BDNF silences GABA synapses onto hypothalamic neuroendocrine cells through a postsynaptic dynamin-mediated mechanism

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Abstract

In the paraventricular nucleus of the hypothalamus (PVN), experimental stress paradigms which suppress GABA inputs to parvocellular neuroendocrine cells (PNCs) also increase the expression of brain derived neurotrophic factor (BDNF). In the adult CNS, BDNF regulates the efficacy of GABAergic transmission, but its contributions to functional changes at inhibitory synapses in the PVN have not been investigated. Analysis of quantal transmission revealed a decrease in the frequency of miniature inhibitory postsynaptic currents (mIPSCs) in response to BDNF with no accompanying changes in their amplitude. These effects were completely blocked by prior inclusion of the TrkB receptor antagonist, K252A in the patch pipette. Inclusion of a dynamin inhibitory peptide in the patch pipette also blocked the effects of BDNF, consistent with an all-or-none removal of clusters of postsynaptic GABA_\text{A} receptors. Finally, to confirm a decrease in the availability of postsynaptic GABA_\text{A} receptors, we tested the effects of BDNF on focal application of the GABA_\text{A} agonist, muscimol. Postsynaptic responses to muscimol were reduced following BDNF. Collectively, these data indicate that BDNF re-models functional synaptic contacts putatively by reducing the surface expression of postsynaptic GABA_\text{A} receptors.
The neurotrophins are a family of molecules that are critical for normal development of the central nervous system (Tapia-Arancibia et al. 2004). They play a vital role in facilitating the formation and maturation of the precise neural circuitry during the developmental phase through the strengthening of some synapses and pruning of others (Lu 2003; Tapia-Arancibia et al. 2004). Our knowledge of their scope of action has been expanded recently by the demonstrations that neurotrophins, and in particular, brain-derived neurotrophic factor (BDNF), can modify synaptic efficacy in neural circuitry derived from adult brain (Lu 2003). While the focus, in the adult, has been on the ability of BDNF to alter synaptic strength, it may also play an important role in changing the relative weightings of individual synapses onto target neurons.

In response to stress paradigms, the expression of BDNF mRNA and protein increases in the adult hypothalamus (Givalois et al. 2004; Rage et al. 2002; Smith et al. 1995). This is particularly evident in the neuroendocrine neurons in the paraventricular nucleus of the hypothalamus (PVN) and is paralleled by decreases in inhibitory synaptic drive to these cells (Verkuyl et al. 2005; Verkuyl et al. 2004). BDNF can alter synaptic inhibition by decreasing the surface stability and expression of GABA<sub>A</sub> receptors through activation of postsynaptic TrkB receptors (Brunig et al. 2001; Jovanovic et al. 2004; Tanaka et al. 1997). An alteration in the surface stability of the receptor may underlie the demonstration that BDNF can promote loss of receptors at some sites and increased clustering at other sites (Elmariah et al. 2004), resulting in a re-modeling of inhibitory synapses through changes in the localization of GABA<sub>A</sub> receptors.

In order to test the effects of BDNF on stress-relevant inhibitory circuitry, we obtained whole-cell patch clamp recordings from p21-28 rat parvocellular PVN neurons in acute coronal
slices. We recorded from putative parvocellular neuroendocrine cells (PNCs) in the medial region of the nucleus and examined the effects of BDNF on miniature IPSCs (mIPSCs). Our findings indicate that BDNF selectively inhibits a subpopulation of GABA synapses through a dynamin-mediated endocytosis of postsynaptic GABA_A receptors. These limited, yet precise actions of BDNF unveil a mechanism by which neurons meet physiological demands by selectively altering the balance of inhibitory drive to fine tune synaptic outputs.

**Methods**

All experiments were performed according to protocols approved by the University of Calgary animal care and use committee in accordance with guidelines established by the Canadian Council on Animal Care.

