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Role of nitric oxide in the induction of the behavioral and cellular changes produced by a common aversive stimulus in *Aplysia*

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Abstract

Although it is well documented that exposure to aversive stimuli induces modulation of neural circuits and subsequent behavioral changes, the means by which an aversive stimulus concomitantly alters behaviors of different natures (e.g., defensive and appetitive) remains unclear. Here, we addressed this issue by using the learning-induced concurrent modulation of defensive and appetitive behaviors that occurs when the mollusk *Aplysia* is exposed to aversive stimuli. In *Aplysia*, aversive stimuli concomitantly enhance withdrawal reflexes (i.e., sensitization) and suppress feeding. Sensitization and feeding suppression, which are expressed in the short term and long term, depending on the training protocol, are accompanied by increased excitability of the tail sensory neurons (TSNs) controlling the withdrawal reflexes, and by decreased excitability of feeding decision-making neuron B51, respectively. Serotonin (5-HT) has been shown to mediate sensitization, but not feeding suppression. In this study, we examined which other neurotransmitter might be responsible for feeding suppression and its underlying cellular changes. Our results indicate that nitric oxide (NO) contributes to both short-term and long-term feeding suppression, as well as to the underlying decreased B51 excitability. NO was also necessary for the induction of long-term sensitization and for the expression of short-term increased TSN excitability *in vitro*, revealing a previously undocumented interaction between 5-HT and NO signaling cascades in sensitization. Overall, these results revealed a scenario in which multiple modulators contribute to the widespread changes induced by sensitizing stimuli in *Aplysia*.

Keywords

Feeding suppression; neuronal plasticity; nitric oxide; sensitization; serotonin

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Author contributions

J.F., M.L.W. and R.M. conceived the study and developed the experimental design. J.F., L.L., J.A., M.L.W. and R.M. conducted experiments and data analysis. R.M. and M.L.W. wrote the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors have no actual or potential conflicts of interest.

1. Introduction

It is well documented that exposure to aversive stimuli induces persisting modulation of neural circuits leading to changes in individual behaviors [1–3]. However, the means by which an aversive stimulus concomitantly alters behaviors of different natures (e.g., defensive and appetitive) remains unclear. One such example is the modulation of defensive and non-defensive responses that occurs when the mollusk *Aplysia* is exposed to a common aversive stimulus.

In *Aplysia*, nonassociative application of noxious electrical stimuli, mimicking a predator attack [4], leads to a learned concurrent enhancement of defensive withdrawal reflexes (i.e., sensitization) and suppression of feeding [5,6]. These behavioral changes are sustained by modifications occurring within the corresponding neural circuits [2,6,7]. In particular, sensitizing stimuli, delivered both *in vivo* and *in vitro*, induce increased excitability in the sensory neurons and facilitation of the sensorimotor synapses mediating withdrawal reflexes [2,8,9], as well as decreased excitability of decision-making neuron B51 in the feeding neural circuit [6,7,9].

How does the same sensitizing stimulus differentially alter distinct neural circuits and subsequent behaviors? Until recently, studies in *Aplysia* have focused almost exclusively on sensitization of individual defensive behaviors (e.g., withdrawal reflexes [2] and escape response [10]). In these cases, it is well known that the neurotransmitter serotonin (5-HT) contributes to sensitization and its cellular correlates, including increased sensory neuron excitability and synaptic facilitation [2,8]. The co-expression of sensitization and feeding suppression offers a unique opportunity to investigate the mechanisms of diffuse modulation of multiple neural circuits by individual stimuli. Previous findings demonstrated that 5-HT does not alter feeding or the activity of decision-making neuron B51 [6,9], suggesting that a one-pathway model does not support the effects of sensitizing stimuli on multiple neural circuits and behaviors in *Aplysia*. This study began to investigate which signaling pathway(s) translates sensitizing stimuli into suppression of feeding and decreased excitability of B51.

The gaseous neurotransmitter nitric oxide (NO) is a candidate to serve as the training-induced modulatory signal that mediates suppression of feeding and decreased B51 excitability. NO has been shown to modulate behaviors and the function of neural circuits in both vertebrates and invertebrates [11–14]. In *Aplysia*, NO modulates the activity of defensive neural circuits [15,16] and feeding behavior [17,18]. In particular, NO mediates increased excitability of tail sensory neurons [15] and decreased excitability of B51 [19]. Finally, in *Aplysia*, NO mediates a form of nociceptive sensitization [15,20] and a form of learning in which the animals learn that food is not edible and downregulate the expression of feeding [21,22].

