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A novel nitric oxide (NO)-dependent 'molecular switch' mediates LTP in the Octopus vulgaris brain through persistent activation of nitric oxide synthase (NOS)

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1 **Title:** A novel nitric oxide (NO)-dependent 'molecular switch' mediates
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14

Abstract

Cephalopod mollusks provide a striking example of the independent evolution of advanced cognitive abilities in invertebrates. In octopuses, learning and memory are mediated by the vertical lobe (VL), whose visual input synapses undergo robust activity-dependent long-term potentiation (LTP). Here, we used *Octopus vulgaris* of either sex to show that persistent activation of nitric oxide synthase (NOS) mediates both LTP expression and maintenance, independently of *de novo* protein synthesis. Contrary to the prevailing view, NO signaling is not required for LTP induction, as LTP can still be induced in the presence of NOS inhibitors. Remarkably, however, NOS inhibitors blocked presynaptic LTP expression, while NO donors induced only short-term potentiation, together suggesting that LTP expression depends on persistently elevated NO levels. The effective NO concentrations, estimated both pharmacologically and amperometrically, were more than three orders of magnitude higher than those required for activation of the canonical sGC–cGMP pathway, suggesting that NO acts through direct covalent modification of proteins, such as S-nitrosylation. In a subset of synapses, the NOS inhibitor L-NNA rapidly and completely erased established LTP; however, following washout, full potentiation could be reinstated by renewed tetanic stimulation. We therefore propose a ‘molecular switch’ mechanism in which an NO-dependent positive feedback loop persistently reactivates NOS, thereby maintaining LTP. These findings reveal an unprecedented form of convergent evolution in which molecular innovations equip the octopus VL network with the computational properties required for associative learning.

Significance Statement

Long-term potentiation (LTP) is a central cellular mechanism of learning and memory. While extensively studied in vertebrates, its mechanisms in octopuses—animals that independently evolved sophisticated cognitive abilities—remain poorly understood. Here, we uncover a novel form of LTP involving activity-dependent activation of nitric oxide synthase (NOS) in the postsynaptic cell. The produced nitric oxide (NO) mediates LTP maintenance through persistent NO-dependent reactivation of NOS (a “molecular switch”) and simultaneously mediates LTP expression by retrogradely facilitating presynaptic

transmitter release. These findings reveal a striking example of convergent evolution in which distinct molecular mechanisms independently evolved to implement associative (Hebbian) learning rules within a neural network.

Introduction

Octopuses and other coleoid cephalopods (squid and cuttlefish) are outstanding examples for the independent evolution of complex motor (Nesher et al., 2020; Hochner and Zullo et al., 2023) and cognitive behaviors (Hanlon and Messenger 2018; Turchetti-Maia et al., 2017; Shomrat et al., 2015; Wells 1978; Amodio et al. 2019). As such, they possess the largest nervous system among invertebrates, containing ~500 million neurons (Young 1971). The vertical lobe (VL), in their central brain controls learning and memory (Figs. 1A,B), exhibits the connectivity scheme of fan-out/fan-in association/classification networks (Shomrat et al., 2011; Aso et al., 2014; Litwin-Kumar et al., 2017). The fan-out layer consists of axons projecting out of 1.8 million superior frontal lobe neurons (SFLn) (Figs. 1B,C,G); these axons innervate *en passant* 25 million small amacrine interneurons (AM). A connectome study (Bidel and Meirovitch et al., 2023) found that in contrast to previous views (Gray 1970; Young 1971), the AMs are not a single homogeneous group of interneurons. The large majority (89.3% of the 25 million VL neurons) are “simple” AMs (SAMs) projecting a single non-bifurcating neurite into the neuropil, each receiving a single SFLn input through a large axonal varicosity (Fig. 1G). A very small population (~1.6%) of “complex” amacrine (CAMs) receive convergent inputs onto their “complex” dendritic tree from multiple SFL axons and from SAMs (see Fig. 1C). The SAMs and CAMs then converge onto the dendritic processes of the VL output layer, comprising 65,000 large neurons (LN) (Fig. 1C). These connectome findings, along with a comprehensive histolabeling study (Stern-Mentch et al., 2022), led Bidet and Meirovitch et al., (2023) to propose that the VL circuitry is organized in two parallel and interconnected feedforward networks: an excitatory network emerging from the cholinergic SAMs and an inhibitory network emerging from the GABAergic CAMs.

In 2003 Hochner et al. developed VL slice preparations (see Supplemental Fig.1A) and demonstrated a robust, vertebrate-like, activity-dependent long-term potentiation (LTP) at the SFL-to-AM synapses. This LTP is NMDA receptor-independent and is expressed

presynaptically through facilitation of transmitter release (Hochner et al., 2003; Shomrat et al., 2011). Approximately half of the synapses exhibit a key Hebbian property, as LTP induction was blocked when postsynaptic response was inhibited by kainate-like receptor antagonists (Hochner et al., 2003). Importantly, VL LTP was shown to be important for learning, as global tetanization-induced “saturation” of VL LTP, impaired long-term memory acquisition (Shomrat et al., 2008).

In the present work, we summarize our years-long efforts to decipher the three canonical processes underlying LTP: activity-dependent **induction**, which initiates it; **maintenance**, which preserves it over time; and **expression** which implements the increase in synaptic strength (e.g., Bliss et al., 2018).

The postsynaptic-dependent induction and the presynaptic expression of LTP, led us to hypothesize that NO may act as a retrograde messenger diffusing from the post- to the presynaptic side of the synapse. Indeed, NO has been implicated in visual and tactile learning in *Octopus vulgaris* (Robertson et al., 1994, 1995, 1996). In addition, NO is a well-established mediator of synaptic plasticity in mollusks, particularly those associated with complex forms of learning (Briskin-Luchinsky et al., 2018; Kemenes et al., 2002; Korshunova and Balaban, 2014; Susswein et al., 2004).

We uncovered an unprecedented form of NO-dependent LTP. Contrary to prevailing dogma, relatively high concentrations of NOS inhibitors did not block LTP *induction*, but instead interfered with LTP *expression* and, in some synapses, also blocked LTP *maintenance*. These findings suggest a profound adaptation of the molluscan nitroergic system (Wright, 2019; Moroz and Kohn, 2011), in which NO signaling relies not on the canonical NO–sGC–cGMP pathway but likely on direct covalent protein modifications such as S-nitrosylation. We propose a NO-dependent “molecular switch” in which a persistent NO-dependent NOS reactivation *maintains* LTP, while retrograde NO signaling mediates presynaptic facilitation underlying LTP *expression*. In the Discussion, we outline how this mechanism may operate within the recently resolved VL connectome to provide the Hebbian learning rules of *input-(synaptic)-specificity* and *synaptic-associativity*.

Materials and Methods

Animals and electrophysiological recordings

Mature *Octopus vulgaris* of both sexes were collected by local fishermen on the Israeli Mediterranean coast. Animals were held and handled according to the guidelines for the EU Directive 2010/63/EU for cephalopod welfare. The conditions under which the animals were kept, the slice preparation procedures, and the extracellular local field potential (LFP) recording methods followed Shomrat et al., (2011) and are further detailed in Supplemental Figure 1.

The postsynaptic field potential (fPSP) of the synapses between SFL axons and the AMs was evoked by stimulating the SFL tract at 30 s intervals. LTP was induced by high frequency (HF) stimulation (4 trains of 20 stimuli at 50 Hz, 10 s inter-train interval). We routinely used paired pulses (20 ms apart) as test stimulation to study the paired pulse facilitation (PPF) ratio between the amplitudes of first and second postsynaptic field potentials (fPSPs) (see Supplemental Fig. 1). Unless otherwise indicated, the results are displayed as normalized response (fPSP2/TP2), given that in most experiments under control conditions the first fPSP (fPSP1) was often indistinguishable from noise.

Histochemistry

NOS activity was detected using NADPH diaphorase histochemistry, as described in detail in Stern-Mentch et al., (2022).

To evaluate anisomycin suppression of *de novo* protein synthesis in the octopus brain, we adapted the method of fluorescent noncanonical amino acid tagging (FUNCAT) using L-azidohomoalanine (Thermo Fisher Scientific) described in detail for hippocampal slices (tom Dieck et al., 2012). The adaptations aimed to guarantee marine invertebrate physiological conditions prior to fixation. The slices were obtained as for electrophysiological recordings. We used Alexa Fluor 488 azide (Thermo Fisher Scientific) as fluorophore-alkyne-tag. Control slices were incubated with L-azidohomoalanine in the absence of anisomycin. Confocal imaging of the treated slices was performed using the Nikon C1 confocal system mounted on a Nikon TE-2000 Eclipse microscope system with a Nikon plan-Apo 60X NA 1.4 objective. Images were collected and processed using EZ-C1 software (Nikon). The Alexa Fluor 488 azide was excited with

the 488 nm laser and the emission was collected with a 515 ± 30 nm filter. Images were prepared using NIH ImageJ software (Bethesda, MD, USA).

Amperometry

NO was measured directly in the VL using electrochemical methods with 30 μ m polypropylene-insulated carbon fiber microelectrodes (CFMs) (ProCFE Dagan Corporation) coated by dipping the electrode tip five times into Nafion® 117 solution and drying the electrode for 10 min in 85 °C (Dias et al., 2016; Friedemann et al., 1996). This procedure was repeated ten times. The CFMs were then coated by electro-polymerization with o-phenylenediamine dihydrochloride (Friedemann et al., 1996) and cellulose (Marinesco et al., 2003). All these coatings were found to be essential for increasing the sensitivity and specificity of the CFM to NO in ASW. To test the electrodes, we measured the release of NO from a known concentration of NO donor (stock solution of SNAP prepared in 100 μ M EDTA). 10 μ M CuCl_2 was added to the ASW to catalyze SNAP decomposition to NO and disulfide by-product (Zhang et al., 2000). This concentration of Cu^{2+} had no effect on the physiological responses. For testing the peak oxidation voltage of NO in ASW, a slowly rising (~ 2.3 s) voltage ramp from a DC holding voltage of 0.5 V to 1.1 V was used as a command for clamping the CFM potential with a Chem-Clamp Voltmeter-Amperometer (Dagan Corporation). Adding 10 μ M SNAP to ASW containing 10 μ M CuCl_2 caused a maximal positive current response close to 750 mV. Hence, for the *in vitro* measurements of NO during physiological experiments the electrode potential was clamped at 750 mV. Because the oxidation current declined gradually in the presence of constant NO concentration, we used a micrometric manipulator to insert the CFMs into the tissue (~ 100 μ m deep) every 5 min for only 90 s. Amperometric measurements were stored together with the recording of the LFP responses generated by stimulation of the SFL tract.

