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NMDA receptors control vagal afferent excitability in the nucleus of the solitary tract

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Abstract

Previous behavioral studies have demonstrated that presynaptic N-methyl-D-aspartate (NMDA) receptors expressed on vagal afferent terminals are involved in food intake and satiety. Therefore, using *in vitro* live cell calcium imaging of prelabeled rat hindbrain slices, we characterized which NMDA receptor GluN2 subunits may regulate vagal afferent activity. The nonselective NMDA receptor antagonist D,L-2-amino-5-phosphonopentanoic acid (D,L-AP5) significantly inhibited vagal terminal calcium influx, while the excitatory amino acid reuptake inhibitor D,L-threo- β -benzyloxyaspartic acid (TBOA), significantly increased terminal calcium levels following pharmacological stimulation with ATP. Subunit-specific NMDA receptor antagonists and potentiators were used to identify which GluN2 subunits mediate the NMDA receptor response on the vagal afferent terminals. The GluN2B-selective antagonist, ifenprodil, selectively reduced vagal calcium influx with stimulation compared to the time control. The GluN2A-selective antagonist, 3-chloro-4-fluoro-N-[4-[[2-(phenylcarbonyl)hydrazino] carbonyl]benzyl]benzenesulfonamide (TCN 201) produced smaller but not statistically significant effects. Furthermore, the GluN2A/B-selective potentiator (pregnenolone sulfate) and the GluN2C/D-selective potentiator [(3-chlorophenyl)(6,7-dimethoxy-1-((4-methoxyphenoxy)methyl)-3,4-dihydroisoquinolin-2(1 H)-yl)methanone; (CIQ)] enhanced vagal afferent calcium influx during stimulation. These data suggest that presynaptic NMDA receptors with GluN2B, GluN2C, and GluN2D subunits may predominantly control vagal afferent excitability in the nucleus of the solitary tract.

Keywords

NST; calcium imaging; presynaptic; GluN2B; GluN2C; GluN2D

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1. Introduction

The nucleus of the solitary tract (NST) in the dorsal medulla processes a vast array of visceral afferent information that, ultimately, controls a number of homeostatic and behavioral functions, including feeding and gastric motility. Glutamatergic visceral vagal afferents have cell bodies in the nodose ganglion and carry physiological and nutrient information from the gut to the brain. These glutamatergic vagal afferents synapse upon second-order NST neurons (Aylwin et al., 1998; Leone and Gordon, 1989; Ohta and Talman, 1994) which, in turn, control homeostasis through ascending GABAergic, catecholaminergic, and glutamatergic connections (Blessing, 1997; Rogers and Hermann, 2006).

NMDA receptors are members of a class of ionotropic glutamate receptors subdivided by pharmacology and sequence homology that also includes the α -amino-3-hydroxy-5-methyl-4-propionic acid (AMPA), kainate (KA), and delta receptors (Mayer, 2005; Traynelis et al., 2010). Ionotropic glutamate receptors are composed of four multi-domain subunits that combine to form the ion channel pore. NMDA receptors typically are comprised of two GluN1 subunits and two GluN2 subunits and are unique among ionotropic glutamate receptors in that concurrent binding of glycine to the GluN1 subunit and glutamate to the GluN2 subunit is required for receptor activation (Furukawa et al., 2005; Johnson and Ascher, 1987; Kleckner and Dingledine, 1988). Unlike AMPA and kainate receptors, NMDA receptors also must be relieved of voltage-dependent magnesium block prior to activation (Ascher et al., 1988; Mayer et al., 1984; Nowak et al., 1984). Four separate GluN2 subunits, GluN2A-GluN2D, have been identified and are thought to determine a majority of the pharmacological and kinetic properties of the receptor (Monyer et al., 1994; Stern et al., 1992; Vicini et al., 1998). NMDA receptors mediate the slow, calcium-permeable component of excitatory postsynaptic currents, as they activate and deactivate significantly more slowly than AMPA or kainate receptors (Traynelis et al., 2010).

