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Nitric Oxide Alters GABAergic Synaptic Transmission in Cultured Hippocampal Neurons

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Abstract

Nitric oxide (NO) production increases during hypoxia/ischemia-reperfusion in the immature brain and is associated with neurotoxicity. NO at physiologic concentrations has been shown to modulate GABAergic (gamma-aminobutyric acid) synaptic transmission in the adult brain. However, the effects of neurotoxic concentrations of NO (relevant to hypoxia-ischemia) on GABAergic synaptic transmission remain unknown. The present study tests the hypothesis that nNOS is expressed at GABAergic synapses and that exposure to neurotoxic concentrations of NO results in enhanced GABAergic synaptic transmission in cultured hippocampal neurons (days-in-vitro 10-14) prepared from fetal rats. Using double immunocytochemistry techniques, we were able to demonstrate that nNOS is co-localized to both presynaptic and postsynaptic markers of GABAergic synapses. The effects of NO on GABAergic synaptic transmission were then studied using whole cell patch-clamp electrophysiology. Spontaneous and miniature inhibitory postsynaptic currents (sIPSCs and mIPSCs) were recorded prior to and after exposure to 250 μ M of the NO donor diethylenetriamine/nitric oxide adduct (DETA-NO). Exposure to DETA-NO resulted in increased sIPSCs and mIPSCs frequency, indicating that neurotoxic concentrations of NO enhance GABAergic synaptic transmission in cultured hippocampal neurons. Because GABA synapses appear to be excitatory in the immature brain, this effect may contribute to overall enhanced synaptic transmission and hyperexcitability. We speculate that NO represents one of the mechanisms by which hypoxia-ischemia increases seizure susceptibility in the immature brain.

Keywords

Nitric oxide; GABA; synaptic transmission; nNOS; electrophysiology

1- INTRODUCTION

Hypoxic-ischemic brain injury represents the most common cause of seizures in the neonatal period[61]. Mounting experimental evidence suggests that neonatal seizures are not only a marker of severe underlying disorder but also can contribute to brain injury[17;25;35;50]. Previous studies have shown that nitric oxide (NO) concentrations increase from a baseline of

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less than 1 nM to 20 nM to 1-4 μ M in the brain of adult rats exposed to ischemia/reperfusion [14;32]. Hypoxia-ischemia has also been shown to result in increased NO production in the immature brain[3;16]. NO is synthesized by a NO synthase (NOS)-catalyzed reaction of which neuronal NOS (nNOS) is the main isoform, accounting for 95% of all NOS catalytic activity in the brain [21;62]. NO has been shown to play an important role in neurotoxicity after hypoxia-ischemia in the immature brain[22;24;36;39]. The mechanisms of NO-mediated neuronal injury include: energy depletion, lipid and protein peroxidation, protein nitration and DNA damage as well as mitochondrial movement impairment and remodeling[37;63].

In addition, NO also plays an important role in signal transduction the CNS[11;12], including mediation of synaptic dynamics and plasticity; synapse formation and patterning; growth cone behavior as well as induction of long-term potentiation and long-term depression[10;13;53]. NO signal decays rapidly with distance from the site of production, suggesting that NO signaling is synapse-specific[23;40]. Its role in retrograde signaling at glutamatergic synapses has been described and its role at GABAergic synapses is under investigation[1]. Recent evidence suggest that nNOS is present in hippocampal GABAergic synapses in adult animals [58]. Further, physiologic concentrations of NO can lead to potentiation of GABAergic synapses [20;42;49;49;55].

However, the effects of higher concentrations of NO (μ M) relevant to that observed during a hypoxia-ischemia events have not been described. Therefore, the present study was designed to first describe the synaptic location of nNOS at GABAergic synapses and second investigate the effects of μ M concentrations of NO on GABAergic synaptic transmission in a cultured hippocampal neuron model. Using electrophysiology techniques, we demonstrate for the first time an acute effect of neurotoxic concentrations of NO on GABAergic synaptic transmission.

