording. In general, PTX pre-treatment uncouples $G_{i/o}$ G-proteins from GPCRs by ADP-ribosylating a cysteine residue near the carboxyl-terminus of susceptible $G\alpha$ subunits. The actions of CTX are more complex. Under *in vivo* conditions, CTX ADP-ribosylates an arginine residue in $G_s$ and $G_{olf}$ (GNAL) containing G-proteins. Initially, this produces an activation of $G\tilde{\alpha}$ subunit leading to stimulation of adenylate cyclase and elevations in cAMP levels. However, the modified $G\tilde{\alpha}$ subunit is rapidly degraded leading to functional uncoupling from GPCR (Zhu and Ikeda 1994). We examined the GABAbR and 5-HTR responses because the consistent response (Fig. 7C) facilitated comparisons of toxin-treated and control neuron populations.

Examples of $I_{Ca}$ traces in the presence or absence of 5-HT for control, PTX-treated, and CTX-treated R-B neuron are shown in Fig 8A. PTX-treatment appeared to attenuate inhibition although a clear voltage-dependent component remained. However,
CTX-treatment abolished the majority of the inhibition. Pretreatment with either toxin did not alter the mean $I_{Ca}$ amplitude when compared with untreated neurons (one-way ANOVA, Dunnett’s test, $P > 0.05$). Similar effects were seen with the baclofen-mediated response (traces not shown).

A summary of the toxin pre-treatment experiments are shown in Fig. 8B and C. In PTX pre-incubated neurons, $I_{Ca}$ inhibition by baclofen was reduced from $40.3 \pm 2.1$ (n = 17) to $25.4 \pm 3.0\%$ (n = 17). Treatment with CTX, however, reduced $I_{Ca}$ inhibition further to $7.9 \pm 1.6\%$ (n = 16). A very similar profile was seen with 5-HT-mediated inhibition which was reduced from $45.7 \pm 2.3$ (n = 6) to $28.0 \pm 3.8$ (n = 8) and $5.4 \pm 3.5$ (n = 6)$\%$, respectively, for PTX- and CTX-treated neurons. PTX-treatment did not affect the agonist-induced increase in FR for either baclofen or 5-HT (Fig. 8C). However, CTX-treatment reduced the FR to nearly basal levels for both agonists consistent with the near ablation of modulation. These results suggest that both metabotropic 5-HT and GABAb receptors inhibit N-type Ca$^{2+}$ channels of zebrafish R-B neurons via a process dominated by CTX-sensitive G$\alpha$-subunits.

DISCUSSION

**Zebrafish R-B neurons as model system for investigating voltage-gated Ca$^{2+}$ channels**

At present, the transient nature of morpholino antisense knockdown of gene translation and heterologous expression of proteins from cRNA microinjection limits the usefulness of these techniques to the first few days following fertilization. Accordingly, we chose R-B neurons as a platform to investigate VGCC properties as these neurons appear
rapidly following fertilization and form functional circuits within hours (Rossi et al. 2009; Saint-Amant 2006). Unlike in R-B neurons of *Xenopus* larvae and lamprey (el Manira and Bussières 1997; el Manira et al. 1996), voltage-clamp studies of zebrafish R-B neurons are few (Ribera and Nüsslein-Volhard 1998) due to the difficulty in isolating identified neurons for electrophysiology. Here, we demonstrate the suitability of using enzymatically dissociated single R-B neurons from zebrafish expressing *Isl2b*-promoter driven EGFP as marker for primary sensory neurons (Pittman et al. 2008; Tokumoto et al. 1995). Dissociated single R-B neurons were amenable to conventional whole-cell patch-clamp studies, and the spherical geometry and lack of neurites facilitated spatial voltage control. Although R-B neurons are smaller than rodent peripheral neurons (e.g., 10 vs. 20–30 μm), the HVA-\(I_{Ca}\) density (78 pA/pF) was approximately 2–3 fold greater than what we measure in adult rat sympathetic neurons thus providing a favorable signal-to-noise ratio.

