Kv1.3 channels regulate synaptic transmission in the nucleus of solitary tract

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Running head: Kv1.3 modulates sensory afferent-nTS synapse

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ABSTRACT

The voltage-gated potassium channel, Kv1.3 has been reported to regulate transmitter release in select central and peripheral neurons. In this study we evaluated its role at the synapse between visceral sensory afferents and secondary neurons in the nucleus of the solitary tract (nTS). We identified mRNA and protein for Kv1.3 in rat nodose ganglia (NG) using RT-PCR and Western blot analysis. In immunohistochemical studies, anti-Kv1.3 immunoreactivity was very strong in internal organelles in the soma of nodose neurons with weaker distribution near the plasma membrane. Anti-Kv1.3 was also identified in the axonal branches that project centrally including their presynaptic terminals in the medial and commissural nTS. In current clamp studies margatoxin (MgTx), a high affinity blocker of Kv1.3, produced an increase in action potential duration in C-type but not in A-or Ah-type neurons. To evaluate the role of Kv1.3 at the presynaptic terminal we examined the effect of MgTx on tract evoked monosynaptic excitatory postsynaptic currents (eEPSCs) in brain slices of the nTS. MgTx increased the amplitude of the eEPSC in a subset of neurons with the major increase occurring during the first stimuli in a 20 Hz train. These data together with the results from somal
recordings support the hypothesis that Kv1.3 regulates the duration of the action potential in presynaptic terminal of C fibers limiting transmitter release to the postsynaptic cell.

Key Words: nodose, margatoxin, potassium channels, cumulative inactivation
INTRODUCTION

The voltage-gated potassium channel Kv1.3, best known for its role in immunological responses in lymphocytes, is also present in select populations of central and peripheral neurons. Kv1.3 is reported to be targeted primarily to axons (Veh et al., 1995; Rivera et al., 2005) and to be involved in regulation of transmitter release (Ohno-Shosaku 1996, Shoudai et al., 2007; Doczi et al., 2008). A recent study has identified Kv1.3 in presynaptic terminals in calyx of Held (Gazula et al., 2010). In nodose ganglia (NG) visceral sensory neurons a variety of voltage-gated potassium channels contribute to excitability including the members of the Kv1 family, Kv1.1, Kv1.2 and Kv1.6 (Glazebrook et al., 2002), the KCNQ family (Wladyka and Kunze, 2006) and Kv2.1 (unpublished observations). While we find that these currents collectively account for the majority (>80%) of delayed rectifying and slowly inactivating outward potassium current in these neurons, a component of this potassium current remains unblocked in the presence of α-dendrotoxin to block Kv1.1, Kv1.2 and Kv1.6, XE991 to block KCNQ2, 3 and 5 and an intracellular Kv2.1 blocking antibody. In surveying the mRNA expression of the potassium channels in NG neurons we detected the presence of
another member of the Kv1 family, Kv1.3. In this study we asked where Kv1.3 protein is expressed in NG neurons and their axons and whether it plays a role in excitability at central terminals. Specifically we asked whether Kv1.3 might regulate the duration of the action potential and, thus, potentially alter transmitter release at a presynaptic terminal.

Visceral sensory neurons have been subdivided according to various criteria. Historically the population was divided into three groups based on the conduction velocity of the axons, action potential duration and the resistance of the voltage-gated sodium current to tetrodotoxin (TTX-R) (Belmonte and Gallego, 1983; Bossu and Feltz, 1984; Stansfeld and Wallis, 1985). The first group of neurons (A-type) has axons that conduct in the range of fast myelinated fibers, have narrow action potentials and their sodium currents are blocked by TTX. The second group (Ah-type) displays broader action potentials than A-type neurons, exhibits a small hump on the falling phase of the action potential, and have axons that conduct in the slow myelinated fiber range (Stansfeld and Wallis, 1985, Li and Schild, 2007b). While the Ah-type group has not been examined specifically in NG neurons for TTX-sensitivity, a comparable group of
Ah-type neurons in dorsal root ganglion expresses TTX-resistant sodium current as well as TTX-sensitive current (Villière and McLachlan, 1996). These first two cell groups with myelinated fibers are estimated to make up only 10-25% of the total population based on anatomical and physiological studies (Evans and Murray 1954; Agostoni et al., 1957; Mei et al., 1980; Higashi and Nishi, 1982; Yamasaki et al., 2004; Li and Schild 2007b).

Finally, the third group (C-type) has the longest duration action potentials and their voltage-gated sodium currents are poorly blocked by TTX (Stansfeld and Wallis, 1985; Bossu and Feltz, 1984; Schild and Kunze, 1997). These neurons have axons conducting in the range of unmyelinated fibers. In the present study we classified the response of the nodose neurons to MgTx, a blocker of Kv1.3, based on the duration of the action potential and the presence or absence of TTX-R current.

METHODOLOGY

Animal Protocols. Animal protocols were approved by Institutional Animal Care and Use Committees at Case Western Reserve University and University of Missouri and handled according to their guidelines. With the exception of postnatal day one (P1)
animals included in the Western blot study, all animals were male Sprague-Dawley rats between 4 and 7 weeks of age. P1 Sprague-Dawley rats were mixed gender.

