Neuroscience Letters 590 (2015) 29–34

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Enhancement of tonic and phasic GABAergic currents following nitric oxide synthase inhibition in hippocampal CA1 pyramidal neurons

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HIGHLIGHTS

- Tonic and phasic $GABA_A$ receptor activity was measured in hippocampal slices.
- Effects of nitric oxide depletion on $GABA_A$ receptor mediated responses were examined.
- Tonic and phasic $GABA_A$ receptor responses were potentiated after NOS inhibition.
- $GABA_A$ receptors in CA1 hippocampal neurons are modulated by basal NO levels.

ARTICLE INFO

Article history:
Received 25 November 2014
Received in revised form 21 January 2015
Accepted 22 January 2015
Available online 27 January 2015

Keywords:
$GABA_A$ receptors
Tonic inhibition
Phasic inhibition
Nitric oxide synthase
Hippocampus

ABSTRACT

Nitric oxide (NO) is involved in synaptic plasticity in the hippocampus through different presynaptic and postsynaptic mechanisms that include the modulation of the GABAergic neurotransmission. Inhibitory synapses on hippocampal pyramidal neurons are known to possess the molecular machinery for retrograde NO-signaling, but the modulation of $GABA_A$Rs function by NO in these neurons and the mechanisms of action involved have not been fully characterized. Here we show that suppression of the endogenous NO generation by the nitric oxide synthase (NOS) inhibitor L-NAME produces significant and reversible increases in the magnitude of both tonic and phasic GABAergic currents in CA1 hippocampal pyramidal neurons. GABA-evoked chloride currents were measured in the presence or absence of L-NAME using whole-cell patch-clamp recordings in acute hippocampal slices from young adult mice. Enhancement of the tonic GABA responses induced by L-NAME was insensitive to TTX and decreased by co-incubation with the NO donor DEA/NO. Applications of DEA/NO alone did not produce significant effects on tonic GABA responses. L-NAME treatment also increased the amplitude of phasic GABAergic currents evoked by GABA-puffs. Our results indicate that the extent of tonic and phasic inhibition mediated by $GABA_A$ receptors in CA1 hippocampal pyramidal neurons is affected by endogenous NO production.

1. Introduction

Nitric oxide (NO) is a diffusible and ubiquitous signaling molecule involved in the regulation of cellular functions under both physiological and pathological conditions [1]. NO is generated by oxidation of L-Arginine mediated by a family of nitric oxide synthases (NOS), namely the neuronal, endothelial and inducible isoforms (nNOS, eNOS and iNOS, respectively). nNOS represents the major source of NO in the brain [2] and NO functions in several types of synaptic plasticity, including hippocampal long-term potentiation in which it may serve as a retrograde messenger after postsynaptic NMDA receptor activation [3,4].

Reactive nitrogen species were also found to play different roles in the modulation of the inhibitory neurotransmission [1] which is mostly mediated in the central nervous system (CNS) by the ionotropic γ-aminobutyric acid receptors ($GABA_A$Rs). $GABA_A$Rs are pentameric chloride (Cl^-) channels made up by combinations of different subunits ($\alpha 1-6$, $\beta 1-3$, $\gamma 1-3$, δ, ϵ, π, θ, $\rho 1-3$) that confer distinct physiological, pharmacological and biophysical properties [5]. Fast and transient (phasic) neuronal inhibition is mediated by synaptic $GABA_A$Rs, whereas slow and persistent (tonic) inhibition arises from activation of extrasynaptic $GABA_A$Rs

Abbreviations: ACSF, Fartificial cerebrospinal fluid; BIM, bicuculine methiodide; CNS, central nervous system; DEA/NO, DEA/NONoate, 1,1-Diethyl-2-hydroxy-2-nitroso-hydrazine sodium; GABA, γ-aminobutyric acid; $_A$Rs, γ-aminobutyric acid A receptors; L-NAME, nitro-L-arginine-methyl ester; NO, nitric oxide; NOS, nitric oxide synthase.