**Ca\(^{2+}\) channel currents in zebrafish R-B neurons.**

Based on the presence or absence of LVA-\(I_{Ca}\) component, zebrafish R-B neurons were divided into two subpopulations. Thirty percent of the R-B neurons recorded from displayed an LVA-\(I_{Ca}\) component. However, no overt characteristic examined differentiated the two R-B neuron populations. Previous studies have reported that approximately 40% of R-B neurons at 24 hpf express PKC isoforms (Patten et al. 2007; Slatter et al. 2005) and thus the presence of LVA-\(I_{Ca}\) and expression of PKC isoforms may be correlated although this relationship was not examined. The predominant \(I_{Ca}\) component was HVA and present in all R-B neurons tested. Pharmacological dissection of the HVA-\(I_{Ca}\) revealed that \(\omega\)-conotoxin GVIA-sensitive N-type Ca\(^{2+}\) channels
accounted for 90% of the current amplitude. A residual $I_{Ca}$ component (ca. 6%) resistant to the subtype-specific Ca$^{2+}$ channel antagonists but blocked by Cd$^{2+}$ was observed. The toxins used in these experiments, especially $\omega$-conotoxin GVIA, have the advantage of identifying functional channels as well as specific subtypes of VGCCs (Adams et al. 1993; Feng et al. 2001; Kerr and Yoshikami 1984; Randall and Tsien 1995). A tacit assumption underlying our interpretations is that pharmacological specificity in zebrafish was comparable to that documented for mammalian/avian preparations. The lack of heterogeneity in HVA-Ca$^{2+}$ channel subtype composition within and between cells is an advantage for studying GPCR-mediated modulation and N-type channels are more highly modulated than other CaV2.x subtypes. Mammalian DRG neurons are known to express a wide variety of different HVA-Ca$^{2+}$ channel subtypes which varies greatly from cell to cell (Scroggs and Fox 1992a; b). Amongst R-B neurons, *Xenopus* larvae have an HVA-$I_{Ca}$ composition of N- (70%), P/Q- (25.5%) and L-type (<5.5%) channels (Sun and Dale 1997) while mechanosensory dorsal cells in lamprey HVA-$I_{Ca}$ is comprised of N- (70%), P/Q- (6%) and L-type (12%) Ca$^{2+}$ channels (el Manira and Bussières 1997). Conversely, acutely dissociated embryonic (11–12 d) avian DRG, like zebrafish R-B neurons, exhibit HVA-$I_{Ca}$ comprised of nearly pure population of N-type channels (Cox and Dunlap 1992).

The precise composition of R-B neuron N-type Ca$^{2+}$ channel is unclear at this point. Each of the major subunits families, i.e., $\alpha_1$, $\beta$, $\alpha_2$-$\delta$ has undergone gene duplication in *Danio rerio* leading to a somewhat complex picture. In terms of $\alpha_1$ subunits (CACNA1), 16 genes are listed in the Ensembl database with multiple members from CaV1.x, 2.x, and 3.x represented. Two genes corresponding to CaV2.2 (CACNA1B) $\alpha_1$ subunits are annotated; however it is unclear whether both are functional or correctly
annotated at this point. It seems likely the ω-conotoxin GVIA sensitive $I_{Ca}$ component arises from one or both of these genes. Likewise, the zebrafish Ca$^{2+}$ channel α-δ family (CACNA2) is complex with eight members likely representing duplication of each of the four α-δ genes found in mammals (Davies et al. 2007). The zebrafish Ca$^{2+}$ channel β-subunit (CACNB) family (β1–4) has also been duplicated with the exception of β1 (CACNB1)—hence there are seven members. Expression patterns of Ca$^{2+}$ channel β-subunit in zebrafish embryos determined using in situ hybridization (Zhou et al. 2008) demonstrate that orthologs of mammalian CACNB3, cacnb3a and cacnb3b, are expressed in R-B neurons. Based on these data, cacnb3a/b likely contribute to the channel phenotype observed in our study.

**G-protein modulation of Ca$^{2+}$ channels in R-B neurons**

Modulation of VGCCs, especially N- and P/Q-type Ca$^{2+}$ channels, by GPCRs has been intensively studied for many years (Dolphin 2003; Elmslie 2003; Hille 1994; Ikeda 1991a; Ikeda 1996; Ikeda and Dunlap 1999; Jeong and Ikeda 2000; Tedford and Zamponi 2006b; Zamponi and Snutch 1998; Zhu and Ikeda 1994). Activation of heterotrimeric G-proteins produces two classes of $I_{Ca}$ inhibition, voltage-dependent and voltage-independent, which are not mutually exclusive. All of the signature properties of voltage-dependent pathway, usually attributed to Gβγ were observed with both direct G-protein activation and GPCR-mediated (GABAbR and metabotropic 5-HTR) inhibition in R-B neurons. In contrast, DAMGO, presumably acting via μ-opioid receptors, produced a pure voltage-independent block of $I_{Ca}$ as indicated by the absence of these characteristics during agonist application. These interpretations are predicated on the assumption of
similarity between zebrafish and mammalian/avian N-type Ca\(^{2+}\) channels with respect to the biophysical characteristics of modulation.