**Antibodies.** Two commercial antibodies against Kv1.3 were used in these studies. A polyclonal antibody (Alomone Labs, Jerusalem, Israel, APC-002, lot AN-03,) generated against a GSF fusion protein corresponding to residues 471-523 of human Kv1.3 protein recognizes a single band (~65kDa) on a Western blot (Figure 1). The antibody preabsorbed with the immunizing peptide was tested in nTS sections and gave no signal. We also used a monoclonal antibody (NeuroMab, UC Davis/NIH NeuroMab Facility, Davis, CA, 75-009, lot #413-5RR-07 Clone L23.27IgG2a) generated against synthetic peptide corresponding to rat sequence 485-506. This antibody recognizes a band of approximately 70kDa and has been tested in Kv1.3 knockout mice for specificity (NeuroMab). In addition, we used a monoclonal anti-vesicular glutamate transporter antibody (VGlut2, NeuroMab clone N29/29, fusion protein amino acids 501-582), a goat polyclonal anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA sc-1616; lot #H0608) and a mouse monoclonal anti-myelin basic protein antibody (Chemicon, Millipore, Billerica, MA, MAB 381, amino acids 119-131).
**PCR amplification of Kv1.3 a-subunit cDNA fragment**  mRNA from adult rat NG and brain was isolated using the microPoly (A+) Pure Kit (Ambion). Poly-(A+) mRNA was quantitated by spectrophotometric absorbance at 260 nm and stored at –80°C until used. Primers used to amplify the cDNA fragment corresponding to a region of the rat Kv1.3 gene (accession number: RATRGK5) by RT-PCR were: 5’-AGG ACG TGT TTG AGG CTG CCA ACA AC –3’ (695-721 bp; sense primer) and 5’- CCT CTT CGA TCA TAT ACT CCG AC-3’ (1487-1463 bp; antisense primer). RT-PCR was performing as previously described (Glazebrook et al., 2002). Channel-specific PCR products were identified by hybridization using a radiolabeled internal oligonucleotide specific for the Kv1.3 potassium channel. The internal oligonucleotide was: 5’-GAC AAC TGT CGG TTA TGG TGA TAT GC-3’ (nucleotide 1209-1235). Southern blots were performed as previously described (Glazebrook et al., 2002; Doan et al., 2004).

**Western blots.** Rat pups were anesthetized in a saturated CO2 chamber before the collection of the ganglia. NG from 10 newborn rats (P1) were pooled and frozen at -80°C. Adult rats were anesthetized with 5% isoflurane and decapitated. NG were isolated, frozen in liquid nitrogen and kept at -80°C. The experiment was repeated in
two separate groups of animals. Frozen NG were homogenized in RIPA buffer (1% NP-40, 0.5% DOC, 0.1% SDS, 0.15M NaCl, 50mM Tris/HCl and 2.5mM EDTA) complemented with protease inhibitors (Complete, mini-EDTA-free tablets; Roche, Indianapolis, IN) and phosphatase inhibitors (set I and set II, Calbiochem, La Jolla, Ca). Samples were incubated on ice for 2 hrs and then centrifuged at 14,000xg for 15 min at 4C. Protein concentration of the supernatant was measured by the BCA method (Pierce, Rockford, IL). Equal amount of protein were separated on 4%-20% Tris-Glycine gel (Invitrogen) and transferred to PVDF membranes. Western blots were performed as described previously (Kline et al., 2007). Primary antibodies anti-Kv1.3 (1:500; rabbit polyclonal, Alomone, Jerusalem, Israel) and anti-actin (1:2000) were used to immunoblot.

**Immunocytochemistry.** Adult rats were deeply anesthetized with 5% isoflurane and decapitated. NG, medulla and the aortic depressor nerve (ADN) were isolated, quick frozen and cryosectioned. Serial sections of NG, ADN and medulla, 8 µM thick, were collected on glass slides and fixed with cold 4% paraformaldehyde for 30 min. Sections were blocked with PBS containing 1% bovine serum albumin (BSA), 10% normal
donkey serum (NDS), 0.3% Triton (TX)-100 for at least 30 minutes followed by incubation in the presence of primary antibody for 3 hr at room temperature. The slides were rinsed in phosphate buffered saline (PBS) and secondary antibodies were added for 90 min. Control sections were incubated with PBS and appropriate secondary antibodies. Sections were rinsed, mounted in Vectashield (Vector laboratories) with DAPI (4',6-diamidino-2-phenylindole) and acquired using a Nikon E600 microscope and a Spot camera or a Leica TCS Confocal microscope. Anti-Kv1.3 labeled neurons were counted in one entire ganglion, sectioned at 8 µm. DAPI labeled nuclei used to identify neurons in each section were followed in adjacent sections to minimize double counting of cells. DAPI staining of neuronal nuclei was distinct from glial cell nuclei based on intensity and shape. Metamorph (Molecular Devices, Sunnyvale, CA) was used for analysis of Kv1.3 immunoreactivity and measuring NG soma. Sections from an additional ganglion were co-labeled with anti-Kv1.3 and anti-MBP antibodies. Horizontal nTS sections (8 µm) containing the solitary tract and the medial nTS from four animals were examined for co-labeling of Kv1.3 and vesicular glutamate transporter 2 (vGlut2) using confocal microscopy. The ADN sections were labeled for Kv1.3 and
myelin basic protein (MBP). For all immunohistochemistry, photomicrographs were
imported into Photoshop and only adjusted for brightness and contrast for clarity.