*Slice Preparation.* Hypothalamic slices containing the PVN were prepared from postnatal day 21 (P21) to P27 male Sprague Dawley rats. Animals were anesthetized with sodium pentobarbital (30-50mg/kg) and decapitated, and the brains were rapidly removed into ice-cold high sucrose slicing solution (saturated with 95% O_2-5%CO_2) and allowed to cool for ~3min. The slicing solution contained the following (in mM): 87 NaCl, 2.5 KCl, 25 NaHCO_3, 0.5 CaCl_2, 7 MgCl_2, 1.25 NaH_2PO_4, 25 glucose, and 75 sucrose. The brain was then blocked, mounted on a vibrating slicer (Leica, Nussloch, Germany), and submerged in slicing solution that was constantly bubbled with 95%O_2-5%CO_2. The brain was cut in the coronal plane, and hemisected slices of 300μM thickness, containing the hypothalamus, were incubated at 32.5°C in a submerged chamber of oxygenated artificial CSF (aCSF) for a minimum of 90 min before recording. The aCSF contained the following (in mM): 126 NaCl, 2.5 KCl, 26 NaHCO_3, 2 CaCl_2, 2 MgCl_2, 1.25 NaH_2PO_4, and 10 glucose.

*Electrophysiological recordings.* Whole-cell recordings were obtained from medial parvocellular cells visually identified using an upright microscope (Olympus Optical (Tokyo, Japan) BX51W)
fitted with infrared differential interference contrast optics. All recordings were obtained at 32.5°C using borosilicate glass microelectrodes (tip resistance of 3-7 MΩ) filled with intracellular solution containing the following (in mM): 150 CsCl, 1 EGTA, 10 HEPES, 0.1 CaCl₂, 4.6 MgCl₂, 2 Mg-ATP, 0.3 NA-GTP. The internal solution was filtered before use. In preliminary experiments, recordings were obtained with a potassium gluconate based internal solution to ascertain the phenotype of these cells based on their electrical fingerprint. Previous work has demonstrated that a lack of inward rectification and low-threshold spikes are consistent with a neuroendocrine phenotype (Luther et al. 2002). Once we were confident of our ability to record from neuroendocrine cells faithfully, the remaining recordings were performed with a Cs-based internal solution to increase driving force for Cl⁻ ions, and inhibit postsynaptic voltage-gated K⁺ currents. All experiments were performed in voltage-clamp mode, with cells held at -80mV (to block any NMDA currents) and recordings were accepted when access resistance changes were limited to <15%.

For all experiments, the perfusate solution contained 10µM DNQX (Tocris Cookson, Ellisville, MO) to block excitatory AMPA-mediated synaptic currents and tetrodotoxin (TTX) 1µM, to ensure only spontaneous miniature inhibitory postsynaptic currents (mIPSCs) through GABAₐ receptors were recorded. Signals were amplified with the Multiclamp 700A amplifier (Axon Instruments, Foster City, CA), low-pass filtered at 1 kHz, and digitized at 5-10kHz using the Digidata 1322 (Axon Instruments).

Previous investigators have reported multifaceted effects of BDNF that were a function of the duration for which BDNF was applied (Jovanovic et al. 2004). In order to avoid this complication, we applied BDNF for two minutes and consistently analyzed our data from 6-12 minutes after the end of the BDNF application.
Data analysis. Data were digitized (pClamp 9; Axon Instruments) and stored on computer for offline analysis. Events were detected when they crossed a threshold set at three times the baseline noise. The detected events were confirmed as synaptic events by eye. The frequency and amplitude distributions of mIPSCs in individual cells were compared before and after drug administration using a Kolmogorov-Smirnov statistic. Comparisons between drug administrations are shown as mean ± SEM. Data points were compared using ANOVA with a post hoc Newman-Keuls test for multiple groups. Student’s t test was used to compare two groups.

Drugs. TTX and K252B were purchased from Alamone Labs, bicuculline methiodide, dynamin inhibitory peptide and basic lab salts from Sigma-Aldrich, BDNF from R & D Systems, K252A, dynamin inhibitory peptide, dynamin inhibitory peptide myristoylated, muscimol and DNQX from Tocris Cookson.
Results