In this study, we characterized the contribution of NO to both feeding suppression and decreased B51 excitability induced by aversive stimuli. In order to have a comprehensive picture of the widespread effects of NO beyond the feeding neural circuit, we also analyzed the possible role of the NO pathway in the sensitization of the defensive tail-induced siphon

withdrawal reflex (TSWR) and increased excitability of the tail sensory neurons mediating the reflex [2,9]. We used a combination of previously-established *in vivo* [5] and *in vitro* [9] training protocols to compare and contrast the role of NO in the behavioral and cellular modifications induced by sensitizing stimuli in short-term and long-term temporal domains.

2. Materials and methods

2.1. Animals

Adult specimens of *Aplysia californica* were purchased from South Coast Bio-Marine (San Pedro, CA), individually housed in two aquaria (Aquatic Enterprises Inc., WA) of continuously circulating 15 °C aquarium seawater (Instant Ocean), and maintained on a 12-h light/dark cycle. Animals were fed one strip of dried seaweed (3 × 19 cm; 0.5 g; Emerald Cove® Organic Pacific Nori; Great Eastern Sun, Asheville, NC) three times per week [5]. Animals were food deprived for 48 h before the beginning of an experiment [5].

2.2. Behavioral methods

In this study, we used previously-established procedures for animal preparation, handling, and behavioral testing and training protocols [5,6,23–25]. We tested two behaviors (the tail-induced siphon withdrawal reflex and feeding [5,25]) and used two sensitization training protocols to induce short-term (15 min) and long-term (24 h) changes, respectively [5,25].

2.2.1. Measurement of the tail-induced siphon withdrawal reflex—As a readout of a defensive response, we used the tail-induced siphon withdrawal reflex (TSWR), which was assessed using previously-established testing protocols [5,23,24]. Prior to the beginning of behavioral testing, animals underwent two surgical procedures: 1) bilateral removal of the posterior portions of the parapodia (parapodectomy) performed one week before pre-tests to permit full visualization of the siphon withdrawal [5,23,24]; 2) implantation of a pair of Teflon-coated silver wire electrodes (36 Gauge; Medwire Cat# Ag5T, A-M Systems Cat# 786000) into one randomly-chosen side of the tail three days before pre-tests to deliver test stimuli [5,23,24]. Both procedures were conducted in animals that were anaesthetized by placing them under ice for about 20 min [5]. In the cases in which animals secreted ink and/or opaline during or after each of the above procedures, they were excluded from the study (9% of animals).

For each animal, the TSWR was elicited by 20-ms AC electrical stimulation delivered via the two implanted electrodes. The stimulus current intensity was set at two times the threshold required to elicit a detectable siphon withdrawal [5,23]. The TSWR was measured as the duration of the siphon contraction from the onset of the contraction to the onset of relaxation [5,23]. In all the experiments, a baseline for the TSWR was established by delivering five test stimuli at 10-min intervals (pre-test in Figs. 1A1, 2A). The five evoked responses were averaged to determine the pre-test TSWR duration [5,23]. Animals were excluded from the study if they had a pre-test TSWR duration greater than 10 s (6%), or if they inked and/or secreted opaline before training (1%), as these conditions may be indicative of an animal that is already sensitized or unhealthy [5,23].

2.2.2. Measurement of feeding behavior—Feeding was assessed by examining the elicitation of bites, defined as openings of the jaws followed by complete cycles of protraction, closure and retraction of the radula [26]. Individual animals were placed in a pedestal glass bowl containing a seaweed extract solution [5,27], and the number of bites was counted during a 5-min period [5,27]. In all experiments, feeding tests were conducted identically prior to (i.e., pre-test) and at different time points after training (i.e., post-tests; Figs. 1B1, 2A) [5,6]. A requirement of at least five bites during the pre-test was applied to provide a baseline level of feeding behavior that could be further modified by training [5,6]. Four percent of the animals tested did not meet this criterion and were excluded from the study.