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http://dx.doi.org/10.1016/j.neulet.2015.01.058
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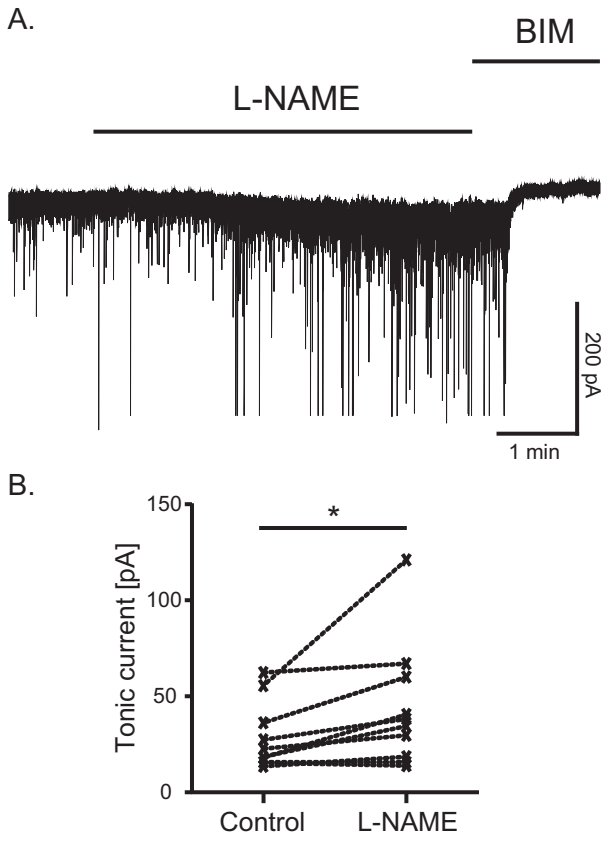

Fig. 1. Tonic inhibitory GABAergic currents in hippocampal CA1 pyramidal neurons were enhanced during NOS inhibition. A. Representative trace of the holding current before and during application of the NOS blocker L-NAME (100 μM) followed by BIM (100 μM). Cells were voltage-clamped at −70 mV in the presence 5 μM GABA and 20 μM CNQX. Horizontal lines over the recording trace indicate the period of drug application. L-NAME increased the holding current (inward shift) and the baseline variance. BIM strongly reduced the holding current amplitude and variance by blocking the $GABA_A$Rs-mediated tonic current. B. Scatterplot illustrates tonic current amplitudes obtained from the all-point histograms of the holding current (see Methods) before and after the application of L-NAME on each cell ($n = 10$) (*$p < 0.05$).

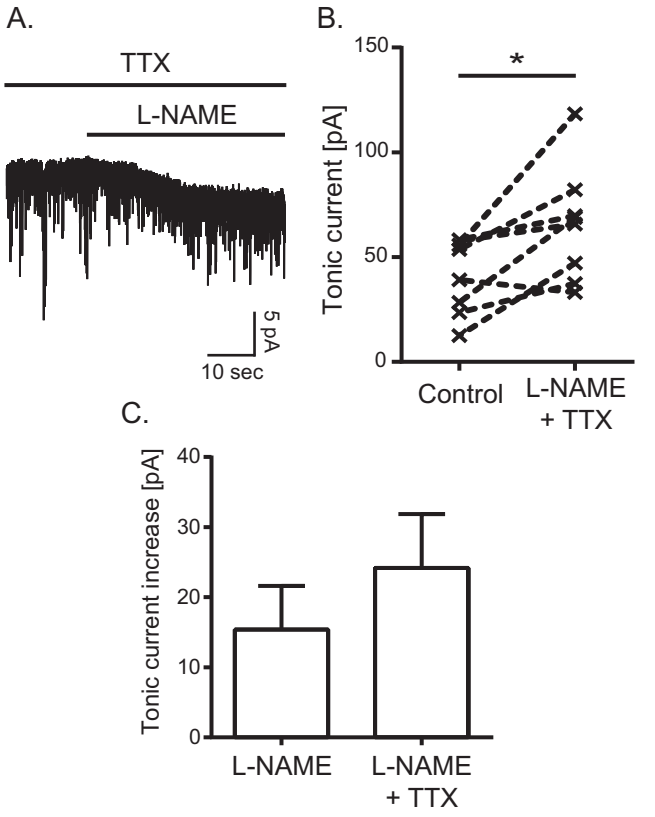

Fig. 2. Enhancement of the tonic inhibitory GABAergic currents induced during NOS inhibition was not blocked by TTX. A. Representative trace of the holding current recorded in the presence of TTX, before and during L-NAME application. B. Scatterplot of the tonic current amplitudes measured before and after the application of 100 μM L-NAME in the presence of TTX ($n = 8$) (*$p < 0.05$). C. Bar graph summarizing the change in the tonic current after L-NAME application in the presence and the absence of TTX. Increases in the tonic current amplitudes were not significantly different.

by low concentrations of ambient GABA [6–8]. A number of studies have shown that NO can act presynaptically to control GABA release, as well as postsynaptically to directly or indirectly modulate the activity of $GABA_A$Rs [9–13]. Despite the fact that inhibitory synapses on hippocampal pyramidal neurons are known to possess the molecular machinery for retrograde NO-signaling [9,14,15], NO modulation of $GABA_A$Rs function was not studied in detail and the underlying mechanisms still need further characterization.