Recordings were obtained from parvocellular neurons in medial PVN. We initially targeted putative PNCs based on their location and morphology. These cells are located in the medial aspects of PVN (Bains and Ferguson 1997b; Cowley et al. 1999; Luther et al. 2002; Luther and Tasker 2000; Pronchuk et al. 2002; Verkuyl et al. 2005), and unlike the magnocellular neurons, which are large and spherical in appearance, parvocellular neurons typically have a small, ovoid cell body. To further ascertain their identity, we obtained preliminary recordings from these cells using a potassium gluconate internal solution. This, we reasoned, would allow us to further identify PNCs on the basis of their electrical fingerprint. In this recording configuration, parvocellular neurons differ from the magnocellular neurons in that they do not exhibit a delay to first spike when depolarized from negative potentials (Bains and Ferguson 1997a; Hoffman et al. 1991; Stern and Armstrong 1995; Tasker et al. 1991). Within the parvocellular subgroups, we can further differentiate between PNCs (no rebound low threshold spikes) (Luther et al. 2002), and pre-autonomic neurons (rebound LTS or spike burst) (Stern 2001). Once we were satisfied that the cells we were patching in the medial region were always PNCs, we conducted the rest of the experiments with CsCl internal as described above. All subsequent experiments were conducted in voltage-clamp mode at a holding potential of -80mV.

BDNF decreases frequency of mISPCs

In addition to the action-potential driven release of GABA, PNCs in PVN are bombarded by a high tonic level of quantal, non-action potential driven mIPSCs. The mean mIPSC frequency recorded from these neurons was 3.99±0.22 Hz, n=40 while the mean amplitude was 76.36±1.11 pA, n=40. The mIPSCs were blocked reversibly by the GABA$_A$ receptor antagonist, bicuculline
methiodide, 10 µM (data not shown) thereby confirming that these events were mediated by the activation of postsynaptic GABA<sub>A</sub> receptors.

To test the effects of BDNF on GABAergic inputs, we bath applied 2 nM BDNF for two minutes and assessed changes in the frequency and amplitude of mISPCs. Previous investigators have reported multifaceted effects of BDNF that were a function of the duration for which BDNF was applied (Jovanovic et al. 2004). We failed to see any evidence for an increase in quantal amplitude or frequency in response to BDNF (n=20). In response to BDNF, we observed a slowly developing decrease in the frequency of mISPCs which reached a new, stable level approximately six minutes after the termination of the BDNF application. Consequently, we only examined changes in synaptic strength during this stable period (six – twelve minutes after BDNF) and compared these values to the control time period prior to BDNF application. BDNF elicited a significant decrease in mIPSC frequency (74.09±3.87% of control, p<0.01, n=20, Figure 1A,B,C) but had no effect on amplitude (101.40±2.91% of control, p>0.05, n=20, Figure 1A,C). No further decrease in frequency was observed beyond this time period and the effects were not reversible up to 35 minutes after the termination of BDNF application.

**BDNF acts through postsynaptic TrKB receptors**

Although a decrease in the frequency of mIPSCs is classically taken as an indicator of a change in vesicular release probability, manipulations that remove entire clusters of postsynaptic receptors can also cause a decrease in quantal frequency (Beattie et al. 2000; Oliet et al. 1996). BDNF can decrease the surface stability of the GABA<sub>A</sub> receptor within the membrane (Brunig et al. 2001; Jovanovic et al. 2004; Tanaka et al. 1997) and alter the postsynaptic localization of GABA<sub>A</sub> receptors (Elmariah et al. 2004) through interactions with postsynaptic TrkB receptors. We
examined the possibility that BDNF decreases inhibitory signaling through the activation of postsynaptic TrKB receptors. In order to test this hypothesis directly, we included the TrkB receptor inhibitor, K252A (200 nM) (Cheng and Yeh 2003; Cheng and Yeh 2005; Patapoutian and Reichardt 2001; Tanaka et al. 1997) in the patch pipette and repeated the experiments with BDNF. During the fifteen minutes of control to allow for infusion of K252A into the cell, we did not see any consistent changes in event frequency or amplitude (data not shown). In the presence of K252A, BDNF had no effect on either the frequency or the amplitude of mIPSCs (frequency: 102.10±4.06% of control, p>0.05, n=6, Figure 2A; amplitude: 102.20±4.32% of control, p>0.05, Figure 2B). To confirm that the effects of BDNF were the result of specific TrKB receptor activation, we repeated these experiments in the presence of K252B (200nM), a compound that acts as a weak, non-specific inhibitor of the Trk-type kinase and protein kinase C, and which is commonly used as a control for K252A (Tanaka et al. 1997). No frequency or amplitude effects were observed during the fifteen minute control period during which the drug concentration reached a steady-state. In the presence of K252B, BDNF elicited a small, but significant decrease in mIPSC frequency (85.30±4.83% of control, p<0.05, n=8, Figure 2A), with no effects on event amplitude (101.10±4.84% of control, p>0.05, Figure 2B). This decrease in mIPSC frequency was not as robust as that observed in BDNF alone (p<0.05). To further assess the intracellular pathways mediating the effects of BDNF on mIPSCs, we conducted additional experiments with the calcium chelator EGTA (10mM) in the patch pipette. Under these conditions, BDNF decreased mIPSC frequency (83.66±3.97% of control, p<0.05, n=6, Figure 2A) and again had no effect on amplitude (95.23±3.92% of control, p>0.05, Figure 2B). Like K252b, this change in frequency was also significantly different from that observed in BDNF alone (p<0.05). From these observations, it appears that TrkB receptor activation is
necessary for the actions of BDNF and that downstream effectors such as PKC and intracellular Ca\textsuperscript{2+} may be involved in an important, yet limited fashion.