2- RESULTS

nNOS is expressed at GABAergic synapses in cultured hippocampal neurons

Because NO signals decay rapidly suggesting synapse specific NO effects, we performed double-labeling immunocytochemistry experiments to investigate the synaptic location of nNOS at GABAergic synapses. As shown in Figure 1, punctate nNOS immunoreactivity was observed in neuronal processes whereas a more diffuse staining was observed in the cell bodies of pyramidal neurons. GAD65 containing terminals were identified around the cell soma and neuronal processes. Colocalization of nNOS immunoreactivity to GAD65 immunoreactivity was measured as described in the method section. $31.1 \pm 2.3\%$ of nNOS punctate immunoreactivity colocalized with GAD65 immunoreactivity (n=14 cells). Conversely, $51.1 \pm 4.2\%$ of GAD65 punctate immunoreactivity colocalized to nNOS clusters. These results suggest that nNOS is present at GABAergic synapses in cultured hippocampal neurons. To confirm these finding, labeling with the postsynaptic marker $\alpha 2$ was also performed. As shown in Figure 1, $\alpha 2$ immunoreactivity in cultured hippocampal neurons was characterized by punctate staining of neuronal processes and dense staining of the cell body. Colocalization measurements indicated that $35.4 \pm 1.7\%$ (n=14 cells) nNOS punctate immunoreactivity colocalized to $\alpha 2$ clusters in cultured hippocampal neurons at DIV 14. And similarly to what we observed with a presynaptic marker, $50.3 \pm 2.4\%$ of $\alpha 2$ punctate immunoreactivity displayed nNOS colocalization. A representative microphotograph of nNOS colocalization to $\alpha 2$ is shown in Figure 1, panels D-F. Together these results indicate the presence of nNOS in about 50% of GABAergic synapses in our cultured hippocampal model.

NO alters GABAergic synaptic transmission in cultured hippocampal neurons

The effects of NO at concentrations relevant to those measured during ischemia-reperfusion on GABAergic synaptic transmission were studied using whole-cell patch clamp

electrophysiology. sIPSCs were recorded at baseline and during exposure to the NO donor DETA-NO (250 μ M) in cultured hippocampal neurons at DIV 10-14. In Figure 2A, traces recorded at baseline and during DETA-NO exposure (250 μ M, 10 min) are displayed demonstrating increased sIPSCs frequency. In the example shown, exposure to DETA-NO (250 μ M, 10 min) resulted in increased sIPSCs frequency from 0.59 Hz at baseline to 0.88 Hz ($P < 0.05$ Kolmogorov-Smirnov test, Figure 2B). This increase in frequency was not accompanied by a change in sIPSCs amplitude: 38.9 pA at baseline versus 35.1 pA after DETA-NO exposure. To confirm these results, sIPSCs were recorded in 7 additional cells. Significant variability in baseline frequencies was noted in our culture model. Overall, we found a 42% increase in sIPSCs frequency: from 0.65 ± 0.25 Hz at baseline to 0.92 ± 0.33 Hz ($n=8$, $p < 0.05$ vs. baseline, paired t-test). There was no change in amplitude: 45.7 ± 3.3 pA vs. 41.5 ± 3.0 pA in baseline and DETA-NO, respectively (Figure 2C and D). Further, the frequency distribution was similar in both conditions (Figure 2E). These results indicate that exposure to NO leads to enhanced GABAergic synaptic transmission in cultured hippocampal neurons.

NO enhances GABAergic synaptic transmission

In order to determine if the increase in sIPSCs frequency during NO exposure is due to increased action potential frequency or increase in GABA release mechanisms, further experiments were conducted in the presence of tetrodotoxin to block action potentials. mIPSCs were recorded at baseline and during DETA-NO exposure in cultured hippocampal neurons at DIV 10-14. In Figure 3A, traces recorded at baseline and during DETA-NO exposure (250 μ M, 10 min) are displayed demonstrating increased mIPSCs frequency. In the example shown, mIPSC frequency increased from 0.19 Hz at baseline to 0.44 Hz after 10 min exposure to 250 μ M DETA-NO. Comparison of cumulative probability plots of frequencies revealed a significantly shorter interevent interval during DETA-NO exposure ($p < 0.05$, Kolmogorov-Smirnov test, Figure 3B). This increase in mIPSCs frequency was accompanied by significantly lower amplitudes after DETA-NO exposure: from 49.6 pA at baseline to 41.8 pA after DETA-NO exposure ($p < 0.05$, Kolmogorov-Smirnov test, Figure 3C-E). To confirm these results mIPSCs were recorded in an additional 13 cells. As was noted for sIPSCs recording, we noted variability of baseline mIPSC frequency in our cultured neurons model. Overall, there was a 26% increase in mIPSC frequency following DETA-NO exposure, from 0.38 ± 0.09 Hz at baseline to 0.48 ± 0.09 Hz after exposure to DETA-NO ($N=14$, $P < 0.001$ vs. baseline, paired t-test). This increase in frequency was accompanied by a small reduction (11%) in mIPSCs amplitude: from 44.0 ± 3.6 pA at baseline vs. 39.4 ± 3.6 pA after DETA-NO exposure ($N=14$, $P < 0.05$, paired t-test). mIPSCs decay times and 10-90% rise-time were unchanged at baseline compared to DETA-NO (Table 1). Increase in mIPSC frequency can result from modification in presynaptic factors including increased GABA release probability.