The present results show that endogenous NO generation can control the extent of tonic and phasic inhibition mediated by $GABA_A$Rs in hippocampal CA1 pyramidal neurons.

2. Material and methods

All experimental procedures were carried out in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* (NIH Publications No. 80–23) and approved by the CONICET-University of Buenos Aires Animal Care and Use Committee.

2.1. Slice preparation

Balb/c mice (ages: 15–20 days old) were anaesthetized with ketamine (15 mg.mL^{-1})/xylazine (1.5 mg mL^{-1}) and decapitated. Brains were removed into a chilled solution containing the following (in mM): 110 choline-Cl$^-$, 2.5 KCl, 2.0 NaH_2PO_4, 25 $NaHCO_3$, 0.5$CaCl_2$, 7 $MgCl_2$, 20 dextrose, 7 sodium ascorbate, 3 sodium pyruvate, and 2.6 kynurenic acid. Horizontal slices (400 μm thick) were cut in a vibratome (Vibratome 1000 plus, St. Louis, USA) and transferred to a chamber containing standard artificial cerebrospinal fluid (ACSF) (in mM): 125 NaCl, 2.5 KCl, 2.3 NaH_2PO_4, 25 $NaHCO_3$, 2$CaCl_2$, 1.3 $MgCl_2$, 1.3 sodium ascorbate, 3.1 sodium pyruvate, and 10 dextrose (300 mOsm). Slices were bubbled with 95% O_2/5% CO_2 and maintained at 32 °C for at least 1 h before experiments started.

2.2. Electrophysiology

Slices were placed in the chamber for electrophysiological recordings mounted under a Zeiss FS Axioskop (Carl Zeiss, Germany) microscope with DIC and viewed using a 40× water-immersion objective and a camera (Newvicom, Hamamatsu Photonics, Japan). Slices were continuously superfused (1–2 mL min^{-1}) during recordings with ACSF containing CNQX (20 μM) to block AMPA/kainate receptors. Whole-cell patch-clamp recordings were performed at 24 ± 2 °C from visually identified hippocampal pyramidal neurons. Patch pipettes were pulled from borosilicate glass (Corning 7056, Warner Instruments, Hamden, USA) and filled with (in mM): 140CsCl, 5 NaCl, 2 $MgCl_2$, 0.1 EGTA, 10HEPES, 4 Tris-ATP, 0.3 Tris-GTP, 10 phosphocreatine, pH 7.3, and 290 mOsm and had open tip resistances of 3–7 MΩ. As cells were voltage-clamped at −70 mV, $GABA_A$R-mediated currents were inward. Recordings were obtained using an Axopatch 200B

amplifier (Molecular Devices, Foster City, USA), digitized (Digidata 1200, Molecular Devices) and acquired at 10 kHz into a personal computer using the pClamp 8 software (Molecular Devices). Signals were low-pass filtered at 2 kHz. Membrane capacitance and input resistance were obtained from current traces evoked by a 10 mV hyperpolarizing step. Series resistance was not compensated and typically ranged 12–20 MΩ. Experiments were discarded if series resistance was >25MΩ or increased by >25% through the recordings. Tonic GABAergic currents were recorded in ACSF with 5 μM GABA added, to standardize GABA levels present in the extracellular space and to reproduce a more physiological condition [16], and their amplitudes calculated by subtracting the holding current at −70 mV in the presence or absence of 100 μM BIM. Phasic GABAergic currents were evoked by GABA puffing. GABA 30 μM was dissolved in ACSF solution and loaded in a patch pipette of 1.5 mm OD and 1.12 mm ID (WPI, Sarasota, USA) for GABA puffing. After a steady whole-cell recording was achieved the puff-pipette was placed at a distance of 30–100 μm and GABA puffed at a 5–10 psi during 50–100 ms. Under these conditions puff-evoked GABA responses ranged 500–1000 pA. GABA puffs were delivered every 30 s and acquisition of the phasic current recordings was started once at least four steady responses were obtained. All drugs and salts were purchased from Sigma (St. Louis, MO, USA). 1,1-Diethyl-2-hydroxy-2-nitroso-hydrazine sodium (DEA/NO) and nitro-L-arginine-methyl ester (L-NAME) were freshly prepared before each experiment from stock solutions. DEA/NO stock solution (10 mM) was prepared in 0.01 M NaOH and stored on ice for no more than 2 h. L-NAME stock solution (10 mM) was prepared in water.