**BDNF decreases postsynaptic GABA efficacy through a dynamin-dependent mechanism**

We next examined the potential cellular mechanism through which BDNF may exact a removal of GABA\textsubscript{A} receptor clusters. One possibility is that BDNF decreases the surface expression of GABA\textsubscript{A} receptors through an internalization mechanism (Brunig et al. 2001; Cheng and Yeh 2003; Jovanovic et al. 2004). Endocytosis of membrane bound receptors involves their association with adaptor proteins and the subsequent recruitment of clathrin-coated pits. With the receptor bound to an adaptor protein such as AP2, the complex is then able to interact with clathrin and the GTPase, dynamin, both critical elements for the endocytotic process (Herring et al. 2003; Kittler et al. 2000). Since the endocytosis of GABA\textsubscript{A} receptors in the hippocampus is a clathrin-mediated event (Connolly et al. 1999; Herring et al. 2003; Kittler et al. 2000; van et al. 2005), and dynamin is a crucial component to this process, we asked whether dynamin was involved in the postsynaptic actions of BDNF. In order to selectively block dynamin-mediated endocytosis, a 10 amino acid peptide, dynamin inhibitory peptide (Tocris Cookson), 50 \(\mu M\), was added to the internal solution of the patch pipette. This molecule prevents endocytosis by interfering with the binding of amphiphysin with dynamin (Marks and McMahon 1998; Wigge and McMahon 1998). In order to permit sufficient time for the contents of the pipette to fully diffuse into the cell, we obtained control recordings for fifteen minutes prior to application of BDNF. During this time, we did not observe any changes in the frequency or amplitude of mIPSCs. The subsequent application of BDNF did not replicate the changes in mIPSC frequency seen in BDNF alone. We observed no change in mIPSC frequency (95.55±5.55\% of control, \(p>0.05\), \(n=7\), Figure 3A, B) and no change in amplitude
(98.95±5.40% of control, p>0.05, Figure 3A, B). To control for non-specific actions of this inhibitor that may be independent of its actions on dynamin, we conducted additional experiments in which we included a myristoylated scrambled version of the peptide (50µM) in the patch pipette and tested the effects of BDNF on mIPSCs. Under these conditions, BDNF robustly inhibited GABA transmission to a level not significantly different from that seen with BDNF administration alone (mIPSC frequency: 82.11±4.35% of control, p<0.05, n=5; mIPSC amplitude: 92.34±6.42% of control, Figure 3B). These findings provide evidence that BDNF decreases GABAergic signaling by the selective, dynamin-dependent internalization of GABA<sub>A</sub> receptors.

**Agonist-induced inward currents are reduced after BDNF**

To directly measure the consequences of postsynaptic receptor internalization, we examined the effects of BDNF on the inward current induced by focal application of the GABA<sub>A</sub> receptor agonist, muscimol. If BDNF elicits the internalization of GABA<sub>A</sub> receptors, we would predict that fewer receptors would be available for activation after exposure to BNDF and the current induced by direct GABA<sub>A</sub> receptor activation will be reduced accordingly. Muscimol (100 µM) was pressure applied from the tip of a patch pipette which was positioned ~20-30 microns away from the recording electrode in the cell body. Four epochs of muscimol application, spaced one minute apart were delivered. To control for the variability that can arise from responses to exogenous drug application, the value for each epoch represents the averaged response to three consecutive applications spaced four seconds apart. The number of epochs (4) was chosen to provide an adequate baseline prior to BDNF application. Due to the inter-cell variability in the amplitude of the responses to muscimol, data within each cell were normalized to the average control response (Figure 4B). After BDNF, the amplitude of the inward current, in pA, was significantly smaller than in control
(83.01±6.11% of control, p<0.05, n=8, Figure 4 A, B), indicative of a removal of available surface receptors.