3- DISCUSSION

The key findings of this study are that nNOS is co-localized at GABA synapses and that NO at μ M concentrations results in increased GABAergic synaptic transmission in a cultured hippocampal neuron model. This is the first report showing an effect of high concentrations of NO, relevant to those observed after a hypoxic-ischemic event, on GABAergic synaptic transmission. As indicated by the absence of observed current kinetics changes, NO effect on GABA synapses are likely due to presynaptic mechanisms. High concentrations of NO (μ M range) have been shown to be produced during hypoxia-ischemia, thus this effect on GABAergic synaptic transmission may play a role in hypoxia-ischemia pathophysiology.

nNOS containing neurons are widely distributed in the CNS, but areas of highest densities vary with brain developmental stages [6]. In the P10 rat hippocampus, nNOS is predominantly expressed the CA1-3 and dentate gyrus regions. Using double immunocytochemistry

techniques, we demonstrated that nNOS is located at GABAergic synapses in dissociated hippocampal neuron cultures. This finding is significant since the effects of NO are synapse specific. These findings are consistent with a recently published report by Szabadits et al[58]. Using electron microscopy techniques, they demonstrated that nNOS is localized to symmetrical GABAergic synapses on pyramidal cells in the rat hippocampus.

The hippocampus is of particular interest because of its selective neuronal vulnerability to hypoxia-ischemia in the immature brain and its role in the development of epilepsy[8;52;60]. Further, exposure to hypoxia results in acute and chronic increase in hippocampal excitability without macroscopic structural changes[27;28].

The finding that NO enhances GABAergic synaptic transmission is particularly intriguing because in the immature brain the combination of slow GABAergic inhibition maturation and developmentally increased NMDA and AMPA receptor-mediated excitability leads to overall excitation predominance[26]. Studies have shown high levels of NR2B subunits in neuronal NMDA receptor from immature animals, leading to long current decay times and low magnesium sensitivity[5;15;38]. Similarly, AMPA receptors are deficient in GluR2 subunit resulting in increased Ca^{++} -influx into neurons[43;48]. In addition to excitation predominance, GABA_A receptor activation leads to membrane depolarization and excitation, because of the high intracellular chloride concentration[2]. This reversal of the chloride gradient is thought to be secondary to a low expression of the chloride extruding transporter KCC2 as well as the presence of transporters such as NKCC1, that actively carry chloride into the neurons[7;31; 47]. In rats, changes in the chloride gradient and thereby the switch to GABA_A-mediated inhibition occur during the first 2 postnatal weeks, corresponding to term in human gestation. In this setting, a NO-mediated increase in GABAergic synaptic transmission, as shown here, may result in overall hyperexcitability and seizures.

Neonatal seizures have been shown to lead to long-term alterations in the hippocampal network excitability particularly of the CA1 region, in the P10 rat[28]. These changes are not associated with increased neuronal death or morphologic changes as opposed to what is observed in the adult brain. Rats with neonatal seizures had reduced seizure thresholds as well as impairment in learning, memory, and activity level[18;41]. Finally, seizures early in life can lead to increased mossy fibers growth in the supragranular region and CA3 hippocampal subfield, enhanced susceptibility to brain injury later in life and impairment in visual spatial memory [29;51;64]. Although neonatal seizures are common, the mechanisms involved in the increased susceptibility of the immature brain to hypoxia-induced seizures are still incompletely understood.