All the *in vitro* experiments utilized isolated brain preparations comprising the supraesophageal mass of the central brain (see details in Shomrat et al., 2008). At the end of each experiment the CFM sensitivity in ASW was estimated by adding 10 μ M SNAP to the experimental bath containing 10 μ M CuCl_2 .

Drugs

Drugs were administered via the perfusion system, taking ~1 min to reach the recording site. We used the NOS inhibitors L-NNA (*N* ω -Nitro-L-arginine) and L-NAME (*N* ω -Nitro-L-arginine methyl ester hydrochloride), and its inactive enantiomer D-NAME (*N* ω -Nitro-D-arginine methyl ester hydrochloride). We routinely used 10 mM of these drugs because we found that at lower than 5 mM the results were less consistent. We found that 30 min of a continuous stirring is required to dissolve 10 mM L-NNA in ASW. To assess the possible involvement of NO-dependent soluble guanylyl cyclase (sGC)–cGMP cascade we used the cGMP inhibitors ODQ (1H-[1,2,4] Oxadiazolo[4,3-a]quinoxalin-1-one; 50 μ M) and methylene blue (0.1 mM); the cGMP agonists dBcGMP (0.2mM), 8-Br-cGMP (0.1mM), 8-pCPT-cGMP (10-100 μ M); the cGMP phosphodiesterase inhibitor ZAP (Zaprinast; 0.1mM); the cyclic nucleotide phosphodiesterase inhibitor IBMX (3-Isobutyl-1-methylxanthine; 0.5-1mM). To evaluate the involvement of NO we used the NO donors SNAP (S-Nitroso-N-acetyl-DL-penicillamine; 1-100 μ M and up to 10 mM), DETA (Diethylenetriamine NONOate; 0.4 mM), and DEA (Diethylamine NONOate; 0.1mM); the NO scavenger PTIO (2-Phenyl-4,4,5,5 tetramethylimidazoline-1-oxyl 3-oxide; 1 mM). Some drugs were dissolved in ASW and a fresh solution made before the experiments (L-NNA, L-NAME, D-NAME, methylene blue). Stock solution aliquots were prepared in DDW (dBcGMP), in DMSO 0.1-0.25% (ODQ, ZAP, IBMX), in ethanol 1% (PTIO), in NaOH 0.05 mM (DEA), or in 100 μ M EDTA (SNAP), and were diluted in ASW before the experiments in a concentration in which the vehicles had no physiological effects. When required, pH was adjusted to 7.6 using NaOH or HCl. Slices treated with reversible drugs, which could be washed out, could be used a second time in a different recording location to guarantee a different and yet unstimulated local population of cells. All drugs and chemicals were purchased from Sigma-Aldrich, unless stated otherwise.

Data analysis

Electrophysiological data were analyzed as described in Shomrat et al., (2011) and for more details see Supplemental Figure 1. For the analysis of electrophysiological recordings, we measured averages of 5 or 10 test trials (paired pulses, 20 ms apart, delivered at 30 s intervals).

Likely due to the inexcitable properties of the AMs, the postsynaptic field potential (fPSP) does not include 'population spikes' and its waveform is independent of its amplitude (see

Hochner et al., 2003). This property facilitates the accurate measurement of the postsynaptic responses. Furthermore, given the linear relationship between fPSP and tract potential (TP) amplitudes (see Shomrat et al., 2011), the variability across experiments was reduced by normalizing fPSP to TP amplitudes (fPSP/TP).

To estimate presynaptic effects, we quantified the paired pulse facilitation ration (PPF), usually quantified as the ratio of the second to the first fPSP (fPSP2/fPSP1). However, in the VL this ratio is extremely high because in most experiments fPSP1, and under control conditions, was often indistinguishable from noise. Therefore, we used a PPF index defined as $PPF_{index} = (fPSP2/TP2 - fPSP1/TP1)/(fPSP2/TP2)$, where a value of 1 corresponds to maximal PPF (see Supplemental Fig. 1).

Statistical analysis: The exceptionally robust magnitude of VL LTP together with the pronounced effects of NOS inhibitors we are reporting here, allowed us to detect significant differences with a relatively small number of repetitions. Accordingly, group comparisons were performed using Welch's t-test, which does not assume equal variances and is recommended for datasets with relatively small sample sizes (e.g., Ruxton, 2006).

Results

The neuropils of the vertical and sub-frontal lobes are intensely labeled for NOS activity

In a previous study, we tested for the presence of NOS activity in the supraesophageal part of the brain employing histochemical techniques (see Stern-Mentch et al., 2022). Briefly, using the NADPH-diaphorase method (Hope et al., 1991; Moroz, 2000) we discovered a particularly intense labeling in the neuropils of the VL and the subfrontal lobe (SuFL) (Fig. 1D-F). These areas are associated with visual and tactile learning, respectively (see Fig. 1B), and also share a similar anatomical organization (Young 1971). Dense labeling was found in the inner neuropil zones of the five lobuli of the VL (Fig. 1F), where the synaptic connections between the SAMs, CAMs and the LNs reside (Gray 1970; Bidel and Meirovitch et al., 2023; Bidel et al., 2025). These results strongly suggest intense NOS expression in dendritic processes postsynaptically to SFL inputs. The outer neuropil (Fig. 1E) was more sparsely stained, as in this region unlabeled axons run within the SFL

tract, making sparse *en passant* connections with SAMs and CAMs neurites (Gray 1970; Bidel and Meirovitch et al., 2023; Bidel et al., 2025).

The non-bifurcating neurites of monopolar SAM interneurons (Fig. 1G) are likely those that can be seen crossing the tract in faintly stained AMs trunks (arrows; Fig. 1E), suggesting the presence of NOS in the SAMs. Stern-Mentch et al., (2022) reported a similar pattern of immunoreactivity for choline acetyltransferase of the common type (cChAT), consistent with the cholinergic nature of the SAMs. Together, these findings support the supposition that NOS is expressed in the postsynaptic side of the SFL-to-SAM synapses. However, because the small cell bodies of the amacrine cells are largely occupied by the nucleus (Bidel and Meirovitch et al., 2023) and are therefore not clearly labeled for NOS, cChAT, or GABA, NOS expression in other cell types cannot be ruled out (see Stern-Mentch et al., 2022).

NO does not mediate LTP *induction*

To systematically examine the possible involvement of NO in VL LTP, we used slice preparations to test whether NOS inhibitors affect any of the three mechanistically distinct components of LTP: *induction*, *maintenance* and *expression* commonly used to characterize different forms of LTP (see Bliss et al., 2018; Lüscher and Malenka 2012; Caya-Bissonnette and Béïque, 2024).

We used two types of NOS inhibitors, L-NNA and L-NAME. We revealed that these NOS inhibitors were effective at much higher concentrations (5-10 mM), than the commonly used to inhibit NO-dependent sGC-cGMP pathway (Hall and Attwell 2008; Hall and Garthwaite 2009). Because these inhibitors are arginine-based competitive inhibitors (Víteček et al., 2012), millimolar concentration of L-NAME and L-NNA are likely required to suppress the high rates of NO production in the VL. Interestingly, several studies, particularly in marine animals, have reported that NOS inhibitors (e.g., L-NAME) up to mM range are required to effectively inhibit NO-mediated effects (e.g., Ueda et al., 2016; Moroz et al., 1998).

We first tested NO involvement in LTP *induction*. Accordingly, the drugs were administered before and during LTP induction by a high-frequency stimulation (HF; vertical dashed lines). Figure 2A shows that perfusion of 10 mM L-NNA led to a reduction in the already somewhat potentiated normalized synaptic field potential (fPSP2/TP2), indicating that in this set of experiments (and also those shown in Fig. 2B2) some level of potentiation was induced during setting the baseline recording, pointing to the possible involvement of NO in regulation of fPSP amplitude. Yet, HF stimulation in the presence of the drug led to a moderate level of stable potentiation. Washing out the drug gradually revealed further potentiation, and the second HF stimulation did not induce an abrupt potentiation, suggesting that LTP expression and/or gradual buildup of maintenance were curtailed in the presence of L-NNA. Note that the TP amplitude (black circles, and insets) stayed rather constant along the experiments, emphasizing that L-NNA specifically blocked NO-dependent facilitation of synaptic response.

To rigorously assess the specificity of NOS involvement in LTP induction, we repeated these experiments using the stereospecific NOS inhibitor L-NAME in parallel with its inactive enantiomer D-NAME as a stereospecific control (Fig. 2B). In contrast to L-NNA, both 10 mM D-NAME and L-NAME reversibly attenuated TP amplitude (Fig. 2B1), indicating that at this concentration, both drugs exert nonspecific effects on SFL axons excitability. To isolate the specific effect of these drugs on transmitter release, we compensated for the effects of D-NAME and L-NAME on the TP amplitude (see Fig. 2 legend for details).

Figure 2B2 shows that, similar to L-NNA (Fig. 2A), administration of L-NAME (10 mM), but not D-NAME (10 mM), reduced the basal fPSP2/TP2 amplitude, suggesting the presence of some level of NO-dependent potentiation during the setting of baseline recordings. HF stimulation delivered 20 min after drugs application induced a clear short-lived potentiation. However, washout of L-NAME was followed by a markedly larger monotonic increase in fPSP2/TP2 amplitude than that observed after washout of D-NAME. This was quantified as 'relative recovery' = $[(fPSP2/TP2)_{115min} - (fPSP2/TP2)_{75min}] / [(fPSP2/TP2)_{115min}]$ which amounted to 0.641 ± 0.133 SE ($n = 5$) for L-NAME, and 0.201 ± 0.052 SE ($n = 5$) for D-NAME. The difference between the two groups was statistically significant ($P = 0.023$; $t = 3.19$; $df \approx 5.21$ two-tailed Welch's t-test). A second HF stimulation produced no further facilitation, suggesting LTP saturation. Taken together, these results

suggest that LTP induction can occur in the presence of both drugs, but washing out L-NAME have either “unmasked” a greater level of LTP expression and/or enabled a buildup of LTP maintenance.

In summary, the results shown in Fig. 2 are inconsistent with the canonical view that implicates NOS activation in LTP *induction*, prompting us to next test the involvement of NO in LTP *expression*.