**Discussion**

Our data demonstrate that BDNF depresses transmission at inhibitory, GABAergic synapses onto parvocellular neuroendocrine neurons in PVN. This effect is mediated by the postsynaptic activation of TrkB receptors and could be blocked by inhibiting dynamin, a critical component in the clathrin-mediated endocytosis of GABA_A receptors (Herring et al. 2003; Kittler et al. 2000). This decrease in the surface expression of functional GABA_A receptors provides a reasonable mechanism underlying the previously observed decreases in inhibitory signaling at the onset of the stress response when BDNF expression is increased in PVN (Verkuyl et al. 2005; Verkuyl et al. 2004).

Our findings demonstrating the TrKB-mediated inhibition of GABA transmission are consistent with earlier observations (Brunig et al. 2001; Cheng and Yeh 2003; Gottschalk et al. 1998). Although there is also evidence for a BDNF-induced potentiation of GABA_A receptor function (Baldelli et al. 2002) we did not see any evidence of increases in GABAergic drive to PNCs in PVN. These differential effects have been shown to be time-dependent, with an initial potentiation giving way to a depression upon prolonged exposure to BDNF (Jovanovic et al. 2004), but we only observed a slowly developing decrease in the frequency of mIPSCs following application of BDNF.

The most intriguing aspect of our observations is the fact that BDNF appears to selectively target a subset of synapses by removing clusters of GABA_A receptors in an ‘all-or-none’ fashion. Since we observed a decrease in mIPSC frequency, but did not observe any changes in mIPSC amplitude, we hypothesized that BDNF was selectively ‘switching off’ clusters of postsynaptic GABA_A receptors (Beattie et al. 2000; Oliet et al. 1996). Our experiments in which inclusion of the
TrkB inhibitor, K252A in the patch pipette blocked the effects of BDNF are consistent with a postsynaptic locus of action of BDNF. We further propose that this may result from a TrkB driven endocytosis of receptor clusters. GABA_A receptors are recycled at the membrane surface, the rate of which is modulated by a number of factors (Kittler and Moss 2003). BDNF-induced GABA_A receptor internalization has been demonstrated previously, and consistent with our findings, does not occur until approximately five minutes following exposure to BDNF (Cheng and Yeh 2003). The phosphorylation state of the receptor plays a major role in determining the surface stability of the receptor at the membrane and hence the number of receptors present at any one time (Henneberger et al. 2002; Jovanovic et al. 2004; Kittler and Moss 2003; Krishek et al. 1994; Moss et al. 1995). There is extensive evidence that BDNF can alter the phosphorylation state and hence influence the rate of internalization of GABA_A receptors (Brunig et al. 2001; Kittler et al. 2000; Kittler and Moss 2001; Wan et al. 1997). Though there is conflicting evidence for the internalization process for GABA_A receptors, a clathrin mediated, dynamin dependent mechanism is most commonly attributed to this receptor (Connolly et al. 1999; Kittler et al. 2000). While we did not explicitly rule out a role for alterations in the phosphorylation state of the GABA_A receptor, our data are most consistent with a BDNF-mediated internalization of GABA_A receptors through a dynamin dependent mechanism.

The TrkB receptor has been previously linked to the activation of both PKC and intracellular calcium (Henneberger et al. 2002; Tanaka et al. 1997). This prompted us to investigate a role for intracellular calcium using the calcium chelator, EGTA (10 mM) in the patch pipette. With this manipulation, BDNF decreased frequency to a level that was different from control but also much attenuated from its effects when acting alone. Interestingly, we also noted a partial inhibition of the effects of BDNF when K252B, was used. This compound is a derivative of K252A, commonly employed as a control, and while it does not directly interfere with the TrKB receptor, it does, at the
dose used here, negatively affect PKC. Since PKC has also been shown to reduce GABA_A receptor immunoreactivity (Chapell et al. 1998) and BDNF activation of the TrkB receptor has been previously linked to the elevation of intracellular calcium and the activation of PLC (Tanaka et al. 1997), it is not surprising that inhibiting PKC, even partially, should mitigate some of the effects of BDNF observed initially. It appears therefore, that TrkB receptor activation is crucial while the role of these specific downstream effectors is more limited in mediating the BDNF response.