A number of inhibitory neurotransmitters modulate glutamate release from primary sensory neurons onto interneurons in spinal circuit models of lampreys and tadpoles (Grillner 2003; Hale et al. 2001; Sillar and Simmers 1994; Sun and Dale 1997). An analogous circuit might exist in the zebrafish embryo with dorsal spinal 5-HT innervations (Grillner 2003; Hale et al. 2001; Sillar et al. 2002) and four types of GABA-ergic spinal interneurons; KA, DoLA, CoSA and VeLD (Bernhardt et al. 1992; Higashijima et al. 2004). Therefore, our results predict that GABAb or 5-HT receptors may participate in presynaptic modulation of sensory transmission in zebrafish R-B neurons by inhibiting Ca\(^{2+}\) channel involved in synaptic release.

Further examination of the GABAbR and 5-HTR signaling pathway revealed some interesting properties. Both responses were partially blocked by pre-treatment with PTX but nearly completely blocked by CTX pre-treatment. As 5-HT is a non-selective agonist and there are numerous subtypes of metabotropic 5-HT that coupled with different G-proteins families, a complex signaling pathway might be anticipated. However, the GABAbR response was unexpected as analogous modulation in mammalian neurons is predominately mediated by PTX-sensitive G-proteins (Menon-Johansson et al. 1993; Scholz and Miller 1991). The nearly complete block of the modulation by CTX pre-treatment has several possible explanations. First, GABAb receptors in zebrafish may, unlike their mammalian counterparts, primarily couple to CTX-sensitive G\(_{i}\) family G-proteins including G\(_{i}\) and G\(_{olf}\). Previous studies of VIP-type receptors in mammalian neurons show voltage-dependent N-type \(I_{Ca}\) inhibition mediated...
by a CTX-sensitive pathway (Zhu and Ikeda 1994). Second, G\(\alpha\) subunits in zebrafish may not respond to bacterial toxins identically to their mammalian orthologs. Although PTX requires a cysteine residue near the C-terminus to be effective, the arginine residue modified by CTX is found in nearly all G\(\alpha\) subunits. What controls the specificity of CTX in terms of ADP-ribosylating particular G\(\alpha\) subunits is unclear and somewhat dependent on experimental conditions (e.g., \textit{in vivo} vs. \textit{in vitro}). Finally, the diversity of G\(\alpha\) subunits in zebrafish is actually greater than that of mammals. Recently a fifth class of G\(\alpha\) protein, termed G\(\upsilon\), was identified in zebrafish (Oka et al. 2009). Although related to the G\textsubscript{i} family, this class of G-proteins appears to be under stringent negative selection and is found in only a few organisms. The G\(\upsilon\) proteins lack the cysteine required to be a PTX substrate but possess an arginine that might be a substrate for CTX. Thus a novel G\(\alpha\) containing G-protein may couple with GABAbR in zebrafish to produce voltage-dependent modulation (which we assume occurs via the G\(\beta\gamma\) subunit); a scenario that could provide insight into the specificity of GPCR coupling.

**Conclusions and summary**

In terms of investigating modulation of Ca\(^{2+}\) channels, what are the advantages using zebrafish R-B neurons? First, single identified R-B neurons can be isolated from transgenic fish lines, here \textit{Isl2b:EGFP}, and are suitable for patch-clamp recording of Ca\(^{2+}\) and other voltage-gated channels. Second, the R-B neurons appear and are functional (i.e., participate in a macroscopic phenotype) during the brief period when transient gene manipulation is convenient and feasible in this model organism. Hence, linking Ca\(^{2+}\) channel modulation to integrative function might be possible. Third, R-B neurons are present during an early stage of development when zebrafish embryos are transparent and
easily imaged. Since we have demonstrated intact G-protein coupling at this early stage, R-B neurons might represent a platform for the testing genetically-based optical sensors for G-protein activation in a living organism. Fourth, the neurons are relatively homogenous, when compared with mammalian DRG neurons, in terms of both HVA Ca$^{2+}$ channel composition, consisting primarily of N-type channels, and some GPCR responses. Finally, the genetic divergence in orthologous genes provides insight into conserved domains underlying the numerous similarities between mammalian and zebrafish Ca$^{2+}$ channel modulation. On the other hand, there are challenges to using zebrafish R-B neurons not present in conventional mammalian preparations. First, molecular complexity in terms of signaling component numbers is, paradoxically, greater in the zebrafish system. This arises not only from gene duplication but also, unexpectedly, from divergent evolutionary paths. For example, as mentioned earlier, zebrafish have an additional G$\alpha$ subunit family not found in the majority of vertebrate species. Second, attempts to ablate specific components during early development can have unexpected, albeit interesting, consequences. For example, knockdown of zebrafish CACNB4 (Ca$^{2+}$ channel $\beta$4 subunit) blocks initiation of epiboly in a manner independent of Ca$^{2+}$ channel function resulting in an embryonic lethal phenotype (Ebert et al. 2008). Therefore, novel developmental properties for proteins with seemingly well-defined functions (in the adult) may be uncovered at the expense of diversion from the original intent of the experiment.

