ENDOGENOUS NITRIC OXIDE SYNTHASE ACTIVITY REGULATES SYNAPTIC TRANSMITTER RELEASE

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Abstract. Nitric oxide (NO) signalling contributes to many biological processes involved in activity-dependent fine tuning of neuronal communication. NO is involved in early developmental signalling of the nervous system and is associated with pathological pathways and age-related decline in neuronal function, thus playing a critical role in regulating neuronal function in physiology and disease. Here we assessed the effects of modulating endogenous neuronal nitric oxide synthase (NOS) activity on synaptic function, specifically on neurotransmitter release at the glutamatergic Drosophila neuromuscular junction (NMJ). We found that the absence of NOS activity enhanced synaptic release at the NMJ and conversely, overexpression of NOS diminished transmitter release. The effects of alterations in NO signalling are the consequence of acute signalling at the synapse as we did not observe any developmental changes in NMJ morphology or synaptic parameters, such as expression of the active zone protein, bruchpilot, which could account for changes in release. Ultrastructural analysis did not show any developmental effects in boutons from larvae with reduced NOS activity. Together, our data present evidence for a negative regulation of transmitter release by NO which has implications for physiological synaptic function but also pathological and age-related dysregulation of synaptic signalling.

Keywords: Nitric Oxide, Neurotransmitter Release, Spontaneous Release, Evoked Release, Synapse, Drosophila NMJ

Introduction

Nitric oxide (NO) is involved in a broad variety of signalling pathways contributing to neuronal plasticity, immune responses, vascular signalling, development and survival. It was originally identified as endothelium-derived relaxing factor (EDRF) mediating relaxation of blood vessels (Furchgott & Zawadzki, 1980). In mammals, NO is synthesized by nitric oxide synthases (NOS) through the conversion of L-arginine to NO and L-citrulline (Knowles & Moncada, 1994). Three isoforms of NOS have been identified in the central nervous system (CNS): neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) (Alderton et al., 2001), with the activation of the soluble guanylyl cyclase (sGC) and cGMP production being the canonical pathway and a primary target of NO actions (Garthwaite et al., 1988). Down-stream transduction of NO signalling can be via cGMP regulated cyclic nucleotide-gated ion channels, activation of protein kinase G and protein phosphorylation or direct actions on proteins via post-translational modifications (PTM, S-nitrosylation and 3-nitrosotyrosination) (Hardingham et al., 2013; Garthwaite, 2016). These NO-mediated PTMs in particular have become increasingly recognised as regulators of specific target proteins (Knott & Bossy-Wetzel, 2009). S-nitrosylation is a non-enzymatic and reversible PTM resulting in a covalent addition of an NO group to a cysteine (Cys) thiol/sulfhydryl leading to the generation of S-Nitrosothiols (S-NO), and this reversible PTM resulting in a covalent addition of an NO group to a cysteine (Cys) thiol/sulfhydryl leading to the generation of S-Nitrosothiols (S-NO), and this pathway participates in a huge number of physiological events including cellular trafficking (Ozawa et al., 2008), circulation (Singel & Stamler, 2005), apoptotic pathways and neuronal signalling (Cho et al., 2009). Throughout the nervous system NO modulates synaptic function by multiple mechanisms, including regulation of transmitter release and activity-dependent plasticity phenomena. NO regulates transmitter release (GABA, dopamine, noradrenaline) and, depending on the system and concentrations of NO studied, the effects range from strongly inhibiting to facilitating actions. As such NO can directly modulate SNAP-25 function to enhance release via post-translational modification signalling (Di Stasi et al., 2002) and nitrosylate syntaxin (Wiseman et al., 2011) which leads to reduced transmitter release and exacerbates the detrimental effects of NO on neurotransmission. NMDA receptors are negatively regulated by NO (Choi et al., 2000) leading to a nitricergic negative feedback regulation of excitotoxicity.

Mammalian nNOS exists in different splice variants, namely nNOSβ and nNOSγ, both of which lack the amino terminal PDZ domain (Brenman et al., 1996). Drosophila possesses endogenous NO signalling (Regulski & Tully, 1995; Wildemann & Bicker, 1999; Stasiv et al., 2001) and wild-type Drosophila NOS (dNOS), which has a 43% amino acid sequence identity compared to rat nNOS (Regulski & Tully, 1995) and produces NO in a Ca2+/calmodulin-dependent manner.