**Electrophysiology.** Adult rats were anesthetized with 5% isoflurane and decapitated.
The ganglia were dissected and placed in cold nodose complete media (NCM) that
consisted of DME-F12 media, 5% of fetal bovine serum (FBS) (Hyclone, Logan UT),
0.1% Penicillin-Streptomycin (Invitrogen, Grand Island, NY). The ganglia were
subsequently treated in a 37°C solution of Earl's balanced salt solution containing 0.1%
of collagenase type 2 (Worthington, Lakewood, NJ) for 70 min. After incubation,
enzyme solution was discarded and 1.5 ml of NCM plus 0.15% of bovine serum albumin
(Sigma) was added to the tissue and manually dissociated with a fire-bored Pasteur
pipette. Cells were plated on poly-L-lysine (0.5 mg/ml aqueous solution) coated cover
slips for electrophysiology experiments. To isolate potassium currents from sodium and
calcium currents, cultured neurons (24-72 hrs) were superfused with extracellular
solution containing (in mM): 140 NMDG (N-methyl-D-glucamine), 5.4 KCl, 0.3 CaCl₂, 10
glucose and 10 N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid (HEPES).
Whole-cell patch clamp recordings were performed with glass electrodes of 2-5 MΩ
resistance filled with (in mM): 145 K-Aspartate, 5 HEPES, 10 glucose, 0.3 CaCl$_2$, 1.0 MgCl$_2$ and 2.2 ethylene-glycol-bis(β-amino ethyl ether)-N,N'-tetra-acetic acid (EGTA), pH adjusted to 7.1. For recording of somatic action potentials the extracellular solution consisted of (in mM): 137 NaCl, 5.4 KCl, 1.0 MgCl$_2$, 2.0 CaCl$_2$, 10.0 glucose, 10 HEPES, pH 7.4, and the pipette solution consisted of 145 K-aspartate, 10 HEPES, 0.3 CaCl$_2$, and 2.2 EGTA, pH 7.1. Only cells with a resting membrane potential more negative than -50mV were included for study. All experiments were performed at room temperature. At the end of each experiment neurons were tested for the presence of TTX-resistant Na$^+$ current in a voltage clamp protocol consisting of a series of 10 mV depolarizing voltage steps from -40 to +10mV from a holding potential of -80mV. TTX (1 µM) was added to the extracellular solution with calcium removed to eliminate interference from calcium currents. Margatoxin (Alomone Laboratories, Jerusalem, Israel) a 39 amino acid peptide toxin blocker of Kv1.3 originally isolated from the venom of scorpion Centruroides margaritatus (Garcia-Calvos et al., 1993; Knaus et al., 1995) was used in the range of 50-1000 pM. We selected 500 pM for full block based on the concentration-response curve in the original study by Garcia-Calvo et al., (1993) and Rb
flux studies in HEK or CHO cells transfected with Kv1.3 reporting an IC$_{50}$ of 230 pM and
110 pM respectively (Koschak et al., 1998; Helms et al., 1997). To confirm specificity of
MgTx for Kv1.3 in NG neurons, α-dendrotoxin (10nM) was added to the bathing solution
in a subset of experiments to block Kv1.1, Kv1.2 and Kv1.6 also present in these
neurons, followed by 500 pM MgTx. Membrane current and voltage was recorded using
a patch clamp amplifier (Axopatch-200B, Axon Instruments, Sunnyvale, CA, USA),
digitized on-line (10kHz) with an analog-to-digital interface (DigiData 1200, Axon
Instruments) and filtered at 1.0 kHz. Data were analyzed using pClamp v 8.0 (Axon
instruments).