Our findings demonstrate a novel link between the BDNF activation of TrkB receptors and dynamin-mediated endocytosis of GABA_A receptors in PVN parvocellular neurons. These data indicate that a decrease in frequency is likely due to a loss of postsynaptic sites, further supported by the decreased inward current elicited by direct activation of GABA_A receptors after BDNF application. This reflects a condition whereby release at the presynaptic terminals is unchanged, but there are fewer receptors available to transduce the signal.

**Physiological Significance**

The onset of the stress response is accompanied by an increase in BDNF expression is in PVN CRH cells. Based on the data presented here, we propose that BDNF acts in an autocrine fashion to weaken inhibitory transmission through an internalization of GABA_A receptors at postsynaptic sites. Our data reinforces recent work using acute and chronic stress paradigms (Verkuyl et al. 2005; Verkuyl et al. 2004) which attenuate the frequency of mIPSCs with no effect on amplitude or event kinetics (Verkuyl et al. 2005; Verkuyl et al. 2004). Furthermore, following chronic stress, this decrease in GABA mIPSC frequency was not accompanied by changes in presynaptic release probability (Verkuyl et al. 2004). The authors propose that this reflects a loss of synaptic contacts under the stressed condition. Our data is consistent with this interpretation of a reduction in the number of synaptic contacts and we would further postulate that this is the result of a
BDNF-mediated internalization of receptors at specific synapses. This functional alteration may provide a means through which inputs can be selectively attenuated to increase the specificity of physiologically distinct signals and provide an additional level of control in fine tuning neuroendocrine output.
**Figure legends**

**Figure 1.** BDNF decreases the frequency of mIPSCs with no effect on amplitude.

A) Representative traces (upper panel) during control and after BDNF application (2 nM, 2 minutes). The decrease in frequency with no accompanying change in amplitude is shown in the cumulative fraction plots immediately below. B) Event frequency of all cells tested with BDNF, normalized to control frequency (n=20), shown as a fraction of the control values. Time points taken from two minutes control (dark circles) and from the 6-12 minutes after BDNF (clear circles). C) Summary of the change in event frequency and amplitude following BDNF application. Data were analyzed using Student’s t-test, and are shown as a percent of control values.

**Figure 2.** BDNF effects are mediated through activation of the TrkB receptor.

A) Summary of the change in event frequency as a percent of control values. Experiments were done with either K252A (200nM), K252B (200nM) or EGTA (10mM) included in the patch pipette. BDNF had no effect on frequency in the presence of K252A (p>0.05 vs. control, n=6), but did decrease frequency in both K252B (p<0.05 vs. control, n=8) and EGTA (p<0.05 vs. control, n=6). B) Summary of mIPSC amplitude in BDNF alone or BDNF + K252A, +K252B, +EGTA showing no change from control values. Data were analyzed with a one-way ANOVA with post-hoc Newman-Keuls multiple comparison test.

**Figure 3.** BDNF effects require postsynaptic dynamin.

A) Sample traces (top) from control with dynamin inhibitory peptide (DIP) included in the patch pipette (50µM) and following BDNF application. The effects on mIPSC frequency and amplitude in these cells are quantified immediately below. B) Summary of BDNF effects on frequency (left) and amplitude (right) in the presence of dynamin inhibitory peptide and the scrambled dynamin
inhibitory peptide, 50µM (DIPS). Data were analyzed with a one-way ANOVA with post-hoc Newman-Keuls multiple comparison test.

**Figure 4. Postsynaptic response to focal GABA_A agonist application are attenuated after BDNF.**

A) The GABA_A agonist, muscimol, was focally applied to the soma of the neuron being recorded. Three pressure applied puffs of muscimol were given every four seconds with one minute in between. Sample traces of the inward currents observed in response to three successive puffs (one epoch) are shown both in control (black) and after BDNF (grey) application. B) Summary of the change in the amplitude of the muscimol-evoked inward current inward current from all cells, normalized as a ratio of control as assessed using Student’s t-test.


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