A number of studies have suggested that NMDA, AMPA, and kainate receptors have a role in feeding behavior. Blocking excitatory glutamatergic input into the nucleus accumbens shell with the AMPA and kainate receptor antagonist DNQX stimulates food intake (Kelley and Swanson, 1997; Maldonadoirizarry et al., 1995). Within the lateral hypothalamus, ionotropic glutamate receptors appear to stimulate feeding, as injections of AMPA, NMDA or kainate into the lateral hypothalamus induce feeding (Stanley et al., 1993), and inhibiting these receptors suppresses feeding (Hettes et al., 2007; Hettes et al., 2010; Urstadt et al., 2013). Within the hindbrain, AMPA/KA receptors and NMDA receptors have opposing effects on feeding behavior. Inhibiting AMPA and KA receptors with fourth ventricle or NST injections of the AMPA/KA receptor antagonist NBQX significantly reduces sucrose intake (Zheng et al., 2002). However, NMDA receptor inhibition in the hindbrain with the antagonists MK-801 or D,L-AP5 increases food intake (Campos et al., 2012; Campos et al., 2013; Covasa et al., 2003; Gillespie et al., 2005; Guard et al., 2009a; Guard et al., 2009b; Ritter, 2011).

Immunohistochemical studies show that the GluN1, GluN2B, GluN2C, and GluN2D NMDA receptor subunits are expressed in the nodose neurons or vagal afferent terminals (Aicher et al., 1999; Czaja et al., 2006a; Czaja et al., 2006b; Ritter, 2011); note that expression of the GluN2A subunit has not been specifically evaluated in the rat. Taken together, these immunohistochemical and feeding behavior studies suggest that presynaptic NMDA receptors may enhance the excitability of the vagal afferents. Therefore, we used live cell calcium imaging and available pharmacological tools to determine which GluN2 subunits control vagal afferent calcium levels in the NST.

2. Results

2.1. Vagal afferent calcium signals are modulated by NMDA receptors

Our “time control” experiments demonstrated that repetitive applications of ATP (separated by a 10 min interval during which the slices were exposed to recording solution alone; Fig. 1) produced very similar levels of activation in CGD-labeled vagal varicosities. Specifically, on average, the first ATP challenge evoked a relative increase in CGD fluorescence [(F/F)%] of $28.9 \pm 1.3\%$; the second ATP exposure produced a relative increase in CGD fluorescence of $28.8 \pm 1.5\%$ ($n=172$ ROI; Fig. 1E; $p > 0.05$; paired t-test). Thus, each ROI could act as its own control.

Next, we evaluated whether we could enhance the vagal afferent calcium signals by preventing glutamate reuptake using the excitatory amino acid reuptake inhibitor TBOA. Based on our “time control” experiments discussed above, the magnitude of the first response to ATP was considered “100%”, and the magnitude of the second ATP response was a normalized percentage relative to the first. Bath application of TBOA (100 μ M; 10 min) between the two ATP stimulation periods significantly increased the second response to $122.9 \pm 7.6\%$ of the first ATP response ($n=30$ ROI; $p < 0.05$; one-way ANOVA with Dunnett’s *post hoc* test; Fig. 2). Thus, presynaptic glutamatergic receptors are capable of increasing vagal afferent calcium.

To evaluate whether NMDA receptors control vagal afferent calcium, we bath applied the NMDA receptor antagonist D,L-AP5 (200 μ M; 10 min) between the two ATP application periods. Inhibiting NMDA receptors significantly decreased the vagal afferent calcium response to ATP, as CGD fluorescence evoked by the second ATP application was $83.2 \pm 1.6\%$ of the first ATP response ($n=197$ ROI; $p < 0.05$; one-way ANOVA with Dunnett’s *post hoc* t-test; Fig. 2). Therefore, these glutamatergic presynaptic receptors are NMDA receptors.

2.2. GluN2B and GluN2C/D-containing NMDA receptors control vagal afferent calcium

Subunit-selective NMDA receptor modulators were used to identify which GluN2 subunits control calcium levels in the vagal afferent terminals that synapse upon the NST. In these experiments, slices were exposed to two applications of ATP separated by 10 min bath perfusions of TCN 201, ifenprodil, pregnenolone sulfate, or CIQ (Table 1; Fig. 3).

TCN 201 (10 μ M), a selective antagonist for GluN2A-containing NMDA receptors (Hansen et al., 2012; McKay et al., 2012) produced a small but not statistically significant influence

over vagal terminal calcium (n=108 ROI; $p > 0.05$; one-way ANOVA with Dunnett's *post hoc* test; Table 1). This suggests that if GluN2A-containing NMDA receptors are expressed in the vagal afferents that synapse in the NST, they do not mediate a significant component of the NMDA receptor response.