Persistent NOS activation mediates the long-term expression of the VL LTP

To test the involvement of NO in LTP *expression*, we added the drugs 10 minutes after LTP induction (Fig. 3). The inactive enantiomer D-NAME (Fig. 3A) produced a modest, non-significant, and reversible inhibition of the potentiated response ($23 \pm 20\%$ (SE) ($p > 0.2$; Welch's type t-test)). In contrast, L-NAME (Fig. 3B) and L-NNA (Fig. 3C) strongly inhibited the potentiated response ($63 \pm 16\%$ (SE) and $82 \pm 19\%$ (SE) inhibition, respectively; $p < 0.01$ for both). An HF stimulation given during the maximal inhibition did not overcome the inhibition. A third HF stimulation, delivered after drug washout, did not induce additional LTP. Taken together, these experiments suggest that NOS inhibitors reversibly inhibit LTP expression. Notably, L-NNA exhibits a faster inhibition time course than L-NAME. This may reflect, at least partially, the requirement for intracellular conversion of L-NAME (N ω -nitro-L-arginine methyl ester) to the active inhibitor L-NNA (N ω -nitro-L-arginine) by esterases (Víteček et al., 2012).

The histograms shown to the right of each panel of Fig. 3 represent the PPF-index which estimates the change in fPSP1 amplitude relative to that of fPSP2, serving as an indicator for changes in probability transmitter release (see details in Supplemental Fig. 1). These histograms clearly show that, first, induction of LTP dramatically reduces PPF index, consistent with LTP involving an increase in probability of transmitter release. Second, blocking LTP expression with the specific NOS inhibitors L-NAME and L-NNA was accompanied by a significant rise in the PPF-index, which recovered following the washing out of the drugs. It should be noted that the slight apparent inhibition of fPSP2/TP2 amplitude caused by D-NAME (Fig. 3A) did cause some, yet also insignificant, increase

in PPF-index, which suggests that 10 mM D-NAME may cause some NOS inhibition irrespective of its nonspecific effect on TP amplitude. In summary, the results shown in Fig. 3 support the idea that the presynaptic *expression* of LTP is mediated by NO which is generated through a constitutive activation of NOS.

As L-NNA seems to be more effective and with no apparent effect on TPs amplitude, we used this drug in the remaining experiments.

We next investigated if NO is similarly involved in the later phases of LTP by exposing the VL slices to 10 mM L-NNA for 45 min at different times after LTP induction (Figs. 4A-C). LTP expression was similarly blocked when applied 10, 90, or 240 mins after LTP induction, suggesting that persistent activation of NOS is the mechanism mediating the long-term expression of the VL LTP.

To directly test this possibility, we adopted an amperometric approach for measuring the extracellular concentration of NO (Dias et al., 2016; Ferreira et al., 2005; Friedemann et al., 1996) (Fig. 4D-F). A NO-sensitive carbon fiber microelectrode (CFM, see Methods) was inserted for short periods into the VL neuropil. The experiment shown in Fig. 4D started with three control insertions of the electrode. These insertions likely caused some gradual increase in the NO signals and a concomitant increase in fPSP (Fig. 4D *insets*). After HF stimulation, the clamping current at the NO oxidation potential (750 mV) increased in parallel to the increase in the fPSP amplitude (Fig. 4D,E and *insets*). Note the highly significant linear correlation between NO current signals and fPSP amplitudes (Fig 4E). The rough calibration of the CFM electrode, achieved by releasing NO from a known concentration of the NO donor SNAP, indicated an increase to approximately 0.5 μ M. This concentration is approximately three orders of magnitude higher than the physiological NO levels typically associated with activation of the NO-dependent sGC–cGMP cascade (Hall and Garthwaite, 2009). As is typical for LTP saturation, neither the fPSP amplitude nor the NO signals were further increased by a second HF stimulation administered while the CFM electrode was inside the tissue (Fig. 4D).

Figure 4F shows a summary of five experiments similar to that shown in Fig 4D. The CFM holding current increased substantially when the electrode was inserted into the isolated brain before HF stimulation (tests #1; #2). This increase is likely due to some endogenous redox molecules and/or electrode-evoked NOS activation. Following HF stimulation, the

347 averaged measurements revealed a significant, yet delayed, elevation in NO current
348 signals (see Fig. 4F legend).

349 While the summary of the amperometric results (Fig. 4F) support the notion that LTP
350 induction involves NOS activation, they provide less compelling evidence that persistent
351 elevated NO concentration underlies the long-term stability of LTP expression. Note,
352 however, the strong correspondence between the fPSP/TP amplitudes and the NO current
353 signals in the example shown in Fig. 4D,E. Possibly in this particular experiment a
354 favorable spatial alignment between the CFM and LFP electrodes contributed to the
355 significant correlation between the NO signals and fPSP amplitudes (Fig. 4E).

356 Although we demonstrated that NOS inhibitors blocked the amperometric response along
357 with LTP expression, as expected, we also found that L-NNA blocked the CFM response
358 to NO released from the NO donor SNAP. Consequently, the amperometric
359 measurements could not unequivocally confirm that L-NNA exerts its effect through a
360 reduction in NO concentration.

361 362 **NO-induced presynaptic facilitation requires unusually high NO** 363 **concentrations**

364 To directly test the involvement of NO in LTP expression we used the NO donor SNAP.
365 We found that only mM concentrations of SNAP (in the presence of 20 μ M CuCl₂ to
366 facilitate SNAP decomposition; Zhang et al., 2000) produced an enormous synaptic
367 facilitation (Fig. 5). Figure 5A shows one example out of five experiments, where a robust
368 facilitation was obtained with bath application of 2 mM SNAP. This facilitation is likely due
369 to a marked increase in release probability, as indicated by the reversal of the PPF ratio
370 (i.e., fPSP1 exceeded fPSP2; Fig. 5A, *insets* 1 vs. 2) However, this SNAP concentration
371 also caused a transient increase in TP amplitude, followed by a rapid decline that ended
372 in a complete blockade of TP propagation and consequently fPSP elimination (Fig 5A;
373 *insets* 3 and 4). These latter effects are likely due to the neurotoxic effect of sustained high
374 NO concentrations (Calabrese et al., 2007; 2009).

The results presented in Fig. 5B show that a brief pressure microinjection of a sub-microliter bolus of 10 mM SNAP resulted in short-term facilitation, which subsided after several minutes without causing apparent detrimental effects. Repeated injections produced similar transient facilitation responses. Importantly, these SNAP injections did not lead to a sustained fPSP potentiation, suggesting that NO alone is insufficient to induce LTP. The SNAP-induced facilitation was accompanied by a significant reduction in PPF index (0.826 ± 0.076 relative to 0.964 ± 0.101 in control; $p = 0.02$ paired t-test, $t(6) = 3.1$, $n = 7$), consistent with NO-dependent increase in the probability of transmitter release.

The canonical NO-dependent cGMP signaling pathway appears not to be involved in the VL LTP.

The high concentrations of SNAP required for the induction of synaptic facilitation, together with the levels of NO concentration measured amperometrically, and the high concentrations of NOS inhibitors that were effective, suggest that the NO effects are mediated by much higher NO concentrations than the 100 pM to 5 nM effective in activation of the canonical NO-dependent soluble-guanylyl-cyclase (sGC)-cGMP cascades (Hall and Garthwaite 2009; Hardingham et al., 2013; Garthwaite 2016; 2019; Kleppisch and Feil 2009). These results may explain why our attempts to test the involvement of the cGMP cascade have failed (Turchetti-Maia et al. 2018). Indeed, the NO scavenger (PTIO 1mM), shown to be effective in the cephalopod chromatophore system (Mattiello et al., 2010), did not affect the potentiated fPSPs. This is likely because this NO scavenger is ineffective in significantly reducing the high NO concentration at the synaptic sites. Furthermore, attempts to assess the involvement of cGMP cascade in the VL LTP using a battery of pharmacological tools (listed in Methods) failed to intervene with LTP expression (Turchetti-Maia et al. 2018). Importantly, these drugs were used successfully by Tu and Budelmann (1999; 2000a,b) to demonstrate the involvement of NO-dependent cGMP cascade in the regulation of the cephalopods' statocysts activity.

LTP maintenance does not require *de novo* protein synthesis

Numerous studies indicate that the universal mechanism for maintaining the long-term phases of LTP involves *de novo* protein synthesis (Kandel et al. 2014), including serotonin-induced long-term facilitation in the mollusk *Aplysia* (Montarolo et al., 1986). In contrast, we found that neither LTP induction nor its very long maintenance appears to involve protein synthesis. The administration of 20 μ M anisomycin for 2.5 h, starting 30 min before the induction of LTP, blocked neither induction nor maintenance of LTP for at least 10 h after LTP induction (Fig. 6A). This result contrasts sharply with the hippocampal CA1 region, where a similar protocol blocked the development of the late phase of LTP (e.g., Frey and Morris, 1997; Nguyen et al., 1994). We noticed that anisomycin may have several side-effects, including facilitation of LTP induction even at the slow rate of the test stimulation, a facilitated decline in TP amplitude, and a gradual reduction in fPSP/TP ratio, which recovered following anisomycin washout (Fig. 6A,B). This protein synthesis-independent LTP expression was blocked by L-NNA, indicating that the activity-dependent persistent activation of NOS was not affected by 20 μ M anisomycin (Fig. 6B). Importantly, exposing in vitro slices to 20 μ M anisomycin did suppress protein synthesis, including the intense protein synthesis in the VL (Fig. 6C). (The protein synthesis blocking effect of anisomycin was replicated in five additional experiments conducted in a different project (Bidel et al., unpublished).

The NOS inhibitor L-NNA discriminates between two types of synapses that differ in their LTP *maintenance* mechanisms.

Figures 7A and B present *post-hoc* analysis of the kinetics of L-NNA-induced inhibition of LTP expression. For this analysis, 11 of the 12 experiments shown in Figs. 4A–C were aligned at 10 minutes before the onset of a 42.5-minute exposure to L-NNA (10 mM). (see the excluded outlier experiment in Supplemental Fig 3). A readily noticeable difference was that in some experiments a second high-frequency (HF) stimulation delivered after drug washout, induced an abrupt potentiation (Figs. 7A,B; blue curves), whereas in others, the second HF stimulation did not interfere with the monotonic recovery from inhibition (red curves). The average of this potentiation in the first (blue) group was 2.64 ± 0.38 SE ($n = 5$), (calculated as the ratio of the average fPSP2/TP2 during 5 minutes following HF to that during the 7.5 minutes preceding HF). In contrast, the second (red) group showed

a smaller potentiation of 1.11 ± 0.05 ($n = 6$). The difference between the two groups reached a statistical significance (Welch's t-test, $t = 4.0$, $df \approx 4.1$, $p \approx 0.015$). We confirmed the validity of this classification using an independent permutation test (see Supplemental Fig. 2).