2.3. Data analysis and statistics

Absolute values of the current amplitudes were considered. The mean holding current was determined in 30 s-long segments at three time points: before application of L-NAME or DEA/NO, after 5 min drug treatment and after 3 min 100 μM bicuculline methiodide (BIM) by plotting an all-point histogram. A Gaussian was fitted to the part of the distribution not skewed by synaptic events and the mean of this Gaussian was used as the value of the holding current. Tonic currents in each condition were calculated as the change in holding current after 3 min of treatment with BIM [16]. Whenever it corresponds, the results were reported as average ± SEM. Simple and pairwise comparisons were performed with the appropriate two-tailed Student's t test or an ANOVA followed by Tukey *post hoc* test.

3. Results

3.1. NOS inhibition potentiates tonic GABAergic currents in hippocampal CA1 pyramidal neurons

In order to analyze the effects of the endogenously produced NO on tonic inhibitory currents mediated by $GABA_A$Rs in hippocampal CA1 pyramidal neurons hippocampal slices were incubated with 100 μM L-NAME [17]. The average tonic current in the control condition was 33.0 ± 3.8 pA ($n = 21$), whereas in the presence of this competitive NOS inhibitor both a marked inward shift in the holding current and an increase in the baseline variance were produced (Fig. 1A). After 5 min application of L-NAME tonic currents were significantly enhanced from 28.6 ± 5.5 pA to 44.0 ± 10.2 ($p < 0.05$; $n = 10$; paired two tailed t-test) (Fig. 1B). Concomitantly, the noise variance of the holding current raises from 39.3 ± 7.6 pA^2 to 90.3 ± 25.5 pA^2 ($p < 0.05$; $n = 10$; repeated measured one-way ANOVA) (data not shown).

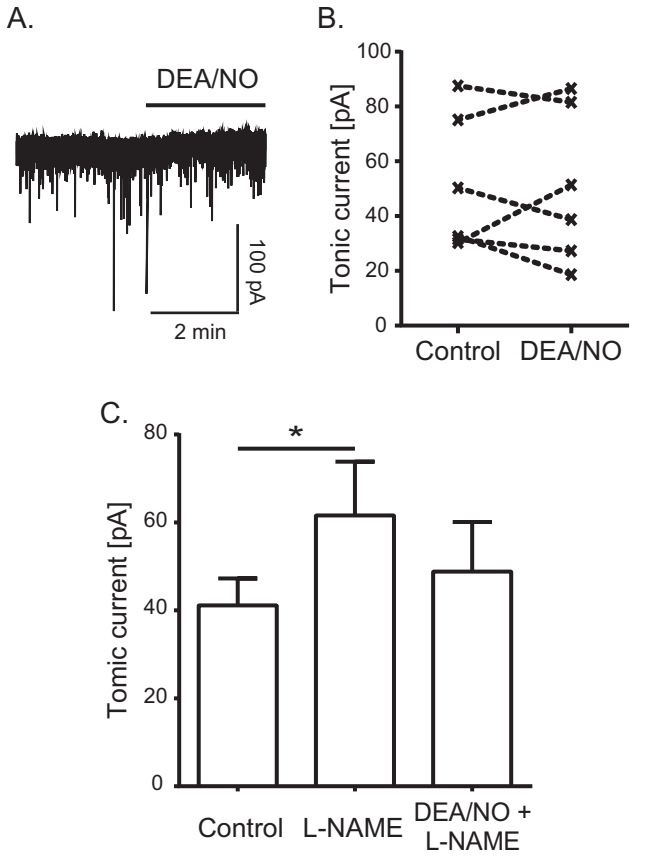

Fig. 3. Enhancement of the tonic inhibitory GABAergic currents induced during NOS inhibition was decreased by NO. A. Representative trace of the holding current recorded before and during the application of the NO donor DEA/NO (10 μM). B. Scatterplot of the tonic current amplitude before and during DEA/NO application. The mean effect was not significant ($n = 6$). C. Bar graph summarizing the tonic GABAergic current recorded in the control condition followed by the sequential application of 100 μM L-NAME and L-NAME + 10 μM DEA/NO in the same cell. DEA/NO partially reversed the increase in tonic current ($n = 8$) (*$p < 0.05$).