Ifenprodil (3 μ M) inhibits GluN2B-containing NMDA receptors (Williams, 1993) and significantly reduced the second ATP response to $83.7 \pm 1.7\%$ of the first response (n = 222 ROI; $p < 0.05$; one-way ANOVA with Dunnett's *post hoc* test; Table 1). Furthermore, pregnenolone sulfate (50 μ M), which potentiates GluN2A/B-containing NMDA receptors (Traynelis et al., 2010), significantly increased the second ATP response to $125.1 \pm 5.3\%$ of the first response (n=186 ROI; $p < 0.05$; one-way ANOVA with Dunnett's *post hoc* test; Table 1). These data suggest that the GluN2B subunit is a component of functional NMDA receptors that control the calcium levels of the vagal afferents.

Finally, the GluN2C- and GluN2D-containing NMDA receptor potentiator CIQ (20 μ M) (Mullasseril et al., 2010) significantly increased the response of the second ATP application to $130.9 \pm 4.6\%$ of the first ATP application (n=121 ROI; $p < 0.05$; one-way ANOVA with Dunnett's *post hoc* test; Table 1). Therefore, GluN2C/D-containing NMDA receptors also mediate a component of the presynaptic vagal afferent NMDA receptor response in the NST.

3. Discussion

Ionotropic glutamate receptors have long been known to mediate a component of excitatory postsynaptic neurotransmission. However, a number of immunohistochemical studies indicate that NMDA receptors also are expressed in presynaptic terminals throughout the brain (Aicher et al., 1999; Charton et al., 1999; Clarke and Bolam, 1998; DeBiasi et al., 1996; Lu et al., 2003; Paquet and Smith, 2000). Furthermore, electrophysiological and calcium imaging studies have shown that presynaptic NMDA receptors positively and negatively regulate glutamate, GABA, and norepinephrine neurotransmitter release in the cortex (Brasier and Feldman, 2008; Buchanan et al., 2012; Fink et al., 1990; Li et al., 2008; Woodhall et al., 2001), cerebellum (Bidoret et al., 2009; Duguid and Smart, 2004; Fiszman et al., 2005; Glitsch and Marty, 1999; Rossi and Collin, 2013), primary sensory neurons in the spinal cord dorsal horn (Bardoni et al., 2004; Yan et al., 2013), and hippocampus (McGuinness et al., 2010; Suarez and Solis, 2006). Indeed, presynaptic NMDA receptors have been shown to have a role in long-term depression and long-term potentiation throughout the brain (Banerjee et al., 2009; Bender et al., 2006; Bidoret et al., 2009; Casado et al., 2002; Larsen et al., 2014; Lien et al., 2006; Rodriguez-Moreno et al., 2011). Here, we report that presynaptic NMDA receptors also are capable of controlling presynaptic vagal afferent calcium levels evoked by pharmacological stimulation with ATP in the nucleus of the solitary tract.

Behavioral studies have suggested that presynaptic NMDA receptors on vagal afferents may control food intake. Systemic or direct injections of the NMDA receptor antagonists MK-801 or D,L-AP5 increase food intake and the size of spontaneously initiated meals; this effect requires intact vagal fibers and caudomedial NST (Burns and Ritter, 1997; Hung et

al., 2006; Jahng and Houpt, 2001; Treece et al., 2000). Extensive behavioral studies by Ritter's laboratory relied on fourth ventricular or intra-NST injections of specific antagonists of different GluN2 subunits (i.e., D-CCPene: an antagonist that is somewhat more selective for GluN2A/B over GluN2C/D; PPDA: an antagonist that preferentially inhibits GluN2C/D over GluN2A/B; or Con-G: an antagonist selective for the GluN2B-D subunits over GluN2A) and suggest that the GluN2B, GluN2C, and GluN2D subunits mediate presynaptic NMDA receptor control of sucrose intake (Guard et al., 2009a). Cholecystokinin (CCK)-induced reduction in sucrose intake also is blocked by pretreatment with systemic D-CCPene (Guard et al., 2009b), further suggesting that GluN2B-containing NMDA receptors are expressed on presynaptic vagal afferents.