As NO has numerous critical functions, it would be expected that a lack of NOS activity would be lethal during developing. However, a role for NO in developmental survival has not yet been demonstrated. Although a genetic analysis of NOS function in vertebrates is complicated by the presence of three NOS genes and different splice variants, mice with a homozygous ablation of any single NOS gene are
viable, animals with two NOS genes knocked out show drastically reduced viability and triple knockout animals have not yet been generated. The use of Drosophila to investigate NO dependent functions is advantageous due to the presence of only one NOS gene. As such, various forms of dominant negative dNOS have been generated to investigate developmental involvement of NO (Enikolopov et al., 1999; Stasiv et al., 2001; Stasiv et al., 2004) and it has been shown that NO has an anti-proliferative function during Drosophila development, controlling the balance between cell proliferation and cell differentiation (Kuzin et al., 1996). Other fly lines with strongly reduced dNOS activity have been generated (NOS<sup>−/−</sup> and NOS<sup>−/+</sup>), both of which show NOS activities comparable to NOS inhibited w<sup>1118</sup> (Regulski et al., 2004; Yakubovich et al., 2010).

In our study we investigated the effects of NO on synaptic release using genetic manipulations of NO signalling and found that enhanced nitrergic activity, induced by an endogenous increase of NOS activity compromises neurotransmitter release at the Drosophila NMJ and conversely, lack of NOS activity enhances release.

**Methods**

**Fly stocks:** Flies were raised on standard maize media at 25°C at a 12h LD cycle. wt<sup>1118</sup> larvae were used as controls (wild-type; WT). NOS<sup>−/−</sup> / NOS<sup>−/−</sup> (Regulski et al., 2004) lines were kindly provided by Patrick O’Farrell (UCSF, San Francisco, California USA). NOS<sup>−/+</sup> deletion removes sequences encoding residues 1–757, encompassing the entire oxygenase domain and including regions that bind the catalytic heme and the substrate rendering the lines NOS ‘null’ (Yakubovich et al., 2010). The dNOS<sub>15</sub> line with dNOS overexpression under the heat-shock promoter was kindly provided by Boris Kuzin (Koltsov Institute of Developmental Biology, Russia) (Kuzin et al., 1996; Stasiv et al., 2001).

**Electrophysiology.** TEVC recordings were performed as described previously (Robinson et al., 2014). Sharp-electrode recordings were made from ventral longitudinal muscle 6 in abdominal segments 2 and 3 of 3rd instar larvae using pClamp 10, an Axoclamp 900A amplifier and Digidata 1440A (Molecular Devices, USA) in hemolymph-like solution 3 (HL-3) (Stewart et al., 1994). Recording electrodes (20-50 MΩ) were filled with 3 M KCl. mEJC amplitudes were calculated as the difference between peak and baseline values. Quantal content (QC) was estimated for each recording by calculating the ratio of eEJC amplitude/average mEJC amplitude followed by averaging recordings across all NMJs for a given genotype. mEJC and eEJC recordings were off-line low-pass filtered at 500 Hz and 1 kHz, respectively. Materials were purchased from Sigma-Aldrich (UK) unless otherwise stated.

**Cumulative postsynaptic current analysis.** The apparent size of the vesicle pool was probed by the method of cumulative eEJC amplitudes (Schneggengerber et al., 1999). Muscles were clamped to −60 mV and eEJC amplitudes during a stimulus train (50 Hz, 500 ms) were calculated as the difference between peak and baseline before stimulus onset of a given eEJC. Receptor desensitisation was not blocked as it did not affect eEJC amplitudes since a comparison of the decay of the first and the last eEJC within a train did not reveal any significant difference in decay kinetics. The number of release-ready vesicles was obtained by back-extrapolating a line fit to the linear phase of the cumulative eEJC plot (the last 200 ms of the train) to time zero. The number of release-ready vesicles was then obtained by dividing the cumulative eEJC amplitude at time zero by the mean eEJC amplitude recorded in the same cell. To calculate the quantal content in the train, we used mean mEJC amplitudes measured before the train.