*Brainstem nTS slices* were prepared from animals anesthetized with 5% isoflurane and
decapitated. The brainstem was removed and placed in ice-cold low calcium-high
magnesium artificial cerebral spinal fluid (aCSF) containing the following (in mM): 124
NaCl, 3 KCl, 1.2 NaH$_2$PO$_4$, 1.2 MgSO$_4$, 25 NaHCO$_3$, 11 D-glucose, 0.4 L-ascorbic acid,
2 MgCl$_2$ and 1 CaCl$_2$, saturated with 95% O$_2$–5% CO$_2$, pH 7.4 (300 mOsm). Horizontal
slices (~290 µm) were cut with a vibrating microtome (Leica VT 1000S). The submerged
sections were secured with a nylon mesh and superfused at a flow rate of 3–4 ml/min
with standard recording aCSF (in mM: 124 NaCl, 3 KCl, 1.2 NaH$_2$PO$_4$, 1.2 MgSO$_4$, 25
NaHCO$_3$, 11 D-glucose, 0.4 L-ascorbic acid, and 2 CaCl$_2$, saturated with 95% O$_2$–5%
CO$_2$, pH 7.4, 300 mOsm) at 31–33°C. nTS neurons were visualized using an Olympus
BX-51WI microscope (40X magnification) equipped with differential interface contrast
(DIC) and an infrared (IR) sensitive camera. The pipette was guided using a
piezoelectric micromanipulator (PCS-6000, Burleigh, Victor, NY). Recording electrodes
(8250 glass, King Precision Glass, Inc, Claremont, CA, 3.5-5.0 MΩ) were filled with a
solution containing (in mM) 10 NaCl, 130 K$^+$ Gluconate, 11 EGTA, 1 CaCl$_2$, 10 HEPES,
1 MgCl$_2$, 2 MgATP, and 0.2 NaGTP (pH of 7.3, 295-300 mOsm). Recordings were
made from cells in the medial and commissural nTS medial to the solitary tract (TS).
Cells with holding currents greater than -50 pA and a resting membrane potential less
than -50 mV upon initial membrane rupture were not considered for further analysis.
Sensory afferent evoked excitatory postsynaptic currents (EPSCs) were generated by
placing a concentric bipolar stimulating electrode (200 μm O.D., 50 μm I.D., FHC Inc,
Bowdoinham, ME) on the TS containing chemosensory and other visceral afferents
(Andresen and Kunze 1994) and stimulating with a negative current pulse (0.01 - 0.3
mA, 0.1 ms duration) with an isolated programmable stimulator (AMPI, Jerusalem, Israel). Recordings were made in voltage clamp mode while the TS was stimulated at 20 Hz. MgTx (20 nM) was dissolved in aCSF and perfused for 7 minutes following 5 minutes of stable baseline recording. The MgTx concentration for brain slice experiments was based on previous studies (Greffrath et al., 1998; Southan & Robertson, 1998; McKay et al., 2005; Casassus et al., 2005; Korngreen et al., 2005; Nakamura & Takahashi, 2007). Data were recorded using a MultiClamp 700B amplifier, filtered at 2 kHz and sampled at 10 kHz using pClamp v10 programs (Axon Instruments).

**Data Analysis.** Second order nTS neurons were identified by jitter analysis, defined as the standard deviation of the latency (Doyle and Andresen, 2001). Neurons with jitter values of <250 μs were considered to be monosynaptic and directly connected to sensory TS neurons. In protocols involving a 20 Hz stimulus train, the percent of synaptic depression in evoked current from the first event was determined. EPSC data points for a given trial were an average of 5-8 EPSC sweeps at 20 Hz. Statistical analysis was performed with SigmaStat (3.5, Systat Software, San Jose, CA) or Origin.
software (Origin Labs). All data are presented as means ± SE. Electrophysiological
data were compared by Student’s t-test and two-way repeated measures ANOVA.
Cumulative probability plots of spontaneous EPSCs were compared by Kolmogorov-
Smirnov two-sample test (K-S, SPSS).

RESULTS

Kv1.3 is expressed in nodose ganglia We examined the NG for the presence of the
Kv1.3 potassium channel. cDNA of the expected size, 792 bp, was amplified in
samples of poly-(A+)-RNA isolated from nodose and brain. Samples run in parallel
using RT(-) gave no amplification (Figure 1A). Western blots confirmed the protein
eexpression of the Kv1.3 in NG. The anti-Kv1.3 antibody (Alomone) recognized a
prominent band with the apparent molecular weight of ~65 kDa size in both P1 and P36
tissue (Figure 1B). We next explored the distribution of the Kv1.3 α-subunit in the soma
of the NG. The Kv1.3 immunostaining of NG was present throughout the neuronal
population and strongly labeled internal organelles (Figure 2A). Immunoreactivity was
weaker at/near the plasma membrane (Figure 2A inset). Ninety six percent of the
neurons (3339/3475) counted in one ganglion were labeled with anti-Kv1.3. Small, medium and large neurons labeled with anti-Kv1.3 (Figure 2B). Myelinated axons within the ganglion identified with anti-myelin basic protein (MBP) co-labeled with anti-Kv1.3 (Figure 2D-F). Axons lightly labeled with anti-Kv1.3 but unlabeled with anti-MBP can also be seen in this image coursing parallel to the myelinated fibers. In cross sections of the ADN afferent sensory nerve with soma in the nodose ganglion, Kv1.3 was identified in MBP-immunoreactive axons as well as clusters of presumed MBP-immunonegative axons (Figure 2C).

Kv1.3 immunoreactivity is present in the presynaptic terminals in nTS

Primary afferent fibers from visceral sensory fibers enter the brain stem nTS as part of the tractus solitarius (TS) and, after exiting the tract, form a synapse with cells in this nucleus. We obtained Kv1.3 immunostaining within the tract and in the medial and commissural nuclei regions of the nTS where afferents from arterial baroreceptors and chemoreceptors terminate (Figure 3A-B). Much of this was punctate labeling surrounding the neurons close to the tract. To demonstrate localization to presynaptic terminals, the nTS region was labeled with both anti-Kv1.3 and anti-vGlut2, the primary
glutamate vesicular transporter in the nTS (Lachamp et al., 2006) (Figure 3C-E). Co-labeling was observed in structures of approximately 2 µM in diameter, consistent with our previously reported size of presynaptic terminals on nTS soma (Drewe et al., 1988). Anti-Kv1.3 immunoreactivity was not present in neuron cell bodies in the region near the tract.