Our imaging studies corroborate these behavioral studies. Specifically, we observed that the GluN2B and GluN2C/D, and to a lesser extent GluN2A, NMDA receptor subunits contribute to vagal afferent calcium levels in response to pharmacological stimulation by ATP. Indeed, subunit-selective NMDA receptor potentiators (i.e., CIQ and pregnenolone sulfate) increased vagal afferent responsiveness. Given that an increase in NST activity is associated with an inhibition of DMN activity and cessation of feeding (Rogers and Hermann, 2012), these observations suggest that it may be possible to inhibit food intake by enhancing NMDA receptor function in the hindbrain.

Most reports in the literature concerning presynaptic NMDA receptor effects on vagal afferents focus on feeding behavior and digestive issues. However, vagal afferents in the NST are also engaged in cardiovascular as well as respiratory reflex control (e.g., (Accorsi-Mendonca et al., 2013; Austgen et al., 2011; Kline et al., 2009; Spencer and Talman, 1986)). Therefore, one might also expect presynaptic vagal control of these functions to be influenced by NMDA receptor action. Still, there is very little direct evidence verifying this claim. One report (Takano and Kato, 1999) suggests that presynaptic NMDA action may modulate vagal influence over respiratory rhythmogenesis.

Our present imaging studies demonstrated a reduction in responsiveness of vagal afferents to ATP stimulation while in the presence of NMDA receptor antagonists. These observations suggest that NMDA receptors expressed on the vagal afferents may be tonically active within the hindbrain. There are several potential sources of glutamate that could activate the presynaptic NMDA receptors in the NST. The vagal afferents synapsing upon the NST are glutamatergic, so it is possible that glutamate spillover following stimulation of the solitary tract activates the presynaptic NMDA receptors (Aylwin et al., 1998; Leone and Gordon, 1989). Additionally, astrocytic glutamate release and presynaptic NMDA receptor activation can enhance presynaptic excitability and synaptic strength (Araque et al., 1998; Jourdain et al., 2007; Perea and Araque, 2007). Finally, glutamate released from the subset of NST neurons that are glutamatergic may be capable of activating vagal NMDA receptors (Fortin and Champagnat, 1993). This arrangement suggests the possibility that vagal afferent neurotransmission could be under some sort of "autoreceptor" feedback control from NST neurons, themselves possessing a robust population of NMDA receptor (Aylwin et al., 1997). Finally, the high density of astrocytes in the NST and their ability to release glutamate (Hermann et al., 2009) suggests that NST glial cells could strongly modulate presynaptic excitability and synaptic strength through the NMDA receptor with significant

functional consequences (Araque et al., 1998; Hermann et al., 2009; Jourdain et al., 2007; Perea and Araque, 2007; Spencer and Talman, 1986).

In summary, our live cell calcium imaging studies suggest that NMDA receptors, particularly GluN2B, GluN2C, and GluN2D-containing NMDA receptors, modulate the calcium levels of presynaptic vagal afferents following pharmacological stimulation with ATP. It remains to be determined whether these subunits mediate vagal calcium following electrical stimulation or whether NMDA receptors expressed on the presynaptic afferents regulate neurotransmitter release in the NST. Nevertheless, these studies, combined with previously published behavioral work, suggest that NMDA receptors can modulate the vagal afferent terminals that regulate physiological processes under autonomic control, including food intake.

4. Experimental Procedure

Long-Evans rats (200–250 g body weight; 11 rats; 34 hindbrain slices) from the breeding colony at Pennington Biomedical Research Center were used for these studies. Animals were housed in a room with a 12 hour light/dark cycle with constant temperature and humidity and had access to food and water *ad libitum*. All experimental protocols were approved by the Institutional Animal Care and Use Committees of Pennington Biomedical Research Center and were performed according to the guidelines determined by the National Institutes of Health.

4.1. Vagal afferent labeling for calcium imaging

Vagal afferents were pre-labeled as previously described (Rogers et al., 2006). Briefly, rats were anesthetized with isoflurane. Using aseptic technique, the right nodose ganglion was accessed through a ventral incision in the neck. A glass microinjection pipette (~75–80 μ m tip diameter) was filled with 20% CalciumGreen 1-dextran 3000 molecular weight conjugate (CGD; Life Technologies) that had been reconstituted in 1% Triton X-100 and distilled water. The micropipette was connected to a Picospritzer (General Valve); CGD calcium reporter dye (~500 nL) was injected into the exposed nodose ganglion. The cervical wound was closed and the dye was allowed to transport for 3–5 days.