Results from this study indicate that NO may represent a novel mechanism by which hypoxia-ischemia alters neurotransmission in the immature brain. The mechanisms of NO-mediated alteration in synaptic transmission are not elucidated in this study. Potential targets for NO action include alterations of mitochondrial function and/or effects on neurotransmitter vesicular release. Indeed, NO has been shown to impair mitochondrial dynamics and morphology[46;63]. Because mitochondria are present in high quantities at synapses, it is possible that hypoxia-induced release of NO modulates synaptic transmission by its action on mitochondria in the presynaptic terminal. Mitochondrial dysfunction secondary to NO exposure may then result in increased Ca^{2+} efflux from mitochondrial stores and represent one of the mechanisms by which NO leads to enhanced presynaptic neurotransmitter release in the immature brain. As suggested by previous studies, a direct effect of NO on neurotransmitter release may also play a role[9;30;44]. Further studies are required to determine the mechanisms by which NO enhances GABAergic synaptic transmission in our model.

In summary, high concentrations of NO lead to enhanced GABAergic synaptic transmission in cultured hippocampal neurons. This may represent a novel mechanism by which NO production during hypoxia-ischemia results in increased excitability and potentially seizures. Studies are underway to confirm these findings in an immature brain model and investigate chronic effects.

4- EXPERIMENTAL PROCEDURE

All procedures involving the use of animals were approved by the UVA Animal Care and Use Committee and strictly follow the NIH guidelines for the use of laboratory animals.

Cultured hippocampal neurons

Primary neuronal/glial cell cultures were prepared from the hippocampi of newborn rats as previously described with some modification. Briefly, hippocampi were dissected, placed in ice cold dissection buffer containing 10 mM Hepes, 10 mM HBSS and incubated in 0.125% trypsin for 30 min at 37°C. Neurons were collected by centrifugation and resuspended in Dulbecco's modified Eagle's medium (DMEM) and F-12 supplement (1:1) (Gibco Invitrogen Corporation) supplemented with 10% fetal bovine serum (heat-inactivated, Hyclone), 2 mM L-glutamine (Sigma), and penicillin (100 U/mL)-streptomycin (100 U/mL). Cells were then plated at a density of 10^4 – 10^5 per 35 mm² on coverslips precoated with poly-L-lysine and kept at 37°C in a 5% CO₂ incubator. After 24 h, the culture medium was changed to DMEM medium containing 2% B27 and 2 mmol/L glutamine. Astrocytes were minimized by treating the culture with cytarabine (10 μ M) on day 3. The medium were replaced with fresh medium every 3 days. All experiments were conducted in neurons DIV 10-14.

Immunocytochemistry

Cultured hippocampal neurons were briefly washed in phosphate-buffered saline (PBS, pH 7.4) and fixed in 4% paraformaldehyde/4% sucrose at room temperature for 15 min. Following washing steps in PBS, neurons were permeabilized in 0.25% Triton-X incubated where applicable and blocked in 5% normal goat serum and 0.5% BSA in PBS for 30 min. at room temperature. Neurons were subsequently incubated with the primary antibodies overnight at 4°C. All double labeling experiments include a specific anti-nNOS antibody and either a presynaptic or a postsynaptic marker. Glutamic acid decarboxylase (GAD65) was used as a presynaptic marker. GAD65 is one of the two isoforms of GABA synthesizing enzyme and is abundant in the hippocampal formation, particularly in axon terminals[19]. The GABA receptor subunit $\alpha 2$ was used as a postsynaptic marker. Studies indicate that the subunit composition of postsynaptic GABA receptors affects its cellular localization. GABA receptors containing $\alpha 2$ subunits have been shown to form clusters at synapses in cultured hippocampal neurons at the age chosen for our experiments (DIV 10-14)[4;34;57]. High resolution studies also support its postsynaptic location[59]. After extensive washing, neurons were then incubated in goat anti-rabbit or goat anti-mouse secondary antibodies conjugated with Alexa Fluor 488 or 594, for 1h at room temperature, in the dark. Neurons were then washed and mounted onto slide using antifade mounting media.