2.2.3. L-NAME preparation and in-vivo injection—The NO synthase (NOS) inhibitor *N*_ω-nitro-L-arginine methyl ester (L-NAME; Sigma-Aldrich, Co., St. Louis, MO) was used to block the NO signaling cascade [28,29]. In all behavioral experiments, animals were injected systemically with 1 mL per 200-g body mass of either L-NAME dissolved at a concentration of 20 mg/mL in artificial seawater (ASW), or ASW (vehicle control). The ASW was composed of 450 mM NaCl, 10 mM KCl, 30 mM MgCl₂, 20 mM MgSO₄, 10 mM CaCl₂, and 10 mM HEPES, with pH adjusted to 7.5 using NaOH [30]. The estimated L-NAME final concentration in the hemolymph was 0.37 mM [29]. The injection site was on the left side of the animal (to avoid the genital groove containing the reproductive organs) just above the foot and perpendicular to the anterior portion of the parapodia [31].

After the completion of the pre-tests, each animal was moved for 1 h into a bowl containing aquarium seawater. This was a modification to the routine procedure that normally used 30 min between the end of the pre-tests and the beginning of sensitization training [5,6]. This alteration was made based on the findings of a pilot study that was conducted to ascertain the behavioral consequences of injecting a solution of L-NAME into an *Aplysia* (see section 2.2.3.1 below).

2.2.3.1. Effects L-NAME injection on spontaneous feeding: Previous findings indicate that *in vivo* injection of the NO donor S-nitroso-*N*-acetyl-penicillamine (SNAP) reduces bites elicitation, suggesting an inhibitory role of NO on feeding in *Aplysia* [17]. Consequently, it was possible that blocking NOS with L-NAME could increase the expression of bites. Therefore, in a pilot study, we examined the effects L-NAME injection on the number of spontaneous bites.

Following a feeding pre-test, as described above, animals were injected with either L-NAME or vehicle and placed in a glass pedestal bowl with 1500 mL aquarium seawater and an air bubbler. Aside from momentary contraction at the injection site upon injection, animals displayed no visible signs of damage nor exhibited any sensitized behavior from the injection (e.g., inking or flaring of the parapodia). Animals were allowed to rest for 10 min, and then were observed for 50 min, with the number of spontaneous bites per 5-min block recorded. The 50-min period was selected based on early observations of when spontaneous biting behavior generally ceased. Spontaneous biting behavior in animals injected with L-NAME was observed to follow a general increasing pattern until the 20-25 min interval and then decreasing to near-zero at the 45-50 min interval. Bites were observed to occur in bouts

as opposed to being evenly distributed throughout each 5 min block. The average amount of spontaneous bites elicited by animals injected with L-NAME solution was significantly different than that elicited by animals injected with normal ASW at the 20-25 minute interval (L-NAME: 8.33 ± 2.64 bites, $n = 9$; ASW: 0.00 ± 0.00 bites, $n = 4$; $p < 0.05$, $U = 4.00$; Mann-Whitney U test), but not at the 45-50 min interval (L-NAME: 2.56 ± 1.25 bites, $n = 13$; ASW: 0.00 ± 0.00 bites, $n = 4$; $p = 0.16$, $U = 10.00$; Mann-Whitney U test).

As a result of this pilot study, the interval between pre-test and sensitization training was increased from 30 min to 1 h to allow spontaneous biting behavior to decrease to baseline/near-zero amounts. This modification ensured that spontaneous biting behavior was at its minimum during sensitization training to avoid the possible formation of associative memory between the sensitization training shocks and biting.

2.2.4. Sensitization training—Sensitization training was administered using trials (single or multiple), each consisting of 10-s trains of electrical stimuli (500-ms pulses, 1 Hz, 60 mA AC) delivered diffusely along the body wall ipsilateral to the side of tail stimulation, using a hand-held probe [5,24]. Because NO signaling may selectively contribute to a specific form of memory (e.g., short-term vs. long-term), we employed two training protocols: single-trial and four-trial training. The single-trial training induces sensitization and feeding suppression lasting 15 min, but not 24 h [5,25,32]. The four-trial training induces concurrent long-term sensitization and feeding suppression lasting 24 h, but not 72 h [5,24,25]. Untrained controls were handled identically to their trained counterparts, but did not receive sensitizing stimuli.

2.2.4.1. Single-trial training: The single-trial training protocol is illustrated in Fig. 1A1, 1B1 [5,25]. Briefly, training consisted of a single trial of sensitizing stimuli. Behaviors were tested prior to and 15 min after training to assess the presence of short-term memory [5,25]. The TSWR may have short-lasting effects on the subsequent expression of feeding, and vice versa, when these two behaviors are evoked in close temporal proximity [33]. Consequently, it was not technically feasible to measure both TSWR and feeding in the same animal at the 15-min post-test without the potential bias of interactions between the two behaviors [5]. Therefore, in this experiment, the TSWR and feeding were examined in two cohorts of animals.