Figures 7A and B further reveal that the two groups differ in three additional characteristics. The second key difference between the two groups lies in the rate of inhibition. Remarkably, LTP expression in the first (blue) group declines much more rapidly than in the second (red) group (Fig. 7B). Accordingly, we hereafter refer to these groups as 'fast' and 'slow' groups, respectively. The estimated exponential decay time constants, fitted to the graphs in Fig. 7B, were 4.44 minutes for the 'fast' group ($R^2 = 0.985$; Pearson correlation, $P = 0.008$) and 19.7 minutes for the 'slow' group ($R^2 = 0.939$; Pearson correlation, $P < 0.001$), indicating that the 'fast' group decay is more than fourfold faster. Comparing the fitted exponential decay time constants across individual experiments confirmed that the 'slow' group exhibited significantly higher values than the 'fast' group (Welch's t-test, $t \approx 6.31$, $df \approx 6.6$, $p < 0.001$). While the slower time constant may reflect the rate of drug perfusion, the rapid and complete suppression of LTP expression in the 'fast' group suggests an accelerated, regenerative deactivation of NOS, strongly implicating that the effect of L-NNA is mediated through disruption of an NO-dependent positive feedback loop (see Discussion).

The third difference, likely related to the second, concerns the extent of inhibition. In the 'fast' group, 10 mM L-NNA almost completely suppressed LTP expression ($97 \pm 5.1\%$ (SE)), bringing back fPSP amplitude to pre-LTP levels (Figs. 7A,B). In contrast, the 'slow' group exhibited a significantly lower level of inhibition ($72.1 \pm 7.3\%$ (SE)). This difference was confirmed by comparing the level of inhibition across individual experiments (Welch's t-test, $t \approx 2.82$, $p \approx 0.021$). These findings suggest that in the 'slow' group, L-NNA reversibly inhibits NO-dependent LTP expression, whereas in the 'fast' group, L-NNA also disrupts LTP maintenance, possibly by returning NOS back to its inactive state. The gradual potentiation observed in the 'fast' group following L-NNA washout likely reflects activity-dependent 'slowly developed potentiation' (SDP), which is induced by the ongoing test stimulation (see below).

And finally, the fourth difference concerns fPSPs amplitudes. The normalized fPSPs (fPSP2/TP2) in the 'slow' group were consistently larger than those in the 'fast' group (Fig

7A). Approximately 3-fold under control conditions (Welch t-test $t \approx 2.97$; $df \approx 6.9$; $p \approx 0.02$) and approximately twofold after LTP induction, (yet with no statistical significance; $p \approx 0.10$).

Notably, the two groups did not differ in their TP amplitudes (1.55 ± 0.25 mV (SE) vs. 1.63 ± 0.32 mV (SE); Welch's t-test, $t \approx -0.22$, $df \approx 8.4$, $p \approx 0.83$), suggesting that the observed differences primarily reflect synaptic properties rather than presynaptic axonal excitability, although small differences in excitability cannot be excluded given the limited sample size.

These two distinct groups of 'slow' and 'fast' inhibition kinetics were also clearly discerned in the *post-hoc* analysis of the experiments in which LTP was induced gradually by a moderate rate of stimulation (Figs. 7C,D). Increasing the rate of the paired-pulse stimulation from 1/30 s to 1/10 s led to a slowly-developing-potential (SDP). Previously, we suggested that SDP shares the same mechanisms with that of HF-induced LTP, because they mutually occlude each other (see Shomrat et al., 2010). The results in Figs. 7C-E support this conclusion by implicating the involvement of NOS also in SDP.

As observed in HF-induced LTP (Figs. 7A,B), the inhibition kinetics induced by 10 mM L-NNA in the SDP experiments (Figs. 7C,D) could be classified into two distinct groups according to the 'fast' and 'slow' inhibition kinetics. The *a priori* classification was based on whether HF stimulation delivered during recovery from inhibition elicited a noticeable potentiation. Accordingly, three of the seven SDP experiments exhibited a clear potentiation in response to the second HF stimulation (Fig. 7E), whereas in the remaining four, the second HF stimulation did not appear to interfere with the monotonic recovery of LTP.

Figures 7C and D further show that, as in HF-induced LTP, these groups differ in three additional characteristics. First, Fig. 7C shows that the normalized fPSP2/TP2 amplitude of the 'slow' group (red) is consistently higher than that of the 'fast' group. The second characteristic is the exceptionally rapid onset of inhibition in the 'fast' group (Figs. 7C-E). Estimation of the average inhibition exponential decay time constant yielded a time constant of only 1.69 min ($R^2 = 0.970$; Pearson correlation, $P = 0.0084$). In contrast, the 'slow' SDP experiments (Figs. 7C,D, red), exhibited a slower time constant of 5.59 min ($R^2 = 0.951$; Pearson correlation, $P < 0.001$). Comparison of decay time constants across individual experiments confirmed that the inhibition time constant of the 'slow' group was

significantly slower than that of the 'fast' group (Welch's t-test, $t = 6.31$, $df = 6.6$, $p < 0.001$). The third characteristic, likely related to the second and consistent with the 'fast' group in HF-induced LTP, is that the 'fast' SDP group exhibits a more-than-complete blockade of SDP expression (Figs. 7C–E). The extremely fast and complete erasure of SDP is consistent with the hypothesis that 10 mM L-NNA abolished also LTP maintenance.

We observed robust and significant differences in inhibition kinetics between HF-induced LTP and that induced in the SDP experiments. In the groups with 'slow' inhibition kinetics, the mean inhibition time constant of the individual SDP experiments (5.92 ± 1.39 min (SD)) was ~2.8-fold shorter than that of the 'slow' HF-induced LTP group (16.4 ± 4.0 min (SD)) (Welch's t-test, $t \approx 5.31$, $df \approx 6.9$, $p \approx 0.0014$). Likewise, the mean inhibition time constant of the SDP group with 'fast' inhibition (1.60 ± 0.69 min (SD)) was ~3.3-fold shorter than that of the 'fast' HF-induced LTP (5.33 ± 1.47 min (SD)) (Welch's t-test, $t \approx 4.36$, $df \approx 5.1$, $p \approx 0.007$).

We suggest that the markedly faster inhibition kinetics observed in SDP protocol may reflect differences in the mode by which potentiation is induced. HF-induced LTP relies on intense stimulation that likely produces a saturating level of NO, whereas in the SDP protocol, NO concentration increases gradually. Consequently, 10 mM L-NNA are likely more effective under conditions of moderate NOS activation. Moreover, if NOS activation is maintained by an NO-dependent positive feedback loop (see Discussion), such a mechanism would be more readily disrupted at lower NO concentrations generated during the gradual induction protocol.

Interestingly, in the 'fast' group, HF stimulation given during complete blockade caused a robust short-term potentiation that was terminated rapidly, likely due to the fast inhibition kinetics of 10 mM L-NNA (Figs. 7C–E). This finding suggests that, under certain conditions, at least in the group of synapses with the 'fast' inhibition kinetics, L-NNA may block induction by inhibiting the transition from the induction to the NO-dependent maintenance phase. In contrast, in the group with the 'slow' inhibition kinetics, L-NNA seems to block only the SDP expression, as HF stimulation in the presence of L-NNA did cause an abrupt and stable potentiation that was fully expressed after L-NNA washout, highlighting again that HF-induced LTP can occur in the presence of NOS inhibitors, at least in the group synapses with 'slow' inhibition kinetics.

The histogram in Fig. 7F shows that the synaptic connection in the medial lobule represents only synapses with the 'fast' inhibition kinetics, while the lateral demonstrates the 'slow' properties. In the lateral/medial, both 'fast' and 'slow' inhibition kinetics could be documented. A Pearson's chi-square test of independence confirmed the highly significant association between anatomical location and kinetic group ($\chi^2 = 11.06$, $df = 2$, $p \approx 0.004$). It should be pointed out that this classification is based on the slice number (typically 5-6 sagittal slices per VL) and therefore the correspondence with specific lobuli is only an approximation.

Discussion

In this paper, we present a series of experiments revealing an unprecedented molecular mechanism that has evolved in the octopus brain to mediate activity-dependent long-term synaptic plasticity. Likely adapted from molluscan nitroergic neuromodulation systems (Wright, 2019; Moroz and Kohn, 2011), this mechanism may provide the octopus VL with cellular processes supporting associative Hebbian plasticity, similar in principle to those provided by NMDA-receptors in the mammalian hippocampus (Malenka 2003; Malenka and Nicoll 1999; Caya-Bissonnette and Béïque, 2024).

The effects of NOS inhibitors on LTP expression and maintenance, particularly in the group with 'fast' and pronounced inhibitory effect of L-NNA, led us to propose that LTP in the octopus VL is mediated by a novel NO-dependent "molecular memory switch". We propose (see Fig. 8A) that activity elevates intracellular Ca^{2+} which triggers LTP *induction* (green arrow) through NOS activation in the postsynaptic neuron (i.e., SAMs). NO then diffuses retrogradely to the presynaptic SFL bouton (blue arrow), where it facilitates transmitter release (i.e., LTP *expression*). In parallel (red arrow), NO mediates LTP *maintenance* through a positive feedback loop that dynamically 'locks' NOS in active states. Two models are proposed (Figs. 8A and B). Both models, assume PKC involvement in LTP *induction*, consistent with our finding that brief exposure to the PKC activator phorbol ester (5 μ M PDBu) induces long-term potentiation that was blocked by L-NNA (Turchetti-Maia et al., 2016 SfN abst). To account for the facilitatory effect of 5-HT on LTP induction (Shomrat et al., 2010), we assume that 5-HT activates phospholipase C

(PLC), generating diacylglycerol (DAG), which acts synergistically with Ca^{2+} to promote PKC activation.

Our results show that LTP *maintenance* and *expression* require unusually high NO concentrations, indicating noncanonical mechanisms involving direct covalent protein modification (e.g., S-nitrosylation) (Santos et al., 2015; Hall and Garthwaite, 2009). We therefore consider two mechanisms for persistent NOS activation. In the first (Fig. 8A), positive feedback loop involves direct NO-dependent reactivation of PKC, whereas in the second (Fig. 8B), it involves direct NO-dependent reactivation of NOS.