In summary, we have used the zebrafish Isl2b:EGFP transgenic line to isolate single identified R-B neurons and obtain whole-cell voltage-clamp recordings of VGCC. R-B neurons contain both LVA- and HVA-$I_{Ca}$ components, the latter comprised almost
entirely of ω-conotoxin GVIA sensitive N-type channels. The N-type channels are modulated by endogenous GPCRs in both a voltage-dependent and -independent manner. Unlike in mammalian systems, GPCR-mediated voltage-dependent modulation of \( I_{Ca} \) appears to be transduced via a CTX-sensitive G\( \alpha \) subunit. These results provide the basis for using the zebrafish model system to understanding Ca\(^{2+} \) channel function, and in turn, how Ca\(^{2+} \) channels contribute to zebrafish mechanosensory function.
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Figure Legends

FIG. 1. Time course of Rohon-Beard (R-B) neuron degeneration in *Isl2b:EGFP* transgenic zebrafish. *A:* EGFP expression driven by *Isl2b* promoter facilitates observation of R-B neurons degeneration concurrent with development of dorsal root ganglia (DRG, arrow heads). All images of the spinal cord are maximum intensity projections of stacks acquired in the lateral plane using confocal microscopy over a period from 24 hours post-fertilization (hpf) to 31 days post-fertilization (dpf). *B:* Merged confocal images (left and right) and schematic representation (middle) of the double-labeled transgenic line (*Isl2b:EGFP/HuC:mCherry*) imaged in the lateral plane. The contrast and transparency of the images were adjusted to emphasize EGFP-labeled sensory neurons. The boundaries of the spinal cord are delineated by the mCherry expression. Note the anterior and posterior axonal projections and location of EGFP-labeled sensory neurons over the dorsal longitudinal fasciculus (DLF) in the double-labeled embryo spinal cord. All scale bars represent 20 μm and all images are oriented with anterior to the left and dorsal to the top.

FIG. 2. Isolation of single R-B neurons from *Isl2b:EGFP* transgenic zebrafish embryos. *A:* At 24 hpf, several clusters of neurons (labeled i–iv) in the cranial region displayed intense EGFP expression. The dashed line represents where embryos were transected prior to enzymatic and mechanical dissociation of the trunk region. The fluorescence in the yolk sac was auto-fluorescence. *B–C:* Phase-contrast (B) and fluorescent (C) photomicrographs of acutely dissociated R-B neurons (arrow heads) from 24 hpf *Isl2b:EGFP* embryos. Scale bar in A (top) represents 1 mm. The remainders of the scale represent 10 μm.
FIG. 3. Distinct subpopulations of R-B neurons based on Ca$^{2+}$ current components. $A$-$B$: Representative traces of inward Ca$^{2+}$ currents ($I_{Ca}$) from dissociated R-B neurons acquired using whole-cell patch-clamp. $I_{Ca}$ were evoked by a 160 ms ramp from -80 to +80 mV (top traces) and test pulses to -30 mV from a holding potential of -90 mV (bottom traces, same neurons as above). R-B neuron with a “hump” in the ramp current displayed a transient Ni$^{2+}$-sensitive (100 μM) LVA T-type $I_{Ca}$ component. $C$: Scatter plot representing membrane capacitances of R-B neurons with (filled circles) and without (open circles) T-type Ca$^{2+}$ channels. The pie graph (bottom) displays the proportion of neurons for each subpopulation.