We have also analyzed if changes in tonic GABAergic currents were due to an increase in the firing activity that could raise the extracellular concentration of GABA [8]. But the effect of L-NAME on the holding current was not abolished in the presence of 1 μM TTX (Fig. 2A). The mean tonic current amplitude was increased by L-NAME in approximately a 54%, while in the presence of TTX increased a 59% (from 41.1 ± 6.3 pA to 65.3 ± 9.7 pA; $p < 0.05$; $n = 8$; paired two tailed *t*-test) (Fig. 2B). L-NAME-induced increase in the tonic current were not significantly different in the two conditions ($\Delta I_{tonicwithTTX} = 24.2 \pm 7.7$ pA, $n = 8$; $\Delta I_{tonicwithoutTTX} = 15.4 \pm 6.2$ pA, $n = 10$; $p = 0.38$; unpaired two tailed *t*-test) (Fig 2C).

Enhancement in tonic current amplitude produced by NOS inhibition raises the question of whether exogenous NO could reduce the current by itself. We applied the NO donor DEA/NO (10 μM) for 5 min, after stabilization of the holding current, in the absence of L-NAME (Fig. 3A). DEA/NO did not produce significant reduction of the tonic GABAergic currents ($I_{toniccontrol} = 51.2 \pm 10.1$ pA; $I_{tonicDEA/NO} = 50.6 \pm 11.5$ pA; $p = 0.93$; $n = 6$; paired two tailed *t*-test) (Fig. 3B). Based on these results we presumed that under our experimental conditions endogenous NO levels might be saturating. If this was the case, the application of exogenous NO during NOS inhibition would totally or partially compensate for NO depletion. Thus, we tested DEA/NO once the effect of L-NAME reached steady state. This significantly increased the mean tonic currents from 41.1 ± 6.1 pA to 61.6 ± 12.2 (matched measures one-way ANOVA followed by Tukey's post-test, $p < 0.05$). After that, the application of