Previous studies demonstrate that NO-dependent covalent modification can modulate both PKC and NOS activity (Balafanova et al., 2002; Choi et al., 2011; Ravi et al., 2004), supporting the mechanistic plausibility of both models.

As the reversible inhibition of *expression* and the irreversible block of *maintenance* are correlated with strong and weak synapses, respectively, it is yet to be explored whether the two blocking modes are based on a single enzymatic cascade, which works differently depending on the strength of the postsynaptic response.

Does persistent, protein synthesis-independent NOS activation underlie long-term memory storage in the octopus?

Although we found that the VL LTP appears to be maintained independently of *de novo* protein synthesis, consistent with the proposed 'molecular switch' mechanism, it is difficult to envision how such a mechanism alone could account for the remarkably long-lasting memory storage exhibited by the octopus.

Several lines of evidence indicate that, although the VL and its LTP are critical for acquiring new memories, the VL itself is unlikely to serve as the long-term storage site. Lesion studies showed that removal of the VL after training did not impair recall of previously learned tasks (Boycott and Young, 1956; Muntz et al., 1962). Likewise, Shomrat et al. (2008) showed that transection of SFL input to the VL, or interfering with VL LTP by global tetanization, impaired acquisition of new memories without abolishing of previously learned tasks. Together, these findings suggest that VL output may regulate *de novo*

protein synthesis-dependent memory consolidation in circuits outside the VL. Consistent with this interpretation, cytochalasin D, which interferes with protein transport, blocked tactile learning in octopuses (Robertson, 1994), and cycloheximide, a protein synthesis inhibitor, impaired the acquisition of long-term memory in cuttlefish (Agin et al., 2003).

NO mediates the presynaptic expression of LTP via a retrograde messenger pathway

As described in the Results, we found that NOS is expressed in the primary, non-bifurcating neurites of the simple amacrine SAMs (Fig. 1E,F). Stern-Mentch et al. (2022) reported a similar pattern of immunolabeling for cChAT. Together, these findings suggest that a substantial proportion, if not all, of the cholinergic SAMs express NOS, thereby supporting the working hypothesis that the ‘molecular switch’ is confined to the postsynaptic side of the SFL-to-SAM synapse (schematized in Fig. 8A).

According to this model a sufficiently strong SFL-evoked postsynaptic response in the SAM, such as during HF stimulation, switches NOS into a persistently active state, leading to a continuous production of NO. NO then diffuses retrogradely to the presynaptic side of the activated synapse, where it facilitates probability of glutamate release (Fig. 8A). Strikingly, Bidel and Meirovitch et al. (2023) found that each SAM receives only a single SFL input via a large presynaptic bouton that synapses onto an enlarged, palm-like postsynaptic specialization on the SAM neurite (Fig. 1G). This prominent glomerular-like structure increases the area of close apposition between the pre- and postsynaptic membranes, thereby spatially constraining NO diffusion into the presynaptic compartment of the synapse.

Can the ‘molecular switch’ confer *input (synaptic) specific* LTP?

To address this question, it is important to recall two key characteristics of VL LTP described by Hochner et al. (2003). First, LTP induction is restricted to the tetanized group of SFL axons (Fig. 4 in Hochner et al., 2003), providing experimental evidence that VL LTP is *input-specific*. Second, during 50 Hz stimulation, the postsynaptic responses display a pronounced initial facilitation followed by depression. Importantly, however, the

total transmitter output—measured as the integrated area of the fPSPs during the stimulus train—remains unchanged following LTP induction (Fig. 3 in Hochner et al., 2003). This finding indicates that LTP most likely involves a robust increase in the probability of transmitter release, while the readily releasable pool remains unchanged. This led Hochner et al., (2003) to conclude that LTP is expressed predominantly, if not entirely, through presynaptic facilitation of transmitter release.

Given that VL LTP is *input-specific* and *presynaptically expressed*, we propose that the high NO concentrations required for NO-dependent signaling, generate steep effective concentration gradients (see Martinez-Ruiz et al., 2013; Garthwaite, 2016) restricting NO action to a single enlarged synaptic glomerulus (Fig. 1G).

By contrast, activation of the canonical NO–sGC–cGMP cascade, which operates at substantially lower NO concentrations, would be expected to permit broader NO diffusion and heterosynaptic spread of synaptic potentiation, similar to that observed at cerebellar parallel fiber–Purkinje cell synapses (Jacoby et al., 2001), and therefore would be incompatible with a mechanism that preserves synaptic specificity.

Although direct neurophysiological evidence assessing the involvement of S-nitrosylation in either pre- or postsynaptic expression of LTP remains scarce, numerous studies have demonstrated S-nitrosylation of proteins associated with both presynaptic and postsynaptic structures (see Santos et al., 2015). Of particular relevance are several proteins of the presynaptic release machinery reported to undergo S-nitrosylation, including, for example, SNAP-25, dynamin, syntaxin-1 and -2, and synaptobrevin (see Bradley and Steinert, 2016 and Lundberg and Weitzberg, 2022).

Can the ‘molecular switch’ provide the mechanism for postsynaptically-dependent (Hebbian) associativity?

While LTP at a single SFL input into each SAMs can provide the essential learning rule of ‘*input (synaptic) specificity*’, it is harder to understand how plasticity at a one-to-one innervation pattern can provide the other fundamental rule of ‘*synaptic associativity*’ (Malenka and Nicoll 1999; Malenka 2003; Caya-Bissonnette and Béique, 2024).

Synaptic associativity requires a mechanism whereby only the synapses that are activated together will facilitate each other's likelihood to undergo LTP. Because we found that NO by itself induces only short-term facilitation (Fig. 5), it suggests that NOS will be "switched ON", only in the SAMs that are both activated and in which the intracellular NO concentration reaches a certain threshold thus providing a 'coincident-detector' mechanism. It is tempting to speculate, therefore, that coactivated SAMs, especially as their neurites are densely packed in an "association module" (Bidel and Meirovitch et al., 2023) could influence each other's NO levels via extrasynaptic volume transmission, leading to an *associative* "switching ON" of NOS.

Bidel and Meirovitch et al. (2023) reported an intriguing arrangement in which several SAMs form adjacent synapses onto a single, likely serotonergic bouton (Shigeno and Ragsdale, 2015; Shomrat et al., 2010). This organization raises the possibility that *synaptic-associativity* is achieved through local 5-HT release evoked by coactivated SAMs. The released 5-HT then, through extrasynaptic volume transmission, facilitates PKC activation, as proposed in Fig. 8, thus promoting the "switching ON" of NOS specifically in SAMs that simultaneously experience depolarization and 5-HT receptor activation.

Preliminary results support the possible involvement of this local serotonergic reciprocal microcircuitry in LTP induction (Katz et al., 2023; SfN abst).

Finally, these scenarios, acting independently or in combination, may provide the "holy grail" constellation, enabling both postsynaptically mediated (*Hebbian*) *synaptic associativity* and *synaptic specificity* through retrograde NO-mediated presynaptic LTP expression.

Conclusions and Perspectives

We propose that the VL evolved molecular and cellular mechanisms enabling *synaptic specificity* and *Hebbian-like associativity*, thereby providing an alternative solution to vertebrate NMDA receptor-dependent associative plasticity.

Strikingly, however, NO-dependent LTP is unlikely to be shared across all modern cephalopods (octopuses, squids, and cuttlefish). Comparison of the VL of *Octopus*

vulgaris and the cuttlefish *Sepia officinalis* reveals a pronounced divergence in nitrergic signaling: the octopus VL exhibits intense NOS activity (Fig. 1D–F), whereas the cuttlefish VL shows no detectable NOS activity (Stereu-Mentch, 2018; Stereu-Mentch et al., 2022). We think that this divergence relates to the dichotomous difference in the synaptic loci of LTP expression in this homologous fan-out/fan-in circuits: in octopus, LTP occurs at the fan-out SFL-to-SAM input layer, whereas in cuttlefish LTP is expressed at the fan-in AM-to-LN output layer (Shomrat et al., 2011).

Interestingly, the octopus VL represents a rare example of activity-dependent LTP at the fan-out input layer, whereas in most biological and computational feed-forward fan-out/fan-in networks, including the cuttlefish VL, LTP occurs at the fan-in output layer. We therefore propose that the nitrergic system in *Octopus vulgaris* evolved to enable LTP specifically at the input layer. Implementing the ‘molecular switch’ at the one-to-one SFL-to-SAM synapses may thus transform each synapse into a “memory unit” tuned to “learn” a particular visual feature conveyed by its specific SFL axon. It is tempting to speculate that each SAM provides a functional analogue of a hippocampal dendritic spine, where localization of voltage- and glutamate-gated NMDA receptors to the spine head restricts synaptic plasticity to the activated spine.

It would be valuable to further explore the molecular novelties enabling this form of LTP in the octopus. Genomic and transcriptomic studies have revealed extensive cephalopods innovations, including genome reorganization, gene-family expansion, and tissue-dependent mRNA editing (Albertin et al., 2015, 2022; Liscovitch-Brauer et al., 2017). These processes may have contributed to adapting basal molluscan synaptic plasticity mechanisms (Hawkins et al., 2006; Kandel, 2001; Kandel et al., 2014; Hochner and Glanzman, 2016; Byrne et al., 2024) for implementation within the octopus VL network, allowing the emergence of network properties underlying the animal’s remarkable cognitive behavior.

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We used OpenAI's ChatGPT to assist in editing the English and improving the clarity of the manuscript. All scientific content, interpretations, and conclusions are the authors' own.

Footnotes

Author Contributions: A.L.T.M., T.S. and B.H. designed the study; A.L.T.M. performed and analyzed the physiological experiments with contribution from T.S. and B.H.; T.S. and B.H. designed and performed the NO-donor experiments; N.S.M., T.S. and N.N. designed and performed the NADPH diaphorase experiments; A.L.T.M., N.S.M., F.B. and N.N. performed the protein synthesis fluorescent assay; B.H. and T.S. performed the amperometric experiments and analyzed the results; I.K. and B.H. performed the permutation statistical analysis. A.L.T.M. T.S and B.H. wrote the paper with comments from all the authors.

The authors declare no competing interests.