High resolution fluorescent images of pyramidal cells were captured as described previously [56]. Neurons were visualized using a Nikon Eclipse TE300 epifluorescent light microscope and high resolution digital images captured with a Roper Scientific Photometrics Cool-SNAPcf CCD camera. Fluorescent images were thresholded using MetaMorph software (vers 6.01) so that punctuate fluorescence was twice that of the background. A puncta was defined as aggregation of 2-1000 pixels corresponding to 0.08-41.7 μ m. Because of intense cell soma labeling, only punctuate labeling on processes was quantitated and analyzed. Each image was thresholded and a binary image created. Binary images were added together by using the

“logical AND” function of the Metamorph software and a third binary image displaying overlapping clusters was created. Binary images were subsequently analyzed using the “integrated morphometric analysis” function of the Metamorph software and number of clusters for each image noted. Percent colocalization was then calculated for each cell. Final images were processed using Adobe Photoshop 6.0 in which overall brightness was increased for final production.

Exposure to NO donor

The NO donor diethyleneamine nitric oxide adduct (DETA-NO) was freshly dissolved in culture medium and pre-incubated for 1h at room temperature before being added to the cells. This incubation period allowed for maximum levels of NO to be reached in the solution. Cultured hippocampal neurons were then exposed *in vitro* to 250 μ M of DETA-NO for 30 min. Deactivated DETA-NO was used to control for NO metabolites effects. DETA-NO was chosen as the NO generator due to its long half-life of approximately 20h. The concentration of NO generated by this concentration of DETA-NO was previously determined to be 4.5 μ M [54].

Patch-clamp electrophysiology

Whole-cell patch-clamp recordings of GABAergic PSCs from cultured hippocampal neurons were performed using standard techniques as described previously[33]. Thick-walled borosilicate patch electrodes (1.5 mmOD, 0.86 mmID) were pulled on a P-97 Flaming-Brown horizontal puller (Sutter Instruments, Novato, CA), using a three-stage pull to a final resistance of 3-8 m Ω . Patch electrodes tips were filled with a sterile internal solution containing 153.3 mM CsCl, 1 mM MgCl₂, 10 mM HEPES, 5 mM EGTA, 4 mM ATP magnesium salt (pH 7.3, 285-295 mOsm). Cultured neurons were perfused with an external solution containing 146 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 10 mM glucose and 10 mM HEPES (pH 7.4, 305-320 mOsm). 6,7-dinitroquinoxaline-2/3-dione (DNQX, 20 μ M) and D(-)-2-amino-5-phosphonovaleric acid (APV, 50 μ M) were used to block AMPA/Kainate and NMDA receptor-mediated currents. For AP-independent mIPSCs recording, tetrodotoxin, (TTX, 1 μ M) was added to the external solution to block action potentials. Synaptic currents were recorded from visually identified pyramidal hippocampal neurons using an inverted Nikon microscope. Recordings were made at a holding potential of -60mV. Series resistance and capacitance were compensated for each neuron. Postsynaptic currents were recorded after input resistance stabilization with a 200A Axopatch amplifier (Axon Instruments, Union City, CA) and low-pass filtered at 2-3 kHz with an 8-pole Bessel filter prior to digitization, storage and display. Each neuron was recorded for 10 min epochs before and after NO exposure.

Off-line analysis was performed using MiniAnalysis software (Synaptosoft, Decatur, GA). Amplitude, frequency, rise 10-90%, rise time and decay were analyzed using a threshold for current detection at 3 times the root mean square of baseline noise. All currents detected were visually confirmed. Decay was analyzed by fitting individual post-synaptic currents with a 10-90% rise time < 3 msec to a 2-exponential curve characterized by 2 time constants (τ_1 and τ_2) and accepted if $r^2 > 0.70$. Weighted decay (τ_w) was calculated as previously described[45] using the formula: $\tau_w = [(\tau_1 \times A_1) + (\tau_2 \times A_2)] / [A_1 + A_2]$, where τ_1 and τ_2 represent the fast and slow decay times, respectively; and A_1 and A_2 represent the amplitude of the fast and slow components, respectively. Decays were analyzed as described until 20 current traces meeting criteria were obtained for each neuron. Because the data is not normally distributed, median are reported.

Statistical methods

For non-normally distributed data (events frequencies) groups (baseline versus DETA-NO exposure) were compared using the nonparametric Kolmogorov-Smirnov (K-S) test. For normally distributed data (events amplitudes and kinetics) groups were compared using paired

t-test. Statistical comparisons were performed using Sigma Stat 3.0 software (Systat Software Inc., Point Richmond, CA). All data are presented as Mean $\pm$ SEM; a value of $P < 0.05$ was considered statistically significant.