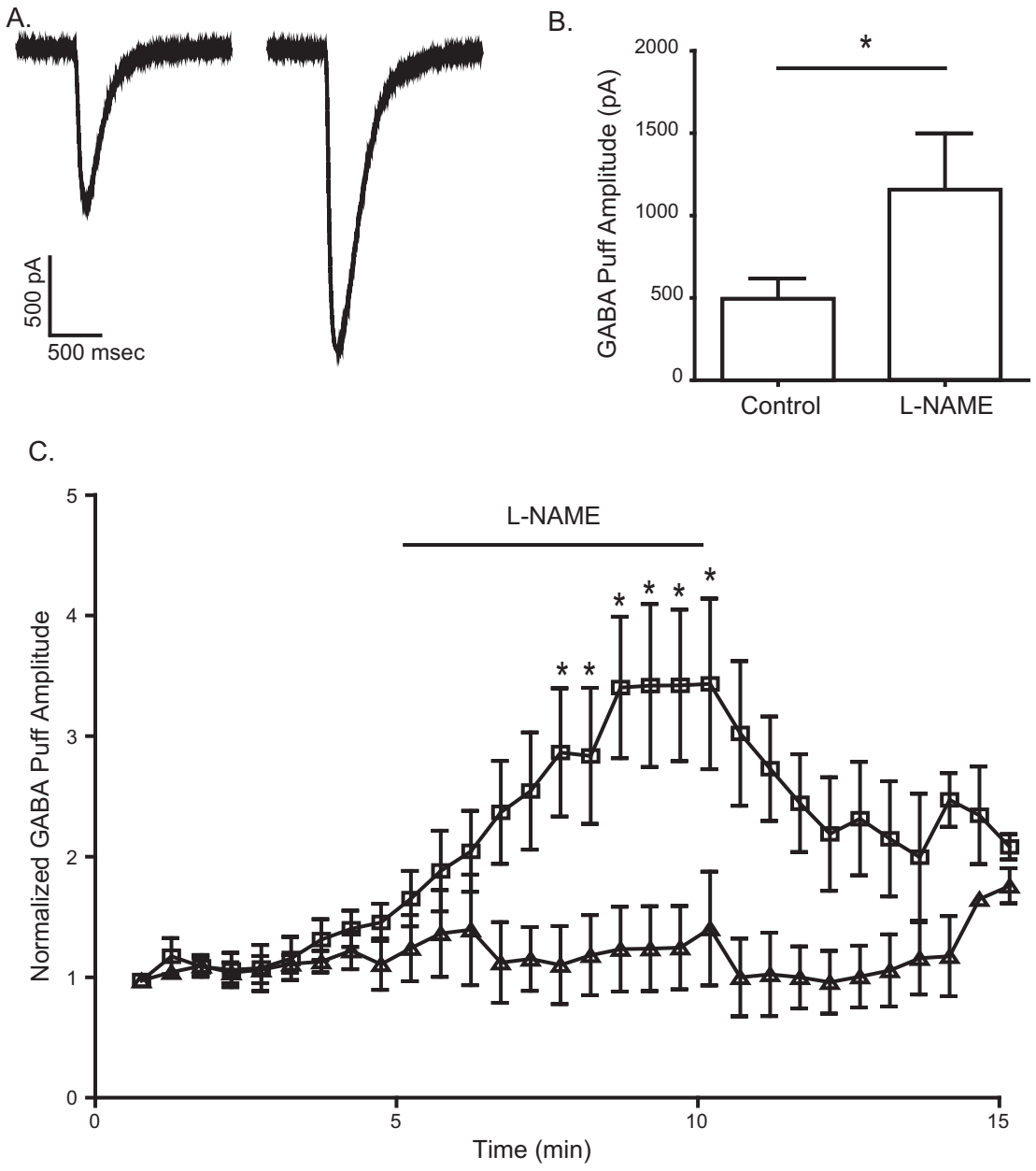

Fig. 4. Phasic inhibitory GABAergic currents in hippocampal CA1 pyramidal neurons were enhanced during NOS inhibition. A. Representative current evoked by a GABA puff (30 μM; 10 ms) before (left) and after (right) 5 min L-NAME (100 μM) application. B. Mean GABA puff amplitude current recorded after 5 min in control condition and after 5 min application of L-NAME ($n = 9$) (*$p < 0.05$). C. Time course of the current amplitude evoked by GABA puff normalized to the first puff. A puff was applied every 30 s during 15 min in control condition (Δ) and with 5 min L-NAME perfusion starting after 5 min of control condition (□) (*$p < 0.05$).

DEA/NO in the continuous presence of L-NAME decreased the mean tonic current to 48.8 ± 11.3 pA ($n = 8$ cells), a value not statistically different from the control ($p = 0.53$) (Fig. 3C).

3.2. NOS inhibition potentiates phasic GABAergic currents in hippocampal CA1 pyramidal neurons

Using a similar approach, we studied the effects of NO depletion on phasic GABAergic currents evoked by GABA puffs (10 ms puffs were delivered every 30 s during 5 min) in hippocampal CA1 pyramidal neurons. Phasic currents were recorded in the absence and presence of 100 μM L-NAME (Fig. 4A). The mean current amplitude underwent an increase of approximately 120% after NOS inhibition, from 537.1 ± 141.6 pA to 1192.0 ± 360.6 ($p < 0.05$; $n = 9$; paired two tailed *t*-test) (Fig 4.B). Fig. 4C illustrates the time course of potentiation. In order to compare the degree of potentiation, the current values were normalized to the first puff amplitude in each condition. Effects became significant 3 min after L-NAME application and lasted for approximately 30 s after wash out ($p < 0.5$; $n = 5$–9; two-way ANOVA).

4. Discussion

We have shown significant potentiation of the tonic and phasic GABAergic currents recorded in CA1 pyramidal neurons of mouse hippocampal slices during acute NOS inhibition.