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Figure Legends

Figure 1. The areas in the octopus brain that are involved in learning and memory are localized in district lobes which express intense NOS activity. (A) The centralized brain of *Octopus vulgaris* in anterior-dorsal view. The supraesophageal brain complex includes the superior frontal lobes (SFL, green) and the vertical lobe (VL, orange), which consists of five cylindrical lobuli (modified from Brusca and Brusca, 1990). (B) A sagittal section of the sub- and supraesophageal brain complexes (modified from Nixon and Young, 2003) showing the location of the SFL-VL system (visual L&M system; red line) and the IFL-SubFL system (chemo-tactile L&M system; blue line) (VL = vertical lobe; SubVL = subvertical lobe; SFL = superior frontal lobe; IFL = inferior frontal lobe; SubFL = subfrontal lobe; SBL = superior basal lobe; aBL = anterior basal lobe; mBL = medial basal lobe; dBL = dorsal basal lobe; BRN = brachial nerve; mPL = medial pedal lobe; PVL = palliovisceral lobe). (C) A schematic wiring diagram of the neural elements of the VL system, showing the basic connectivity (based on Bidet and Meirovitch et al., 2023) (SFLn = SFL neurons; SAM = simple amacrine; CAM = complex amacrine; LN = large efferent neuron). The type of transmitters, the synaptic sites, the number of cells and the site of LTP are indicated by the corresponding colors. (D-F) Histochemical detection of NO-synthase (NOS) activity, by NADPH diaphorase staining in slices of the supraesophageal brain mass (modified from Stern-Mentch et al., 2022). (D) Sagittal section; note intense labeling of the neuropil in VL (1) and SubFL (2). (E) Higher magnification of the respective areas in D. Arrows point to labeled bundles of primary neurites emerging from AMs in the cell body cortex. (F) Transversal section through the five lobuli composing the VL, showing intense labeling of their neuropil. (G) Volume EM reconstruction showing the structure of a simple amacrine neuron (SAM) and an enlargement of the one-to-one SFL-to-SAM synaptic connection (modified from Bidet and Meirovitch et al., 2023).

Figure 2. NOS inhibitors do not block activity-dependent LTP induction. In both panels A and B, fPSP2/TP2 amplitudes were normalized to the final potentiated level measured during the last 5 min of the experiment (n = 10 responses). TP2 amplitudes were normalized to the mean TP2 amplitude (n = 10) recorded during the first 5 min of the control period. (A) Exposure of slice preparations to the NOS inhibitor L-NNA (10 mM; red bar; n = 7) led to a reduction in baseline fPSP2/TP2 amplitude. High-frequency (HF) stimulation (vertical dashed lines) induced stable synaptic potentiation. Insets show averages of 10 LFP traces recorded at the indicated times. Washout of L-NNA resulted in slow recovery to a potentiated fPSP2/TP2 level. A second HF stimulation failed to induce additional potentiation, indicating saturation of LTP. Note that L-NNA had no effect on TP amplitude (filled circles and representative LFP insets). (B1) In contrast to L-NNA, both D-NAME (10 mM; n = 5) and L-NAME (10 mM; n = 5) reversibly attenuated TP amplitude. (B2) Note that the normalized fPSP2/TP2 amplitudes shown in B2 were corrected for drug-induced changes in the normalized TP amplitude shown in B1. (explained below). Application of L-NAME (10 mM; n = 5) produced a significant reduction in baseline fPSP₂/TP₂ amplitude compared with the inactive enantiomer D-NAME (10 mM; n = 5). HF

stimulation in the presence of either drug induced a transient potentiation. Upon washout, L-NAME, but not D-NAME, revealed a higher level of potentiation (see text). To compensate for the drug-induced attenuation of TP amplitude, we assumed that the linear relationship between fPSP and TP amplitudes (Shomrat et al., 2011) is unaffected by the drugs. This allowed us to correct the normalized fPSP2/TP2 values by scaling them with the corresponding normalized TP2, according to: corrected $(fPSP2/TP2)_{norm\ corr} = (fPSP2/TP2)_{norm} \times (TP2)_{norm}$.

Figure 3. NOS inhibitors do block LTP expression. Each panel (A-C) shows the effect of the corresponding drug on fPSP2/TP2 amplitude that were normalized to the LTP levels measured before the administration of the drugs. (A) The inactive enantiomer control D-NAME (10 mM, n=6). (B) The NOS inhibitor L-NAME (10 mM, n=5), and (C) the NOS inhibitor L-NNA (10 mM, n=6). The drug's perfusion (red bar) started 10 min after LTP induction (vertical dashed line). The normalized fPSP2/TP2 in A and B were corrected for the effect of D-NAME and L-NAME on TP2 amplitudes (see legend Fig. 2). A second HF was given 7.5 min before starting the drugs washout. A third HF was given toward the end of the recovery phase to check for residual LTP. The average PPF index is shown in the histograms to the right of each panel. The PPF index estimates the effect of the drugs on paired pulse facilitation ($PPF_{index} = [(fPSP2/TP2) - (fPSP1/TP1)] / (fPSP2/TP2)$) (see Supplemental Figure 1). Each bar shows the average and SE calculated over 10 min time intervals marked in C (*cont.*, *LTP*, *block*, and *recovery*). PPF_{index} values greater than 1 (i.e., $fPSP1 < noise$) were averaged as 1. The numbers between the bars depict the p-value of paired t-test of: *LTP* vs. *block* and *block* vs. *recovery*. Data shown as mean $\pm$ SEM.

Figure 4. Persistent increase in NO concentration mediates the long-term presynaptic LTP expression. (A-C) L-NNA (10 mM) reversibly blocks LTP expression, irrespective of the time window after LTP induction: A, 10 min (n=4); B, 90 min (n=5); and C, 240 min (n=3). fPSP2/TP2 is normalized to LTP level obtained after the first HF stimulation. To detect residual LTP a second HF was delivered 40 min after starting L-NNA washout. (D-F) Amperometric measurement of NO concentration. (D) Current signals obtained by repeated insertions of the carbon fiber microelectrode (CFM) into the VL neuropil at the indicated times (blue dots). The corresponding fPSPs are shown in the upper insets (red lines mark fPSPs amplitude). The second HF stimulation was delivered when the electrode was inside the tissue. (E) A significant correlation (linear regression $R^2 = 0.916$, $p = 5.1 \times 10^{-5}$, $n = 9$) between fPSP/TP amplitude and the amperometric NO signal as expressed by the difference between the currents measured inside and outside the neuropil (the same experiment shown in D). (F) Histogram depicting the averaged responses from five experiments, similar to that shown in D. For each measurement (i.e., electrode insertion), the maximal current measured after electrode insertion (the first blue circle in D) was normalized relative to the holding current measured prior electrode insertion (black circles in D). Measurements were grouped with respect to two tests before, and three tests after HF stimulation (arrow). Data are presented as mean $\pm$ SE. A delayed increase in the NO signal was observed following HF stimulation, as indicated by a significant difference between test #2 and the second post-HF test (#4) (paired t-test: $t(4) = 3.50$, $p = 0.025$), as well as between the averaged pre-HF tests (#1 + #2) and the averaged post-HF tests (#4 + #5) (paired t-test: $t(4) = 4.21$, $p = 0.013$).

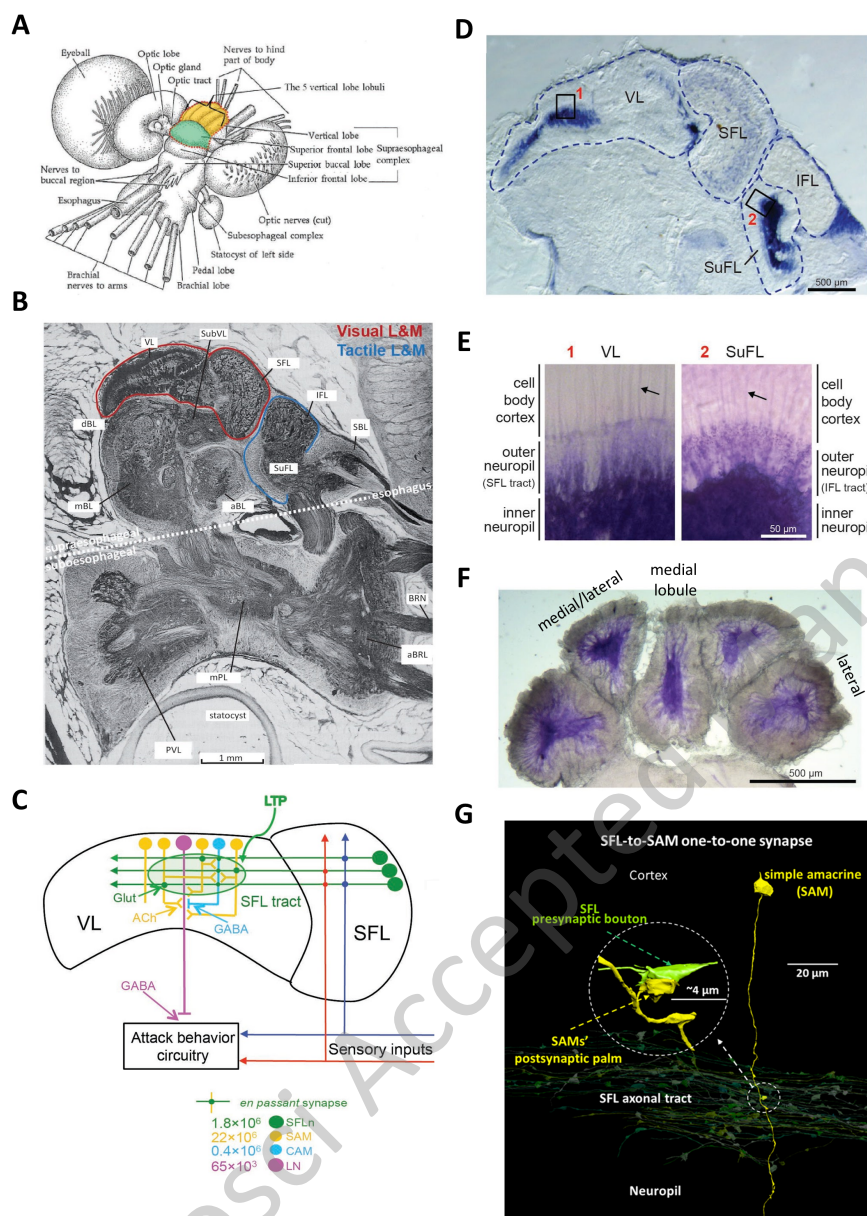
Figure 5. A considerably high range of NO concentrations is required for inducing short-term facilitation of transmitter release. (A) An example of the effects of exposing a slice preparation to millimolar concentrations of the NO donor SNAP (2 mM in the presence of 20 μ M CuCl₂ to facilitate the release of NO) caused a dramatic transient facilitation of fPSP that was terminated due to the neurotoxic effect of NO on the tract action potential (insets numbers correspond to the numbers on the graph). (B) Local pressure injections of sub-microliters bolus of 10 mM SNAP, close to the recording site, induced short-term facilitation of fPSP. fPSP2/TP2 amplitudes are normalized to that in control. The insets show the average $\pm$ SE (gray envelope) of the fPSPs LFPs (the TP's are truncated) recorded in seven experiments (each experiment was first normalized to the amplitude of TP1 and then aligned at the negative peak of the TP's). The calibration bar (0.01 TP), indicates 0.01 of normalized TP amplitude (TP=1). Note that the average $\pm$ SE of fPSP1 amplitude falls significantly below the zero dashed line only during SNAP injection (*inset #2*), whereas fPSP2 shows a transient facilitation. Data shown as mean $\pm$ SE.