**Margatoxin (MgTx) blocks a component of somal outward potassium current**

We next asked whether Kv1.3 is expressed in the surface membrane of the NG soma using a functional assay. We isolated whole cell K+ currents from Na+ and Ca²⁺ currents by replacing Na⁺ in the external solution with equimolar NMDG⁺ and reducing extracellular Ca²⁺ to 0.03 mM. In initial experiments a depolarizing voltage step to +40mV was applied at 20-30 second intervals from a holding potential of -80 mV. When the outward K⁺ current was stable for at least 2 minutes, 500 pM MgTx was added to the bath solution to block Kv1.3 (Garcia-Calvo et al., 1993; Helms et al., 1997), (Figure 4A). As shown in the figure, MgTx blocks a portion of the whole cell K⁺ current. In 8/8 neurons the current decreased, 14.0 ± 3.8 pA/pF (range 3-33 pA/pF) at the peak
and 8.4 ± 2.2 pA/pF (range 4-21 pA/pF) at the end of the 100-150 ms step. The former

corresponded to 10.1 ± 1.8% and the latter to 5.6 ± 1.1% of the total potassium current

at those time points. The inset shows the time course of the MgTx block. Increasing the

concentration of MgTx to 1.0 nM had no further effect (n=3). In a second set of

experiments, a 900 ms ramp stimulus delivered every 30 s from -100 to +80 mV was

used to obtain an I-V relationship which showed the MgTx-sensitive current activated

more positive than -30 mV (Figure 4B). Under current clamp we observed no effect on

the resting membrane potential upon the addition of MgTx (0.5-1 nM), control, -62.0 ±

1.7 mV vs MgTx, -61.8 ± 1.6 mV p>0.3, paired t-test, n=23, consistent with the lack of

MgTx block under voltage clamp at potentials in this voltage range.


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*MgTx alters action potential duration in C- but not A-type neurons*

Somal recordings are used to gain insight to function in regions that are less accessible

such as the central presynaptic terminal. In the present studies we focused on the

contribution of Kv1.3 to action potential duration under current-clamp conditions as an

indicator of a potential role in central transmitter release. The natural stimulus received
by sensory neurons from their peripheral terminals is a series of action potentials whose
frequency is dependent on stimulus intensity at the peripheral receptor terminal. Thus, an appropriate stimulus to evaluate the role of Kv1.3 is a short duration stimulus designed to elicit single action potentials. The neurons were stimulated at 0.5 Hz (0.35-1.5 ms) in current clamp mode. When MgTx was applied to the rare cells subsequently shown to express only TTX-sensitive sodium current (A–type, Stansfeld and Wallis, 1985; Schild and Kunze, 1997; Li and Schild, 2007b,), there was no change in the duration of the action potential measured at 0mV (0.74 ± 0.09 ms vs 0.76 ± 0.1 ms, n=3, p>0.4, paired t-test). In addition, in 3 neurons there were no changes in duration of the action potential in response to 20 Hz repetitive stimulation either in control solution or in the presence of MgTx (Figure 5A-B). To verify that Kv1.3 was, however, functionally expressed in these neurons, we applied a longer duration constant current depolarizing stimulus (150 or 400 ms) at a threshold level for eliciting at least one action potential. Application of MgTx either increased the number of action potentials in response to the stimulus (2/3, example top insert Figure 5A) and/or increased the amount of
depolarization produced by the current injection (1/3, bottom inset Figure 5A), the latter
indicative of a decrease in membrane conductance upon block of Kv1.3.

A second group of neurons (n=6) with intermediate duration action potentials did not
respond to MgTx (action potential duration 1.64 ± 0.12 ms vs 1.65 ± 0.11 ms in MgTx,
n=6, p>0.05) when stimulated at 0.5 Hz and showed no change in duration at 20 Hz
(Figure 5B, p>0.05, paired t-test). The sodium current in this group was incompletely
blocked by TTX. We considered these to represent the Ah class of neurons with more
slowly conducting myelinated fibers and an action potential duration >1.0 ms, as
described by Stansfeld and Wallis (1985) and, more recently, Li et al. (2007b). As with
the previous group, these Ah-type neurons responded to MgTx during a sustained
depolarization with an increase in the number of action potentials to the current injection
(4/7 cells) or a larger depolarization (3/7 cells, range 2-5 mV).

The last group of NG neurons, C-type neurons, have broader initial action potentials
(>2.0 ms) and express TTX-resistant current. These C-type responded to MgTx with an
increase in the duration of the action potential from 2.19 ± 0.24 ms to 2.31 ± 0.23 ms at
0 mV, (p<0.01 paired t-test, n=13) in response to a 0.5 Hz stimulus. This group was
also subjected to 20 Hz stimulation protocol in the absence and presence of MgTx

(Figure 5C-D). MgTx has shown high selectivity for Kv1.3 over other potassium
channels such as Kca channels (Leonard et al., 1992), Kv3.1, Kv1.5, ISK (Calvo-Garcia et al., 1993). The latter group reported weak effect on Kv1.6. Comparable studies have not been done for Kv1.1 and Kv1.2 which are both present in nodose neurons (Glazebrook et al., 2002). Thus, prior to the addition of MgTx, four C-type neurons were incubated with α-dendrotoxin (DTx, 50 nM) to block the Kv1.1, Kv1.2 and Kv1.6 currents. While this increased the duration of their action potentials as expected (Glazebrook et al., 2002) all four subsequently responded to MgTx with a further increase in action potential duration and are included in the grouped 20 Hz data (Figure 5D). The MgTx effect was superimposed on an increase in action potential duration in response to 20 Hz repetitive stimuli (Figure 5C-D), a characteristic of C type neurons as previously reported by Li and Schild (2007a).