Our results are consistent with previous evidence showing inhibitory actions of NO on $GABA_A$Rs in diverse neuronal types. For example, NO donors reduce GABA-induced $^{36}Cl^-$ uptake in microsacs from cerebral cortex [18], puff-evoked phasic GABAergic

currents in cultured cerebellar granule cells and in retinal amacrine cells [19,20] and stimulus-evoked phasic GABAergic postsynaptic currents in postnatal hippocampal CA1 pyramidal neurons [10]. However, the present findings are the first to demonstrate that endogenous levels of NO can modulate tonic GABAergic currents in hippocampal CA1 pyramidal neurons. In addition, we found that the NO donor DEA/NO applied alone was unable to inhibit the tonic GABAergic currents in hippocampal CA1 pyramidal neurons, suggesting that basal levels of NO could exert maximal effects. Previous work showed that the activity of tonic extrasynaptic α6-subunit-containing $GABA_A$Rs in cerebellum granule cells are potentiated by NO depletion [21] and that other tonic $GABA_A$Rs subtypes, such as the retinal GABAρ1Rs, are also modulated by NO [22]. Tonic GABAergic currents in CA1 pyramidal neurons are mainly mediated by α5-subunit-containing $GABA_A$Rs [23]. Thus, these receptors should be the major candidates as target for NO actions. However, the possible mechanisms of action of NO on α5 subunit-containing $GABA_A$Rs in the hippocampus, either direct or mediated by the $_C$GMP/PKG pathway, will require further analysis.

Several studies reported the occurrence of a basal NO-signaling activity [10,24]. eNOS in blood vessels generates a paracrine NO signal that could participate in the modulation of tonic extrasynaptic receptors [24]. In addition, the expression of nNOS at GABAergic synapses was established [9,14,15]. eNOS-derived NO appears to be less space and time specific that nNOS activity dependent NO [25]. In the hippocampus, NO can act on GABAergic targets and the mechanisms by which exerts its actions still are being explored [9,26]. For example, during the early postnatal hippocampal development, while GABAergic transmission is still depolarizing, NO signaling shape the excitability of CA1 pyramidal neurons by depressing the phasic synaptic GABAergic currents through a guanylyl cyclase-dependent presynaptic mechanism [10]. In turn, we found that phasic GABAergic currents evoked by GABA puffs in CA1 pyramidal neurons from young adult mice slices (while GABAergic transmission is hyperpolarizing) are subject to analogous modulatory effects. Interestingly, Dejanovic and col. have recently found that nNOS is associated with gephyrin, the principal scaffolding protein at inhibitory synapses. They demonstrated that gephyrin is *S*-nitrosylated *in vivo* and showed that this post-translational modification can be reversed by nNOS inhibition to induce the formation of larger gephyrin clusters at synaptic sites which eventually increases the cell surface expression of synaptic $GABA_A$ receptors [11]. Based on all these evidences, we speculate that potentiation of the phasic GABAergic currents observed in the present study might be selectively achieved by inhibition of nNOS closely linked to $GABA_A$Rs at postsynaptic sites during L-NAME applications. Thus, it would be interesting to know if NO depletion resulted in a loss of *S*-nitrosylation of gephyrin in our cells, so potentiation of the GABA responses could be mainly attributable to a gain of function induced by receptor clustering. But further studies will be necessary to elucidate the underlying mechanisms of NO modulation. Suffice to say that the potentiation of GABAergic currents in CA1 hippocampal pyramidal neurons during NOS inhibition was reversible, indicating that the activity of tonic and phasic $GABA_A$ receptors can be effectively controlled by variations in the basal concentration of NO in these neurons and that the modulation of tonic GABAergic currents by endogenous NO did not depend on neuronal activity. Considering the presynaptic effects of NO on GABA release [3,12] and on the depolarization-induced suppression of inhibition [13], our results showing a NOS-dependent modulation of tonic and phasic GABAARs provide further evidence indicating that NOS activity could be a key step to regulate the excitatory/inhibitory balance in the hippocampus. On the other hand, as a highly diffusible agent, NO is a good candidate for increasing the synchrony of adjacent ensembles of pyramidal neurons [10]. Finally, in neurons with large fast excitatory inputs as those studied here, a NOS-dependent modulation of the level of tonic inhibition might be important to set the actual gain of the cell, for example by increasing the input required to get a given output firing rate as the NO level decrease [3]. This would have an impact on synaptic integration and could alter firing threshold, firing pattern and network activity [11].

Taken together, our data and the previous findings [10–12] indicate that variations in the basal levels of NO could produce plastic changes in tonic and phasic neurotransmission mediated by $GABA_A$Rs at CA1 hippocampal pyramidal neurons.

Conflict of interest

None.

Acknowledgements

We would like to thank L. Mongiat, A. Marín Burgin and C. Wedemeyer and A.N. Beltrán Gonzalez for discussion. This work was supported by CONICET and FONCyT grants.

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