**Immunohistochemistry (IHC).** 3rd instar larvae were dissected in ice-cold PBS then fixed in 4%
Paraformaldehyde. After permeabilisation with PBS-0.1% Triton (PBS-T) and blocking with PBS-T containing 0.2% BSA and 2% normal goat serum, larval fillets were incubated at 4°C overnight in solutions of primary antibody. The following antibody dilutions were used: NC82 (supernatant) anti-Brp (Bruchpilot; Developmental Studies Hybridoma Bank) 1:200 dilution. After 3x10min washes in PBS-T, larvae were incubated with AlexaFluor 488 goat anti-HRP (Jackson Immuno Research) and AlexaFluor 546 goat anti-mouse 1:500 dilution for 90 min at room temperature. Larvae were mounted using Vectashield mounting medium (Vector Labs) and NMJ 6/7 (segments A2 and A3) images were acquired with a Zeiss laser-scanning confocal microscope (LSM 510, Carl Zeiss). Image analysis was performed of z-stack images with ZEN (Carl Zeiss) and Volocity software and shown in figures as maximal projections.

Western blotting. Protein extracts from adult fly heads were separated by SDS-PAGE followed by immunoblotting with a rabbit dNOS antibody (kind gift from PH. O’Farrell lab) at 1:400 dilution and α-tubulin at 1:1000 dilution.

Electron microscopy. Third instar larvae were ‘filleted’ in phosphate-buffered saline at room temperature and then fixed in 2% (wt/vol) glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) at 4 °C overnight. They were post-fixed with 1% (wt/vol) osmium tetroxide/1% (wt/vol) potassium ferrocyanide for 1h at room temperature and then stained en bloc, overnight, with 5% (wt/vol) aqueous uranyl acetate at 4 °C, dehydrated and embedded in Taab epoxy resin (Taab Laboratories Equipment Ltd, Aldermaston, UK). Semi-thin sections, stained with toluidine blue, were used to identify areas containing synaptic regions (muscle 6/7 in regions A2/A3). Ultra-thin sections were cut from these areas, counterstained with lead citrate and examined in an FEI Talos transmission electron microscope (FEI Company (Thermo Fisher Scientific Inc.), Hillsboro, Oregon USA). Images were recorded using an FEI Ceta-16M CCD camera with 4k X 4k pixels. SV measurements were made using ImageJ software. A total of ~500-600 SVs were measured in 5-10 boutons from three animals per genotype.

Statistics. Statistical analysis was performed with Prism 6 and InStat 3 (Graphpad Software Inc., San Diego, USA). Statistical tests were carried out using an ANOVA test where applicable with a posteriori test (One-Way ANOVA with Tukey’s multiple comparisons test) or unpaired Student’s t-test as indicated. Data are expressed as mean ± SEM where n is the number of boutons, NMJs or fly heads as indicated and significance is shown as *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Results

Glutamatergic transmission is negatively regulated by NO

To examine the impact of NO signalling on glutamatergic transmission we characterised synaptic release at the NMJ of Drosophila 3rd instar larvae, which either overexpress dNOS or do not possess endogenous NOS activity and compared their responses to wild-type animals. As such we used dNOS1 overexpressing larvae (expression induced by heat-shock, HS) (Stasiv et al., 2001), NOSC/ NOS∆15 (Yakubovich et al., 2010) and w1118 wild-type (WT) larvae. In all three genotypes we measured evoked
excitatory synaptic currents (eEJC) and miniature EJCs (mEJC), and characterised the size of available vesicles within the readily releasable pool using the cumulative postsynaptic current analysis (Kuromi & Kidokoro, 2002). We found that eEJC amplitudes are reduced in larvae overexpressing dNOS1 for 24-48 hours compared to wild-type controls or non-heat shocked dNOS1 larvae (Fig. 1). Assuming that single quanta summate linearly in eEJCs and mEJCs and evoked responses arise from the same pool of vesicles at these low stimulus frequencies, the quantal content (QC) – the number of vesicles released per action potential was estimated as the ratio of the eEJC and mEJC amplitudes per NMJ. Analysis revealed that dNOS1 overexpression leads to a reduction of the QC indicating pre-synaptic actions of NO (Fig. 1).