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Abbreviations

DETA-NO	diethyleneamine/nitric oxide adduct
DIV	days-in-vitro
GABA	gamma aminobutyric acid
mIPSC	miniature inhibitory postsynaptic current
NO	nitric oxide
nNOS	neuronal nitric oxide synthase.

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Figure 1. Colocalization of nNOS at GABAergic synapses

Double immunocytochemistry experiments were conducted as described in cultured hippocampal neurons at DIV 10-14. **Panel A:** specific immunostaining for GAD-65 demonstrating punctate clusters and more diffuse immunoreactivity on neuronal processes as well as dense diffuse staining of cell soma. **Panel B:** specific immunostaining for nNOS demonstrating punctate clusters distributed throughout neuronal processes and dense diffuse staining of cell soma. **Panel C-E:** Magnification of boxed area showing specific immunostaining for GAD-65 (red) and nNOS (green). An overlay image showing colocalization of nNOS to GAD-65 in yellow is also shown (E). **Panel F:** $\alpha 2$ immunoreactivity demonstrating punctate clusters on neuronal processes and dense diffuse staining of the cell soma. **Panel G:** nNOS immunoreactivity is shown with punctate clusters on neuronal processes and diffuse stoma staining. **Panel H-J:** Magnification of boxed area showing specific immunostaining for $\alpha 2$ (red) and nNOS (green). An overlay image showing colocalization of nNOS to $\alpha 2$ in yellow is shown in J. Original magnification 60X. Scale bar = 10 μ m.

Figure 2. Effects of NO on sIPSCs in cultured hippocampal neurons

sIPSCs were recorded at baseline and after DETA-NO (250 μ M, 10 min, n=8) bath application using whole-cell patch-clamp electrophysiology. **Panel A:** Representative traces of sIPSCs at baseline and after DETA-NO are shown, demonstrating increased sIPSCs frequency. **Panel B:** A representative inter-event cumulative histogram is shown. * p < 0.05 vs. baseline, K-S test. **Panel C:** Normalized averaged sIPSC traces at baseline and after DETA-NO exposure showing no change in median amplitude. **Panel D:** Cumulative distribution plot of amplitude showing similar amplitude at baseline and during DETA-NO exposure. **Panel E:** Amplitude distribution histogram recorded at baseline (black bars) and after DETA-NO exposure (gray bars).

Figure 3. Effect of NO on mIPSCs in cultured hippocampal neurons

mIPSCs were recorded at baseline and after DETA-NO (250 μ M, 10 min) bath application in cultured hippocampal neurons ($n=14$, DIV 10-14), using whole-cell patch-clamp electrophysiology. **Panel A-B:** Representative traces of mIPSCs at baseline (A) and after DETA-NO (B) are shown, demonstrating increase in frequency. Fragments of the same recording are shown with a higher temporal resolution in **a** and **b**. **Panel C:** A representative inter-event cumulative histogram is shown. * p < 0.01 vs. baseline, K-S test. **Panel D:** Normalized averaged mIPSC traces at baseline and after DETA-NO exposure demonstrating decreased median amplitude. **Panel E:** Cumulative distribution plot of amplitude showing increase in low amplitude currents in DETA-NO. **Panel F:** Amplitude distribution histogram recorded at baseline (black bars) and after DETA-NO exposure (gray bars) showing skewed distribution towards lower amplitude in neurons exposed to DETA-NO.

Table 1

mIPSCs characteristics in cultured hippocampal neurons exposed to exogenous NO

	Baseline	NO (250 μ M)
Frequency (Hz)	0.38 ± 0.09	$0.48 \pm 0.09^*$
Median amplitude (pA)	44.0 ± 3.6	$39.4 \pm 3.6^{**}$
10-90% rise-time (ms)	2.89 ± 0.16	3.15 ± 0.22
τ_1 (ms)	18.7 ± 1.6	22.2 ± 2.2
τ_2 (ms)	45.1 ± 1.5	44.3 ± 2.7
τ_w (ms)	33.0 ± 2.1	34.0 ± 2.7

Values are Mean $\pm$ SEM. See method section for calculation.*
p < 0.001 vs. baseline**
p < 0.05 vs. baseline