4.2. Brainstem slice preparation

Brainstem slices were obtained as described previously (Vance et al., 2014). The animals were deeply anaesthetized with urethane (1.5 g/kg; ethyl carbamate; Sigma) and decapitated. The brainstem was rapidly harvested, isolated, glued to the stage of a vibrating microtome (Leica VT1200), and sliced into 300 μ m thick coronal sections. The cutting chamber of the microtome was filled with cold (4°C) carbogenated (95% O₂ / 5% CO₂) “cutting solution” that contained (in mM) 92 N-methyl-D-glucamine, 30 NaHCO₃, 25 glucose, 20 HEPES, 10 MgSO₄·7H₂O, 5 sodium ascorbate, 3 sodium pyruvate, 2.5 KCl, 2 thiourea, 1.25 NaH₂PO₄, and 0.5 CaCl₂, titrated to pH 7.4 with HCl (Zhao et al., 2011). The brainstem sections were incubated at 32–34°C in the cutting solution for 10–15 min after being cut, after which they were incubated for 1–6 hr at room temperature (22–24°C) in a carbogenated “recording solution” containing (in mM) 124 NaCl, 25 NaHCO₃, 10 glucose, 3 KCl, 2 CaCl₂, 1.5

NaH₂PO₄, and 1 MgSO₄·7H₂O that was supplemented with 5 mM sodium ascorbate, 3 mM sodium pyruvate, and 2 mM thiourea and titrated to pH 7.4 with HCl (Zhao et al., 2011).

4.3. Live cell calcium imaging

Hindbrain slices containing vagal afferent fibers and varicosities prelabeled with CGD were placed in the recording chamber of a Nikon F1 fixed stage upright microscope. Slices were continuously perfused with the carbogenated recording solution (33°C; 2.5 mL/min flow rate). A Prairie Technologies line-scanning laser confocal head equipped with a Photometrics CoolSNAP HQ camera performed time-lapse laser confocal calcium imaging, collecting images at a rate of three frames per second. The CGD-labeled afferent terminals were visualized using a 488 nm excitation/509 nm long pass emission filter.

For each live cell imaging recording, the slices were perfused with control recording solution for 10 min. ATP (100 µM) was bath applied (60 s) to verify the viability of the vagal terminals as reflected by their ability to respond with calcium signals once their P2X₃ ligand-gated cation channels were activated (Jin et al., 2004; Rogers et al., 2006). The slices were then perfused with one of the following solutions for 10–20 min:

1. Recording solution alone (“time control”);
2. 200 µM D,L-2-amino-5-phosphonopentanoic acid (D,L-AP5; Abcam);
3. 100 µM D,L-threo-β-benzyloxyaspartic acid (TBOA; Tocris);
4. 10 µM 3-chloro-4-fluoro-N-[4-[[2-(phenylcarbonyl)hydrazino]carbonyl]benzyl]benzenesulfonamide (TCN 201; Tocris);
5. 3 µM ifenprodil (Abcam);
6. 50 µM pregnenolone sulfate (PS; Sigma); or
7. 20 µM (3-chlorophenyl)(6,7-dimethoxy-1-((4-methoxyphenoxy)methyl)-3,4-dihydroisoquinolin-2(1 H)-yl)methanone (CIQ; Tocris).

ATP (100 µM; 60 s) was then applied for a second time to each slice. Differences in response to the two exposures of ATP could be directly compared. Thus, each varicosity could act as its own control.

4.4. Data Analysis

Nikon Elements AR software was used to analyze the confocal live cell fluorescent signals as previously described (Rogers et al., 2006). Multiple CGD-labeled vagal terminals and varicosities were identified as fluorescent regions of interest (ROI) in each slice. The relative changes in cytoplasmic calcium were calculated after the background fluorescence (measured from a non-active portion of the same field) was subtracted from both the at rest (baseline) and peak fluorescence signals. The relative changes in cytoplasmic calcium for each ROI were calculated as the percentage of change in CGD fluorescence [(F/F)%], where *F* is the baseline intensity of the fluorescence signal before stimulation, and *F* is the difference between the peak fluorescence intensity and baseline intensity. Response

magnitudes to the second exposure to ATP were directly compared to each ROI's first exposure to ATP; the first response was considered "100%". Therefore, the second response was normalized as a percentage of the first.