We further assessed spontaneous miniature responses in larvae with reduced or enhanced NOS expression and detected changes in release frequencies of mEJCs between NOS5 / NOS315 and dNOS1 over-expressors but not in mEJC amplitudes or mEJC decay kinetics (Fig. 2). Reduction of NOS activity further enhanced spontaneous release frequency relative to w1118 WT suggesting that NO negatively influences presynaptic vesicle release.

To assess whether NO can affect the availabilities of release-ready vesicles from presynaptic pools, we estimated the size of ready-releasable pools using cumulative postsynaptic current analysis (Kuromi & Kidokoro, 2002; Weyhersmuller et al., 2011). These estimations showed that the presence of NO reduces the number of releasable vesicles as dNOS1 expressing (+HS) and NOS5 / NOS315 larvae resulted in opposite changes of pool sizes compared to w1118 WT larvae (Fig. 3).

To confirm expression levels of NOS in the genotypes employed in our studies we performed Western blot analysis which showed lack of and strong levels of NOS expression in NOS5 / NOS315 and dNOS1 (+HS) larvae, respectively relative to w1118 WT (Fig. 4). Together, these data suggest that activated NO signalling compromised vesicular release, as seen in reduced numbers of releasable vesicles per incoming action potential as well in the decreased frequency of spontaneously released vesicles under conditions of elevated nitrergic activity. Conversely, reduction of NOS expression leads to enhanced release, which supports the notion of inhibitory NO actions on release.

Altering NOS activity do not cause developmental effects at the NMJ

As NO has been reported to not only affect acute signalling pathways but can also be involved in developmental aspects of neuronal activity (Comes et al., 2007; Lacin et al., 2007; Comes et al., 2007), we sought to determine whether NOS expression levels could affect insect development. Wild-type larvae and larvae overexpressing dNOS1 following a 24-hour heat shock had a similar developmental time to w1118 WT (Fig. 4). This suggests that NO does not have a significant role in developmental processes at the NMJ.
We have tested whether our observed effects in NOS<sup>C</sup> / NOS<sup>Δ15</sup> larvae could be caused by alterations in NMJ morphology. We found no evidence for changes in either total volume of NMJs (HRP labelling) or the total volume of BRP signals as a measure of BRP expression levels at the 3rd instar larvae of the mutant lines (Fig. 5) consistent with previous reports (Yakubovich et al., 2010) and suggesting that the observed nitrergic effects are not caused by developmental changes of the NMJ. To further explore the possibility, that genetic reduction of NOS activity caused ultrastructural changes in vesicle numbers or active zones (AZ) we performed electron microscopy experiments and measured the number of vesicles within a 250 nm semicircle around the AZ in 1b boutons of the NMJs. As we observed no difference in NMJ morphology, we only focused the ultrastructural analysis on two genotypes, w<sup>1118</sup> and NOS<sup>C</sup> to test the developmental effects of lack of NO signalling. This data confirmed that the number of AZs, including T-bar structures, and the number of vesicles located within a 250 nm semicircle are not different between the two genotypes (Fig. 6). Thus our data imply that the observed effects of NO on glutamatergic release are likely to be due to direct nitrergic actions on the release rather than being mediated by developmental effects of NO on synapse structures.

Together, our study provides a comparative data set which illustrates the effects of NOS activity on synaptic physiology and morphology. Interestingly, enhanced NO exposure induced by enhanced dNOS activity is associated with a reduction in glutamatergic transmission at the larval NMJ, whereas reduced NOS activity results in enhanced release.

**Figure 5.** Changes in NOS activity do not cause developmental effects of NMJ morphology. A, Confocal maximal projection images of NMJs of indicated genotypes with HRP (green) and BRP (nc82, bruchpilot, red) labelling. B, Total NMJ volume and BRP volume were calculated from z-stack confocal images, analysis of NMJ and BRP volumes revealed no difference between genotypes, n=36-5 NMJs, p>0.05, ANOVA with post hoc Tukey-Kramer was used for comparisons.