Figure 6. Long-term LTP maintenance is independent of *de novo* protein synthesis. (A-B) Testing the effect of exposure to anisomycin (20 μ M, blue bars) on VL LTP induction and maintenance. (A) Example of a long-duration experiment (10 hr). A second HF stimulation, at 200 min, shows saturated LTP maintenance. (B) Population average of four experiments (mean $\pm$ SEM) showing the reversible effects of anisomycin on fPSP2/TP2 and TP amplitudes. Note that L-NNA (10 mM, red bar) blocked the anisomycin-resistant LTP expression. (C) Confocal microscopy images of *de novo* protein synthesis using the FUNCAT assay (see Methods). C1 and C4: transmission bright-field images of the cell body cortex area. C2 and C3 are fluorescent images showing intense fluorescent signals, reporting protein synthesis, in the control. C5 and C6 show that this signal is largely suppressed by anisomycin. The white squares in C1, C2 and C4, C5 mark the areas of magnification displayed in C3 and C6 respectively.

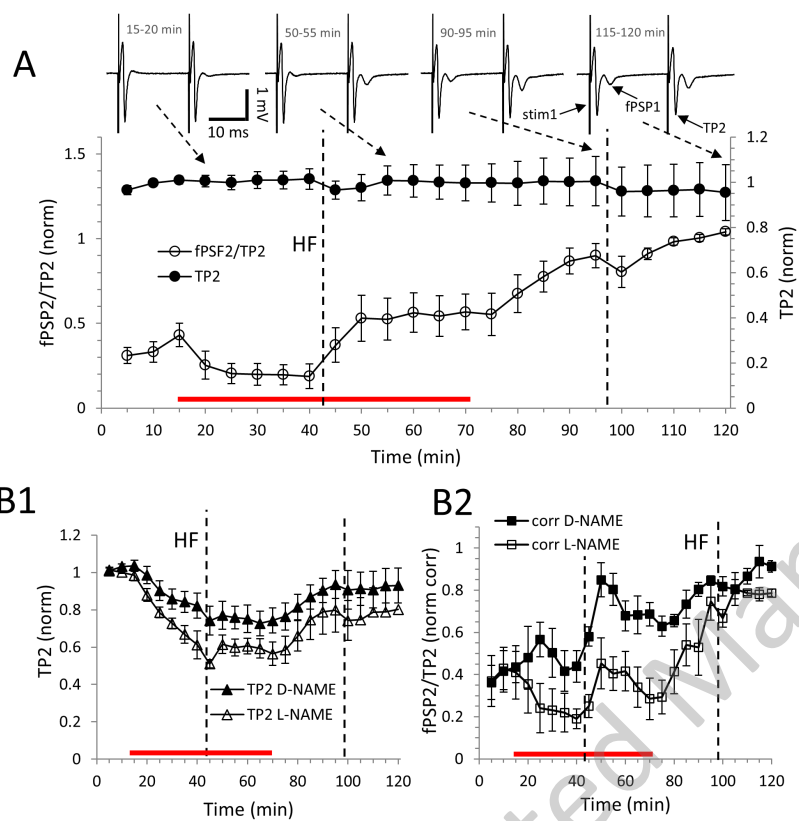
Figure 7. NO is involved in both LTP expression and maintenance. (A and B) Eleven experiments shown in Figure 4A-C in which 10 mM L-NNA was administered 10, 90, and 240 min after LTP induction were aligned at 10 min before the administration of the drug. This enabled the segregation of the inhibition kinetics into two groups (see text). (A) A group of large normalized fPSP (fPSP2/TP2) and a 'slow' inhibition time course (n=6; red curves), and a group of small fPSP and a 'fast' inhibition time course (n=5; blue trace). (B) The experiments shown in A were normalized to the level of LTP before L-NNA application. This depiction highlights the differences between the 'slow' and 'fast' inhibition kinetics and their responses to a second HF, which induced abrupt potentiation in the 'fast' group. (C and D) Testing the effect of L-NNA on activity-dependent 'slowly developing potentiation' (SDP), which is initiated by increasing the rate of the test pulses from 0.033 Hz to 0.1 Hz (first vertical dashed line). This set of experiments could also be segregated into a large fPSP2/TP2, with a slower time-course of inhibition (red; n=4) and a small fPSP2/TP2 (blue; n=3) with a fast and complete block of SDP. HF stimulations were delivered during the steady-state level of inhibition (1st HF) and at the recovery from inhibition (2nd HF). (D) The experiments in C were normalized to the level of LTP obtained after the second HF stimulation. (E) Panel showing the three individual experiments comprising the 'fast group in C and D. (F) Distribution of the two types of LTP across the

five VL lobuli, based on the 18 experiments (11 HF-induced LTP and 7 SDP). The 'fast' inhibition is prevalent in the medial lobule while 'slow' inhibition is prominent in the two lateral lobuli. In the medial/lateral lobuli, both types exist (see Fig. 1F).

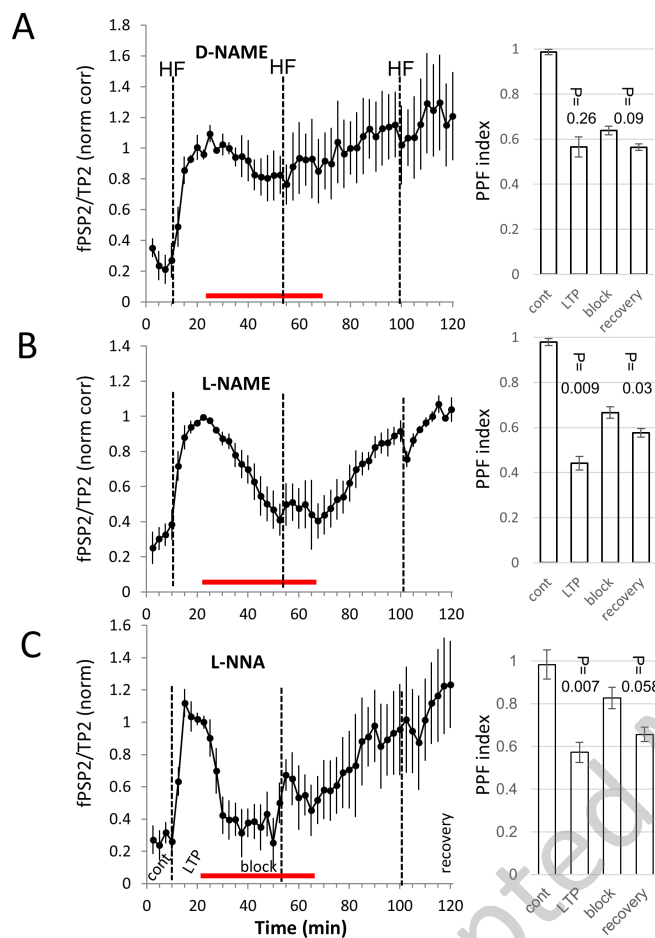
Figure 8. Two possible 'molecular switch' models for the NO-dependent long-term maintenance and presynaptic expression of LTP in the octopus VL. In A, the molecular schemes are divided into those in the large postsynaptic palm-like structure of the SAM (orange) and those in the enlarged presynaptic bouton of the SFL (green) (see Fig. 1G). In both models, LTP induction involves PKC activation triggered by Ca^{2+} influx through voltage-gated Ca^{2+} channels. In addition, 5-HT can facilitate LTP induction through the activation of a G protein-coupled receptor (GPCR), leading to phospholipase C (PLC) activation and the generation of diacylglycerol (DAG), which acts to promote PKC activation. For more details, see text.