FIG. 4. Whole-cell $I_{Ca}$ from R-B neurons with and without a LVA current component. $A$-$B$: Mean normalized (to maximal amplitude) Ca$^{2+}$ current-voltage ($I$-$V$) relationships (left). Each symbol represents the mean ± s.e.m. of 9 (A) or 10 (B) neurons, respectively. Current traces (right) were evoked by a series of voltages steps to potentials ranging between -100 to +60 mV from a holding potential of -90 mV. For illustrative purposes, only traces from -60 mV to -20 mV are shown. Red colored traces in each group represent $I_{Ca}$ components at low-voltage potentials (~ -60 to -30 mV) in $I$-$V$ relationships (red circles). Enlarged LVA-$I_{Ca}$ traces are shown to emphasize transient current trajectory (B, right insert). $C$: Bar graph represents the mean amplitudes of HVA- and LVA-$I_{Ca}$. The number of neurons tested is shown in parentheses.

FIG. 5. Pharmacological dissection of HVA-$I_{Ca}$ in R-B neurons. $A$-$B$: Time courses of $I_{Ca}$ amplitude during serial application of 10μM nifedipine, 0.5 μM ω-agatoxin IVA, 3 μM ω-conotoxin GVIA, and 100 μM CdCl$_2$ (A) or 300 nM SNX-482, 3 μM ω-conotoxin
MVIIA, and 100 μM CdCl₂ (B). \(I_{Ca}\) was evoked every 10 s by 70 ms test pulses to 0 mV from a holding potential of -80 mV. The horizontal bars indicate the duration of drug application. Inset, superimposed current traces obtained at different time points during drug application (labeled as a-e or a-d). C: Bar graph representing the mean \(I_{Ca}\) inhibition (%) produced by application of the indicated antagonists or toxins. The number of neurons tested is indicated in parentheses. D: Summary of relative contribution of N- and other types of currents to total \(I_{Ca}\) in R-B neurons.

FIG. 6. Dialysis of 500 μM Gpp(NH)p into R-B neurons modulates HVA-\(I_{Ca}\). A-B: Time courses of \(I_{Ca}\) (left) and superimposed current traces (right) evoked with the voltage protocol illustrated (inset on the traces in A) from control and Gpp(NH)p dialyzed R-B neurons, respectively. The \(I_{Ca}\) amplitude generated by the pre-pulse (filled circles) and post-pulse (open circles) are plotted. Facilitation ratio (FR) was calculated as the ratio of the post-pulse to pre-pulse \(I_{Ca}\) amplitude (open squares). Cartoons above each figure depict the experimental paradigm. C: Top, Summary graph of the FR just after patch rupture (0 sec, filled circles) or following 200 s of whole-cell recording (open circles) under the conditions indicated. Bottom, Summary for the mean \(I_{Ca}\) inhibition (%) produced by control (open bar) or Gpp(NH)p (filled bar) containing pipette solution after 200 s of whole-cell recording. The numbers in parentheses indicate the tested number of neurons. Error bars represent S.E.M. *** \(P<0.001\) by unpaired \(t\)-test.

FIG. 7. Modulation of HVA-\(I_{Ca}\) by neurotransmitters. A-B: Time courses of \(I_{Ca}\) (left) and superimposed current traces (right) evoked with the double-pulses voltage protocol during application of GABA(100 μM)/baclofen (100 μM) and DAMGO (1 μM),
respectively. The horizontal bars indicate the duration of drugs application. 

*C*: Scatter plots represent inhibition of prepulse $I_{Ca}$ (%) produced by application of GABA, baclofen (Bac), DAMGO, norepinephrine (NE, 10 μM), oxotremorine M (OxoM, 10 μM), L-glutamic acid hydrochloride (L-Glu, 100 μM) and 5-HT (10 μM). Mean and SEM are shown.

*D*: Summary of the mean FR (Post/Pre) before (basal, open bars) or after (filled bars) application of agonists. Data are presented as mean ± S.E.M. * $P<0.05$, *** $P<0.001$ by paired $t$-test.

FIG. 8. Effects of Pertussis and Cholera toxin pretreatment on $I_{Ca}$ modulation in R-B neurons. 

*A*: Superimposed $I_{Ca}$ traces evoked with the double-pulses voltage protocol from control (left), Bordetella pertussis holotoxin (PTX, 500 ng/mL, middle) and Vibrio cholerae holotoxin (CTX, 500 ng/mL, right) pre-incubating R-B neurons. 