A paired t-test was used to evaluate the percentages of change in CGD fluorescence (i.e. (F/F)% values) evoked by the first and second ATP applications to determine if the second ATP application was significantly different than the first ATP application in the time control experiment. One-way ANOVAs with Dunnett's *post hoc* test were used to evaluate normalized calcium imaging data from the second ATP responses for statistical significance (e.g. to compare D,L-AP5 and TBOA to the time control and to compare the subunit-selective NMDA receptor modulators to the time control). Data are reported as mean $\pm$ S.E.M., and statistical significance was set at $p < 0.05$.

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Abbreviations

CIQ	(3-chlorophenyl)(6,7-dimethoxy-1-((4-methoxyphenoxy)methyl)-3,4-dihydroisoquinolin-2(1 H)-yl)methanone
CGD	CalciumGreen 1-dextran
D,L-AP5	D,L-2-amino-5-phosphonopentanoic acid
mEPSCs	miniature postsynaptic excitatory currents
NST	nucleus of the solitary tract
PS	pregnenolone sulfate
ROI	region of interest
TBOA	D,L-threo- β -Benzyloxyaspartic acid
TCN 201	3-chloro-4-fluoro-N-[4-[[2-(phenylcarbonyl)hydrazino]carbonyl]benzyl]benzenesulfonamide

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Highlights

- Presynaptic NMDA receptors on vagal afferents may be involved in food intake.
- We evaluated which NMDA receptor GluN2 subunits regulate vagal afferent activity.
- D,L-AP5 and the GluN2B antagonist ifenprodil decrease vagal calcium signals.
- The GluN2C/D potentiator CIQ enhances vagal calcium levels in prelabeled terminals.
- GluN2B, GluN2C, and GluN2D NMDARs control vagal afferent excitability in the NST.

Figure 1.

Time control applications of ATP produce comparable responses.

A: Field view of a labeled hindbrain slice with a representative vagal terminal ROI outlined by a dotted box. **B:** Representative ROI is shown at rest, prior to bath application of ATP. **C:** The same ROI is shown at the peak activation response to ATP. Scale bars: A=10 μm ; B and C=6 μm . **D:** Plots of change in fluorescence over time for the representative ROI for the first (left) and second (right) ATP applications were separated in time by 10 min. **E:** There was no significant difference between the activation responses produced by two successive applications of ATP separated by a 10 min perfusion of bath alone in the time control; $p > 0.05$; paired t-test.

Figure 2.

NMDA receptors may modulate vagal afferent terminal calcium levels. Bath application of TBOA significantly increased the calcium response to the second ATP application compared to the time control, suggesting that vagal afferent glutamate receptors may amplify vagal calcium signals. The NMDA receptor antagonist D,L-AP5 significantly inhibited the response evoked by the second ATP application. One-way ANOVA with Dunnett's *post hoc* test; * $p < 0.05$.

Figure 3.

Subunit-specific NMDA receptor modulators were used to identify the GluN2 subunits that can control vagal afferent calcium levels. Slices were perfused with recording solution plus TCN 201, ifenprodil, pregnenolone sulfate (PS), and CIQ between the two successive applications of ATP. All NMDA receptor modulators evaluated affected the ATP-evoked responses though the effects of the GluN2A antagonist (TCN 201) were smaller and not statistically significant. One-way ANOVA with Dunnett's *post hoc* test; * $p < 0.05$.

Table 1

NMDA receptor antagonists and potentiators modulate the response to ATP

Experimental condition	% Response	<i>n</i>
Time Control	99.5 ± 2.0	172
TCN 201	88.4 ± 2.6	108
Ifenprodil	83.7 ± 1.7*	222
Pregnenolone Sulfate	125.1 ± 5.3*	186
CIQ	130.9 ± 4.6*	121

Pre-labeled varicosities were first identified as active with a bath application of 100 μ M ATP for 60 s. The varicosities were next exposed to one of the following conditions for 10–15 minutes: Krebs alone (Time Control); D,L-AP5 (200 μ M); TCN 201 (10 μ M); Ifenprodil (3 μ M); pregnenolone sulfate (50 μ M); or CIQ (20 μ M). Finally, the varicosities were exposed to a second application of 100 μ M ATP for 60 s. Data are reported as the percentage of the second response relative to the first response. Data were analyzed for statistical significance (* $p < 0.05$) across all groups using a one-way ANOVA with Dunnett's *post hoc* test.