### Discussion

Nitric Oxide regulates a great number of physiological and pathological signalling pathways in neuronal function. Many physiological actions of NO occur via the activation of the NO-sensitive receptor sGC which generates cGMP. The Drosophila NMJ shows activity- and Ca<sup>2+</sup>-dependent generation of cGMP signalling involving NOS activation (Shakiryanova & Levitan, 2008) and PKG-mediated modulation of oxidative stress responses (Caplan et al., 2013). This suggests functional relevance of the NO-cGMP-PKG cascade at the NMJ. Other activity and kinase-dependent regulatory mechanisms modulating synaptic responses include PKA-mediated presynaptic facilitation of neurotransmitter release (Davis et al., 1998; Steinert et al., 2006; Cho et al., 2015).

In this study, we wanted to establish how regulation of NO levels affects neurotransmitter release in a controlled environment using genetic modifications of endogenous NO release capacity at the well-characterised glutamatergic synapse, the Drosophila NMJ. We employed genetic mutants which either exhibit reduced (NOS<sup>C</sup> / NOS<sup>Δ15</sup> ) or enhanced NOS activity (dNOS<sup>1+HS</sup>) and compared the synaptic responses to wild-type w<sup>1118</sup> NMJs. Our data illustrated that by reducing endogenous NOS activity, the synaptic response increases with respect to spontaneous and evoked vesicular release. In contrast, when enhancing NOS activity, we detected a reduced release of vesicles. Spontaneous release frequency is increased in NOS 'null' larvae; however, enhanced nitrergic activity only induces a tendency to reduce spontaneous release relative to wild-type. This non-significant response could be the...
consequence of a subtle NOS activity in wild-type which already partially suppresses release. More drastic effects of activated NO signalling were observed on evoked release. Quantal content as well as available vesicle pool sizes were strongly reduced following enhanced NOS activity suggesting a robust inhibitory action of NO on evoked release.

Importantly, neuronal development could potentially be responsible for the observed physiological effects in the genetic mutants. It has been reported that NO can affect neuronal development by fine-tuning axon degeneration and regrowth in Drosophila mushroom bodies (Rabinovich et al., 2016). When assessing NMJ morphology, we did not detect any morphological changes of NMJs including the release sites, total NMJ volume or ultra-structural changes within boutons which could account for the changes in transmitter release. Thus, our data strongly suggest that the observed effects on neurotransmitter release are due to acute functional modulations of transmitter release induced by nitrergic signalling.

Together, we have shown that NO reduces the transmitter release at the NMJ which could potentially involve the cGMP signalling cascade. However, it is also well established that NO can lead to thiol S-nitrosylation of cysteine and 3-nitrotyrosination of tyrosine residues of proteins, two NO-mediated post-translational modifications. These modifications can have major impacts on neuronal function and synaptic release (Knott & Bossy-Wetzel, 2009; Bradley & Steinert, 2016). There is discrepancy in the literature regarding the effects of NO on neuronal activity, mainly due to the variable conditions and concentrations of NO applied. S-NO formation of various proteins can lead to opposite effects on neurotransmitter release. Postsynaptic proteins that are negatively affected by S-nitrosylation include mammalian NMDA receptors (NR2A and NR1) (Takahashi et al., 2007) which mediate excitatory neurotransmission and are essential for learning and memory. Nitrosylation of scaffolding proteins postsynaptic density-95 (PSD-95) decreases glutamate receptor clustering at synaptic sites (Ho et al., 2011), further compromising neuronal function. Presynaptic proteins are nitrosylated (e.g. syntaxin) (Wiseman et al., 2011) which reduces exocytosis, all of which can exacerbate detrimental effects of NO on neurotransmission as seen in neurodegenerative conditions. At this point, further experiments are required to investigate the underlying pathways for the observed nitrergic effects in our study which could potentially be mediated via cGMP or PTM signalling at the synapse.

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Conflict of Interests Statement

The authors declare no competing financial interests.
List of Abbreviations

- active zone- AZ
- central nervous system- CNS
- cysteine- Cys
- Drosophila NOS- dNOS
- nitric oxide- NO
- endothelium-derived relaxing factor- EDRF
- heat shock- HS
- neuromuscular junction- NMJ
- nitric oxide synthases- NOS
- post-translational modifications- PTM
- quantal content- QC
- sodium nitroprusside- SNP
- soluble guanylyl cyclase- sGC
- S-Nitrosothiols- S-NO
- SV-synaptic vesicles
- wild-type- WT

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