*MgTx augments synaptic transmission in the nTS.*
We asked whether the inhibition of Kv1.3 by MgTx observed in sensory afferents alters central neurotransmitter release in nTS brain slices. We recorded excitatory postsynaptic currents (EPSCs) from 18 nTS neurons in the medial and commissural nTS within the region of Kv1.3 immunoreactivity. EPSC synaptic latency was 3.7 ± 0.4 ms and its standard deviation (i.e., jitter) was 135 ± 14 μs, suggesting EPSCs were generated from a monosynaptic connection (Doyle and Andresen, 2001). Overall, the initial event of a 20 Hz EPSC train in control solution ranged from 48 to 681 pA, with 15 of 18 cells exhibiting amplitudes less than 300 pA (average, 137 ± 19 pA) with the remainder of cells averaging 536 ± 91 pA (3/18, p < 0.05 vs. smaller events). The neurons with smaller EPSC exhibited sensitivity to MgTx compared to those which exhibited larger initial EPSCs. A representative example of a MgTx-sensitive nTS cell is shown in Figure 6A. In this cell, MgTx increased the amplitude of solitary tract (TS)-evoked EPSCs that were elicited at 20 Hz. Mean data for group of 15 smaller event, MgTx-sensitive cells is plotted in Figure 6B. Bath application of MgTx (20 nM, 6-7 min) increased the amplitude of the initial TS-evoked EPSCs. The amplitude of the first EPSC averaged 137 ± 19 pA in control recordings and increased to 187 ± 26 pA in
MgTx (p = 0.016, paired t-test). Across the TS-EPSC event train, MgTx-sensitive TS-EPSC amplitude was significantly greater at the beginning of the train and reduced at the end (Figure 6B, two-way RM-ANOVA). Spontaneous EPSCs (sEPSCs) in these 15 smaller event, MgTx-sensitive cells were also evaluated. Cumulative probability plots of sEPSC amplitudes and interevent intervals were generated to analyze distribution. MgTx did not alter the amplitude of spontaneous events (Figure 6C, left, p = 0.295, Kolmogorov-Smirnov test) but did reduce the interevent interval indicating a small increase in event frequency (Figure 6C, right, p = 0.02, Kolmogorov-Smirnov test). The second group of larger EPSC amplitude monosynaptic neurons did not respond to MgTx (Figure 6D). Note the amplitude of TS-EPSCs which were sensitive to MgTx were significantly smaller across the stimulus train than MgTx insensitive nTS neurons (two-way RM-ANOVA).
In this study we show, a functional role for Kv1.3 at the first synapse in visceral afferent pathways in the nucleus of the solitary tract. Our results support a role for Kv1.3 modulating transmitter release from presynaptic terminals of C-fibers through effects on the duration of the action potential.

**Kv1.3 as a presynaptic modulator of transmitter release**

Consistent with reports of an axonal distribution for Kv1.3 (Veh et al., 2005; Rivera et al., 2005), we find immunoreactivity in the central axonal branches in the tract extending to presynaptic terminals. Localization of anti-Kv1.3 with the vesicular glutamate transporter, vGlut2 along with the fact that Kv1.3 immunoreactivity was not present in nTS neurons in this region supports a presynaptic site for actions of MgTx. Presynaptic localization has also been reported recently in the calyx of Held (Gazula et al., 2010)

**Kv1.3 modulates transmitter release at the presynaptic terminals.**

We assessed the role of Kv1.3 at the central terminals by monitoring the EPSCs in second-order nTS neurons in medial and commissural nTS receiving monosynaptic
sensory input. In response to tract stimulation the eEPSC amplitude was increased in the presence of MgTx in a subset of neurons. Extrapolating from our data in the nodose soma where the C fiber population, but not the A- or Ah-type neurons, responds to MgTx with an increase in duration of the action potential, we propose that the responding nTS neurons are innervated by C-type fibers and that the increase in duration of the presynaptic action potential in the presence of MgTx augments transmitter release leading to increased amplitude of the synaptic potential (Wheeler et al., 1996; Raffaelli et al., 2004; Stephens and Mochida, 2005). A role for Kv1.3 in transmitter release has been reported in other neuronal studies (Ohno-Shosaku et al., 1996; Shoudai et al., 2007; Doczi et al., 2008). Our data suggest that Kv1.3 in presynaptic terminals, presumably from C-type fibers, would play a significant role in modulating neurotransmitter release at the nTS.

A second group of eEPSCs in nTS neurons was not altered by MgTx. This is consistent with innervation by fibers from A- and Ah-type neurons with short duration action potentials where Kv1.3 would not play a role unless there were sustained depolarization of the terminal. We cannot rule out however, the possibility that Kv1.3 is not present in
the presynaptic terminals of the myelinated fibers although it is present in their soma and axons. Interestingly, the evoked EPSCs which were not altered by MgTx were significantly larger in amplitude than those that were not. Data from Andresen and Peters (2008) support the conclusion that the larger non-responsive EPSCs in our studies were present in neurons innervated by A- or Ah-type fibers. Those authors compared the peak amplitudes of capsaicin-resistant tract evoked EPSCs, presumably innervated by myelinated fibers, with capsaicin-sensitive EPSCs, innervated by C-type unmyelinated fibers. The former were 50% larger than the latter.