*B-C*: Bar graphs summarizing the effects of control, PTX and CTX pre-treatment on the mean $I_{Ca}$ inhibition and FR produced by baclofen or 5-HT in R-B neurons, respectively. Dashed line in bar graph of *C* indicates the mean FR determined in absence of agonists. Data are presented as mean ± S.E.M. The number of neurons tested is indicated in parentheses on the graph. * $P < 0.05$, *** $P < 0.001$ one-way ANOVA followed by Dunnett’s post hoc tests with control.
**Supplementary Figure Legend**

SI FIG. 1. Voltage-dependent Na⁺ current ($I_{Na}$) in R-B neurons. *A:* Left, Na⁺ current-voltage ($I-V$) relationships in presence (open circles) or absence (filled circles) of 300 nM tetrodotoxin (TTX). For recording of $I_{Na}$, the external solution contained (mM) 127 NaCl, 3 KCl, 20 TEA-Cl, 5 MnCl₂, and 5 HEPES (pH 7.4, 300 mOsm/kg H₂O). The pipette solution contained (mM) 120 N-methyl-D-glucamine, 20 TEA-OH, 11 EGTA, 1 CaCl₂, 10 HEPES, 10 glucose, 4 Na₂ATP, 0.3 Na₂GTP, and 5 Tris-creatine phosphate (pH 7.2, 290 mOsm/kg H₂O). $I_{Na}$ were evoked by a series of voltages steps to potentials ranging between -70 and +40 mV from a holding potential of -80 mV. Right, Superimposed current traces recorded from R-B neurons in absence of TTX. For illustrative purposes current traces from -40 to +30 mV are shown. *B:* Voltage-dependent properties of $I_{Na}$ recorded from R-B neurons. *Left,* The voltage-dependence of the Na⁺ conductance ($G_{Na}$, open circles) and steady-state inactivation (filled circles) of $I_{Na}$ from R-B neurons. Conductance ($G$) was calculated as $G_{Na}=I_{Na}/(V_m-V_{rev})$, in which $I_{Na}$ is the peak current, $V_m$ is the voltage, and $V_{rev}$ is the reversal potential for $I_{Na}$. The mean reversal potential, 59.6 ± 3.2 mV (n = 3), was estimated from the linear portion of the $I-V$ relationships. The solid line represents a nonlinear regression fit to the Boltzmann function: $1/(1+\exp[-(V-V_{1/2})/k])$, where $V$ is the step membrane potential, $V_{1/2}$ is the half-activation potential, and $k$ is a slope factor. Voltage-dependence of inactivation was determined using a 200 ms conditioning pulse followed by a test pulse to -10 mV. Test pulse currents are normalized to the maximal value. Solid line is a fit to the Boltzmann equation (see previous text; $k$ is negative for inactivation curve). *Right,* Mean activation and steady-state inactivation parameters.
Fig 3

Graph A shows a plot of cell capacitance with voltage levels of -80 mV and +80 mV, indicating an increase in capacitance over a 10 ms period with a 0.1 nA measurement. Graph B illustrates the same capacitance changes with -90 mV and -30 mV voltage levels. Graph C presents a distribution of cell capacitance values with pie charts indicating percentages of HVA, HVA+LVA, and HVA+LVA combinations.
Fig 4

A

B

C

Normalized ICa

Potential (mV)

Normalized ICa

Potential (mV)

Peak of ICa (nA)

(n = 140)

(n = 42)

-90 mV

-80 to +20 mV

-60 to -30 mV

-90 mV

-60 to +20 mV

-90 mV

10 ms

10 ms

10 ms

0.1 nA

0.2 nA
Fig 5

A

CdCl$_2$

ω-CgTxGVIA

ω-AgaTxIVA

Nifedipine

B

CdCl$_2$

ω-CgTxMVIIA

SNX-482

C

% Inhibition of $I_{Ca}$

D

Relative contribution to total $I_{Ca}$
Fig 7

A

B

C

D

GABA Baclofen

DAMGO

Current (pA) Post/Pre Time (Sec)

DAMGO DAMGO

Current (pA) Post/Pre Time (Sec)

% Inhibition of ICa

Basal FR Agonist FR

*** *
Inhibition of ICa

Fig 8

A

Control

PTX treatment

CTX treatment

0.1 nA
10 ms

0.2 nA
10 ms

B

Control

PTX

CTX

% Inhibition of ICa

***

n.s.

(17)

(16)

(17)

(6)

(8)

Baclofen

5-HT

C

Control

PTX

CTX

Post/Pre

***

n.s.

***

n.s.

***

Baclofen

5-HT