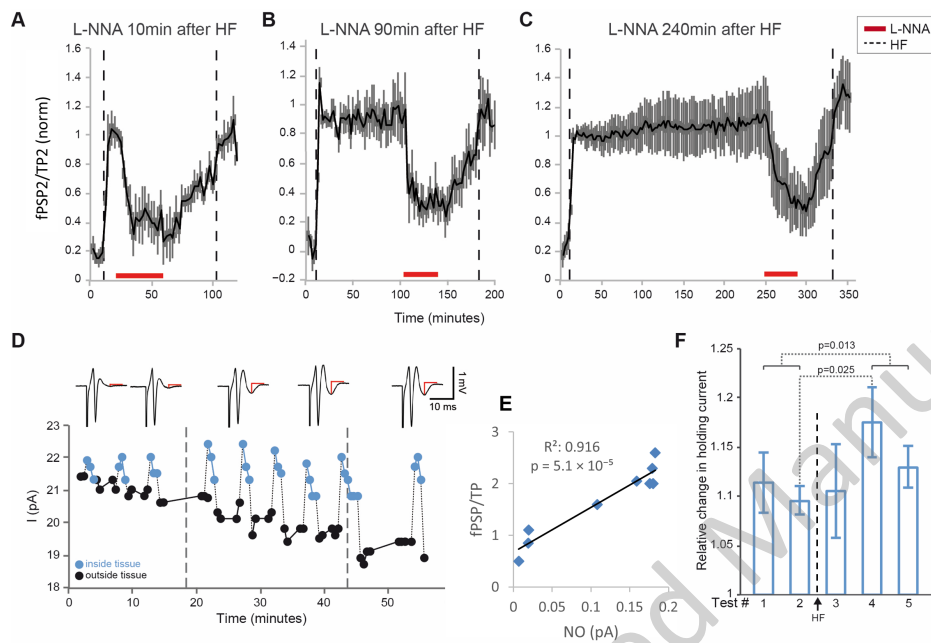
Turchetti-Maia et al. Figure 1



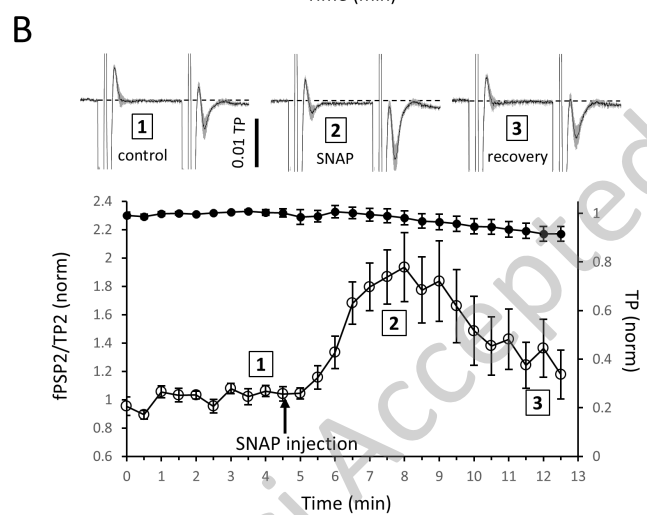
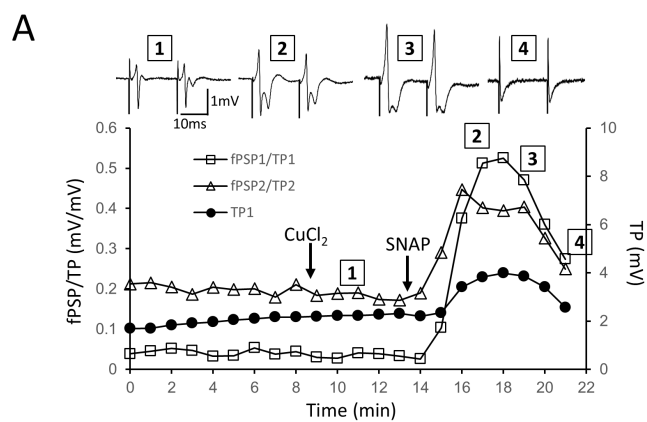
Turchetti-Maia et al. Figure 2



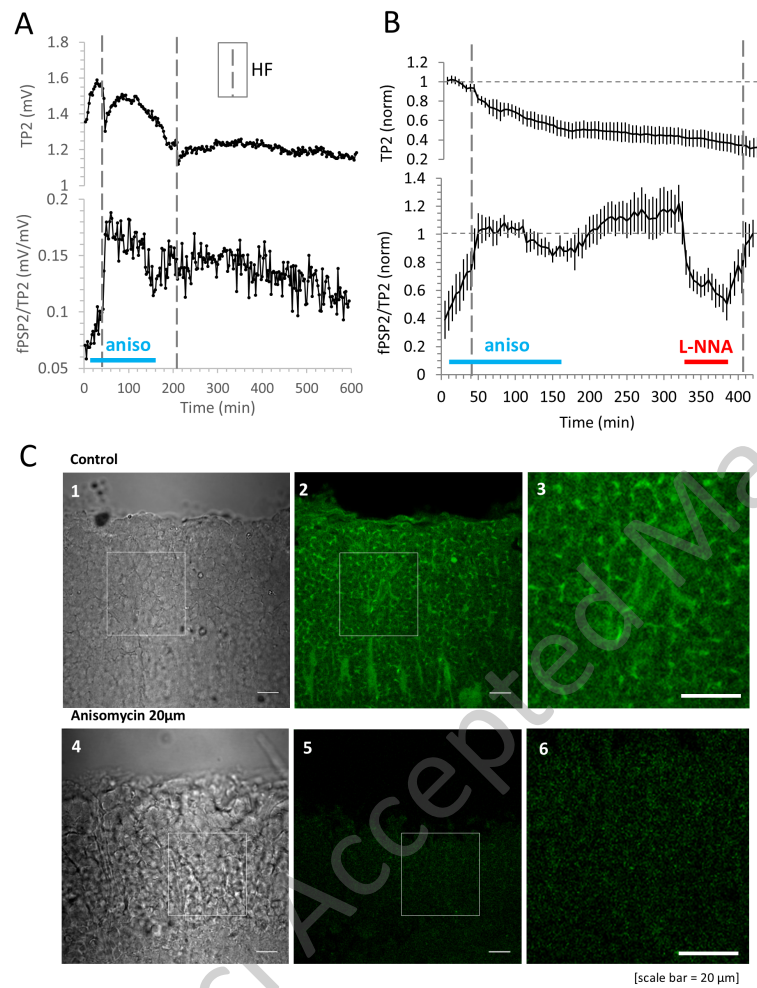
Turchetti-Maia et al. Figure 3



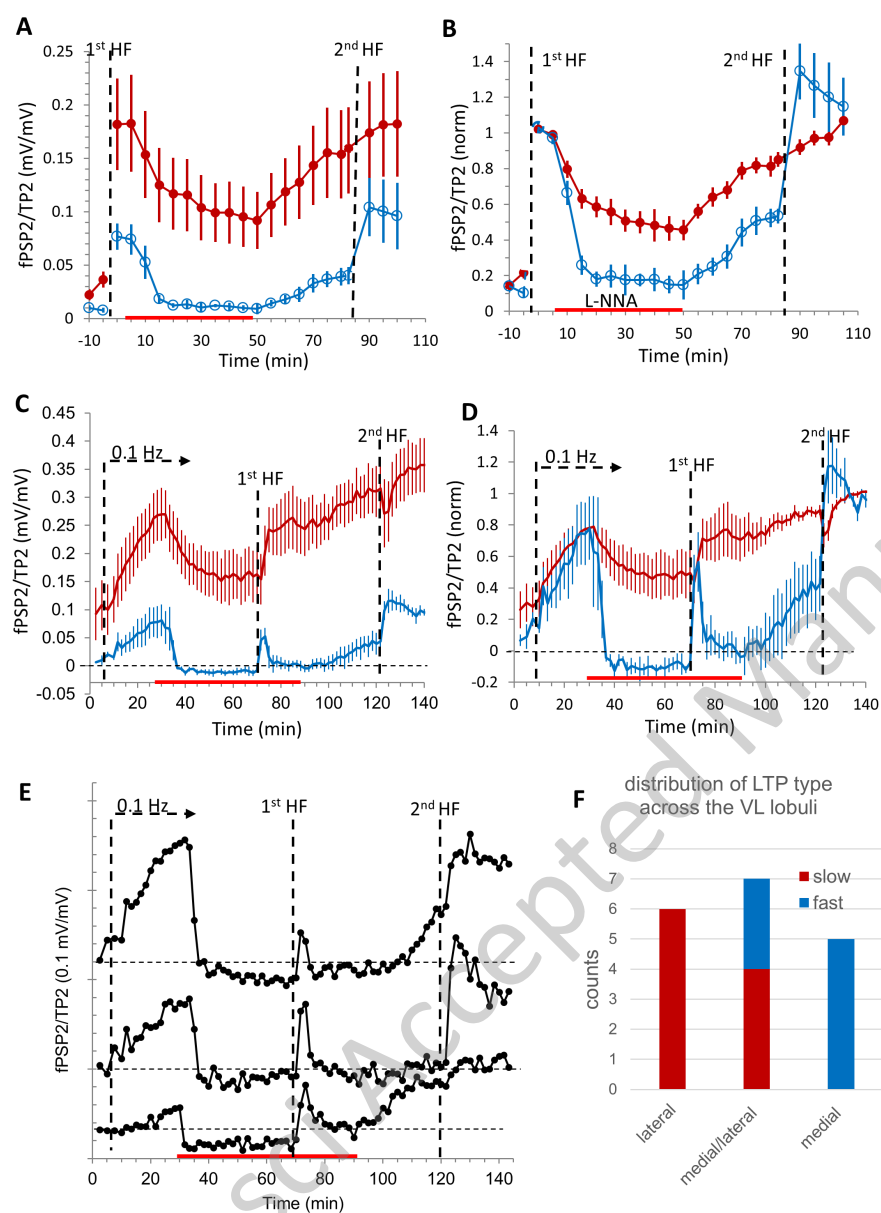
Turchetti-Maia et al. Figure 4



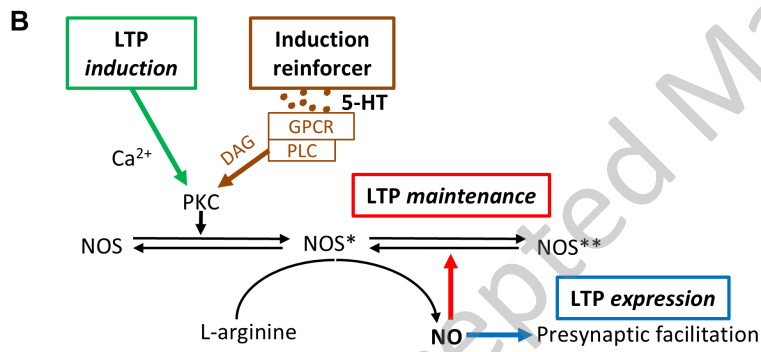
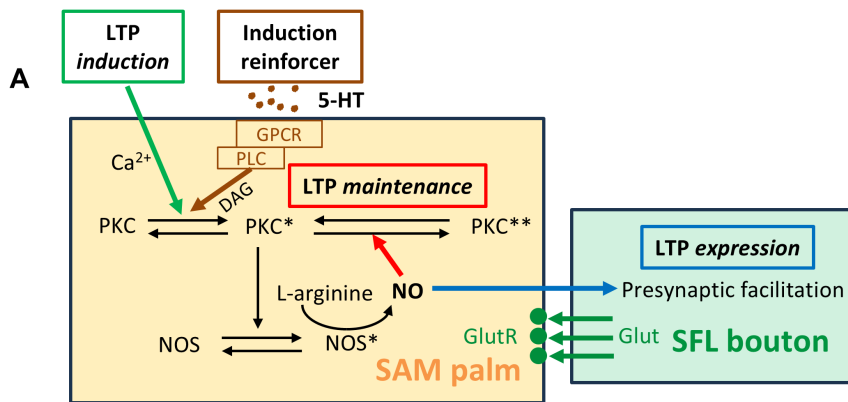
Turchetti-Maia et al. Fig. 5



Turchetti-Maia et al. Figure 6



Turchetti-Maia et al. Figure 7



Turchetti-Maia et al. Figure 8 (Revised)