Frequency dependent depression persists in the presence of MgTx

Frequency dependent depression (FDD) is a hallmark characteristic of the nTS synapse, first described by Miles (1986) and readily demonstrated in Figure 6. FDD is proposed to result from a reduction in the availability of a readily releasable pool of vesicles at the presynaptic site (Schild et al., 1995) and it remains even in the presence of MgTx (Fig. 6). Superimposed on FDD is another factor which could potentially alter transmitter release during repetitive stimulation. This arises from the presence of
presynaptic potassium channels that would limit action potential duration but that undergo cumulative inactivation in response to stimulation. These include Kv1.3 (Cahalan et al. 1985; Maron and Levitan, 1994, Grissmer et al., 1994), the BK calcium-activated potassium channel, KCNMA1 (Shao et al., 1999) and Kv2.1 (Klemic et al., 1998), all channels that are present in NG. One would expect that the broader action potentials toward the end of a stimulus train would lead to an increase in transmitter release. However, it appears that as FDD develops at the central synapse, it overrides the postulated effect of the broader action potentials that we observed during the 20 Hz stimulation in the soma of C-type neurons.

**Physiological significance of presynaptic Kv1.3 in C-type axons**

At the presynaptic site in the nTS, most of the effect of Kv1.3 on transmitter release would occur early in a stimulus train before FDD develops. In our studies, MgTx was most effective on the amplitude of the eESPC in response to the first stimuli in the 20Hz series where the hyperpolarizing effect of Kv1.3 is expected to play a stronger role at a time when competing FDD is still developing. Miles (1986) reported that the amplitude
of the EPSC was reduced to 91% of its control value at a stimulation frequency of 1 Hz,
to 63% at 5 Hz and 39% at 10 Hz. Thus, at low frequencies there is minimum
depression. This is important because the visceral sensory C-fiber population tends to
fire at low frequencies (<1 to 5Hz). For instance, C fiber chemoreceptors generally fire
from ≤ 2 Hz to 4-5Hz although at very strong stimulation of their sensory terminals,
discharge can reach 20 Hz (Sato and Fidone, 1969). The same study showed arterial
baroreceptor C fibers responded to a natural stimulus with only 1-2 spikes per arterial
pressure pulse at a rate of 2-3Hz although under extreme stimulus conditions can reach
15-20Hz. Other studies have confirmed these results (Coleridge et al., 1987; Seagard
et al., 1990; Thoren et al, 1999).

In summary, this is the first study to implicate a specific potassium channel, Kv1.3 in the
evoked release of transmitter at second-order neurons receiving sensory input in the
nTS. The data support the contention that Kv1.3 plays a role in limiting the duration of
C fiber action potentials that is most effective during brief bursts of 2-3 action potentials
with minimal cumulative inactivation and FDD, well within the range of *in vivo* activity of C-fiber populations.
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Figure 1: Detection of Kv1.3 α-subunit

A: PCR products, resulting from the amplification of first-strand cDNA prepared with (+) or without (-) reverse transcriptase (RT) from rat NG or brain poly A+ RNA with Kv1.3 specific oligonucleotides, were separated by electrophoresis and transferred to nylon membranes. After Southern hybridization with $^{32}$P-labeled specific internal oligomer, the autoradiogram showed positive signal from nodose and rat brain in the (+) RT lanes and no signals in the control (-) RT. The oligonucleotide probes amplify a cDNA of 792bp. B: The specificity of the primers was tested using a sample of Kv1.3 cDNA. A single band of the predicted size (792 bp) was amplified from nodose ganglia cDNA (lane 2); Kv1.3 cDNA (lane 3) and nTS cDNA (lane 4). No PCR product was detected in the negative control (H$_2$O, lane 5). Lane1, 1-kb DNA ladder. C: Western blot analysis of the Kv1.3 protein from NG: Kv1.3 receptor was identified in protein lysates from NG dissected from P1 (50 μg ) and P36 (20 μg ) rats. A band about 65 kDa was detected on both lysates. Mobility of the protein correlated with the molecular size already reported for the Kv1.3 α-
subunit (~65 kDa). Western blot analysis showed, also, that Kv1.3 protein in neonates is less abundant than that of older animals.

Figure 2: Immunohistochemical localization of Kv1.3 in NG and ADN

A: Anti-Kv1.3 (Neuromab) immunolabeling in P30 Spraque-Dawley rat nodose slice. The image is a mass z-projection of 5 confocal sections acquired at 0.69 µm intervals. Calibration bar = 30 µm. Inset shows zoomed image to illustrate weaker labeling near/at the membrane. B: Histograms shows the broad distribution of anti-Kv1.3 labeling with respect neuron diameter as compared to the distribution of diameters in the total population. C: A section of the ADN is co-labeled with anti-Kv1.3 (Alomone, red) and anti-myelin basic protein (green) to illustrate the presence of Kv1.3 in myelinated axons. Arrows indicate examples of anti-Kv1.3 label. These results were obtained in ADN from 2 animals. Note the presence of only two large diameter axons. Similar results (2-3 large axons with diameters > 2 µm) were obtained in five other ADNs. Scale bar = 2 µm

D-F: Image of 8 µm nodose section co-labeled with anti-
Kv1.3 (red, D) and anti-MBP (green, E) and overlay (F) shows presence of the Kv1.3 antibody in myelinated and unmyelinated axons in the ganglion. Scale bar = 20 µm.

Figure 3: Confocal Images of the nTS labeled with Kv1.3

A: Confocal image of a horizontal nTS section in the commissural region. Fine fibers in the solitary tract (TS) exit the tract and innervate neurons medial to the tract. B: Confocal image of horizontal section of the nTS, showing the medial nucleus of the nTS (mnTS), site of termination of baroreceptor axons. The immunostaining of both sections with anti-Kv1.3 antibodies (red) show the presence of the protein in the fibers in the tract and in the neuropile but not in cell bodies. Images are stacks of 12-15 z-sections taken at 0.3 µm intervals, scale = 15 µm. C-E: Confocal images of anti-Kv1.3 and anti-vGlut2 label in the tractus solitarius and medial nTS. C: Anti-Kv1.3 is present in the afferent sensory fiber tract (TS) and in discrete bouton-like structures (arrows). D: vGlut2 immunoreactivity is present in the same structures. E: Merged image Kv1.3 (red) and
vGlut2 (green). Each image is a stack of 4 z-sections. Scale bar = 20 μm. Similar anti-
Kv1.3 labeling was obtained in the NTS from 4 other animals.

**Figure 4**: MgTx produces partial block of total potassium current in the soma of nodose neurons

A: Example of total outward potassium current recorded from a neuron depolarized for 100ms from -80 mV to +40 mV every 20 s. When control traces (black solid line) were stable for at least two minutes, 500 pM MgTx was applied and the current decreased to the value shown (red dashed line). The difference (MgTx-sensitive) current is shown as a blue dotted line. The inset show the time course of the MgTx block (↑) and recovery (↓) in another neuron. B: A 900 ms ramp stimulus applied to another nodose neuron in the absence of MgTx is followed by application of 500pM MgTx (↓). The subtracted MgTx-sensitive current activates more positive than -30 mV (inset).

**Figure 5**: Effects of MgTx on neuronal discharge
A: An example of an A-type neuron (sodium current blocked by TTX) responding to 10 brief (0.35-1.5ms) stimuli delivered at 20 Hz. There was no change in the duration of the action potential with frequency nor did MgTx (1nM) have an effect on duration. However, a longer duration stimulus (upper inset) shows increased discharge from one action potential in control (black solid line) to repetitive firing in the presence of MgTx (red dashed line) or increased the amplitude of the depolarization to the constant current stimulus (lower inset). Inset scale bar = 50ms, 10mV. B: The mean duration of the action potential at 0 mV is plotted for three A-type neurons for each of the ten stimuli in control solution (■) and in the presence of MgTx (▲). A group of 6 Ah-type neurons also did not respond MgTx, (Control ■, MgTx ▲). C: MgTx broadened the C-type action potential but did not eliminate the stimulation dependent lengthening of the action potential (20Hz stimulation) as shown in control solution and in the presence of 1.0 nM MgTx. C: Inset left: The first action potential in the 20 Hz train in control solution (black) is compared with the first action potential in the presence of MgTx (red). C: Inset right: The tenth (last) action potential of the series is also shown without (black) and with (red) MgTx. D: The mean duration of the action potential of eight C-types neurons in the
absence (■) and presence (▲) of MgTx is plotted for each of the ten stimuli. Four of these neurons had been previously incubated in DTx to eliminate any effect of MgTx on Kv1.1, Kv1.2 or Kv1.6. ★ = p<0.05, paired t-test.

Figure 6: MgTx augments TS-evoked EPSCs. A: Representative tracings of tractus solitarius (TS) evoked excitatory postsynaptic currents (EPSCs) that were recorded under control conditions (aCSF) and following MgTx (20 nM). The TS was stimulated at 20 Hz. Note the increase in TS-EPSC amplitude, especially the first event. Shown is an average of five current sweeps. B: Synaptic events were grouped according to their initial current amplitude. Data shown are the mean TS-EPSC amplitude for 20 events whose initial amplitude was < 300 pA during control and MgTx application. n=15. * p < 0.05, 2 Way RM ANOVA. MgTx elevated EPSCs primarily at the beginning of the stimulus train. C: Spontaneous EPSC frequency is also elevated in the MgTx-sensitive currents. The cumulative probability of sEPSC amplitude distribution (2 pA bin, left panel) was not altered in MgTx. On the other hand, the cumulative fraction of sEPSC interevent intervals (10 ms bin) illustrates a small leftward shift in the presence of MgTx.
Analysis performed on the entire sample of events. D: Mean TS-EPSC amplitude for 20 events during control and MgTx application for 3 cells whose initial TS-EPSCs > 300 pA and did not respond to MgTx application. Note the amplitude of EPSCs which were sensitive to MgTx (panel B) were significantly smaller than MgTx insensitive nTS neurons.