In one cohort of animals, the TSWR was measured during the pre-test as described above in section 2.2.1 (Fig. 1A1). Animals were then injected with either L-NAME or vehicle and randomly assigned to either the trained or the untrained group. Training began 1 h after the injection. Post-test measurements were conducted 15 min after training, using a single 20-ms stimulus [5,25]. In cases in which one or both electrodes did not remain implanted in the tail between the pre-tests and the post-tests, TSWR could not be assessed at the 15-min time point, and, therefore, the animal was excluded from the study (4% of animals). In the second cohort of animals, feeding was measured during the pre-test as described above in section 2.2.2 (Fig. 1B1). Animals were then injected with either L-NAME or vehicle and randomly assigned to either the trained or the untrained group. Training began 1 h after the injection. Feeding was measured again 15 min after training [5,25].

2.2.4.2. Four-trial training: In this experiment, the TSWR and feeding were assessed in the same animal. The four-trial training is illustrated in Fig. 2A [5,25]. Briefly, the feeding pre-test was conducted 30 min after the end of the TSWR pre-test, as this interval is sufficient to rule out any interaction between TSWR and feeding [5,6]. After the completion of the pre-tests, animals were injected with either L-NAME or vehicle and then randomly assigned to either the trained or the untrained group. Training began 1 h after the injection and consisted of four trials of sensitizing stimuli, with a 30 min inter-trial interval. Post-tests for TSWR and feeding were conducted 24 h after training in a manner identical to pre-tests [6,25]. Measurements of TSWR were suspended for those animals (6%) in which one or both electrodes did not remain implanted in the tail or were damaged between the pre-tests and the post-tests.

2.3. In vitro analog

In order to investigate the contributions of NO to *in vitro* correlates of sensitization and feeding suppression, we employed a ganglion preparation that retains the neural circuits controlling feeding and TSWR [2,34] and expresses *in vitro* short- and long-term excitability changes in TSNs and B51 (i.e., *in vitro* analog) [9] similar to those induced by *in vivo* training [6,24]. Prior to each experiment, the general health and motivational state of the animal was tested by presenting it with a food stimulus. The animal was included in the study only if it generated bites in response to the food stimulus [9].

2.3.1. Ganglia preparation and configuration—*Aplysia* were anesthetized by injecting a volume of isotonic MgCl₂ (equivalent to 50% of the weight of the animal) through the foot [9,24]. The animal was placed ventral side up in a dissecting tray and a longitudinal incision was made along the midline of the foot to expose the central nervous system [9]. The buccal, cerebral, and pleural-pedal ganglia were removed together with their intact inter-ganglionic connectives [9]. The left or right pedal nerves P8 and P9 [35,36] were randomly retained for extracellular nerve stimulation [9,37]. One of the two buccal nerves n. 2,3 was also retained for identification of neuron B51 [9]. All other nerves were cut short.

The isolated ganglia were transferred to a Sylgard-coated Petri dish, containing high-divalent artificial seawater (ASW) composed of 210 mM NaCl, 10 mM KCl, 145 mM MgCl₂, 20 mM MgSO₄, 33 mM CaCl₂, and 10 mM HEPES, with the pH adjusted to 7.5 using NaOH [30]. The sheath on the ventral surface of the pleural ganglion on the side of the retained nerves P8 and P9 was surgically removed using fine forceps and scissors to expose the somata of TSNs in the VC cluster [9,37]. The sheath was also removed from the rostral surface of both buccal hemiganglia to expose the somata of B51 [38]. Individual bipolar extracellular stimulating electrodes were placed along P8, P9 and n.2,3 and were isolated from the bath with Vaseline [30]. The high-divalent ASW was replaced by either ASW containing 0.37 mM L-NAME [29] or ASW (vehicle control). Preparations were then rested for 30 min. The recording chamber was maintained at 15 °C throughout the electrophysiological recordings and experimental treatments with a feedback-controlled cooling device (Model BTC-100/BTC-S, Bioscience Tools, CA).

2.3.2. Intracellular recordings—Intracellular recordings from TSNs and B51 were conducted in current-clamp mode using a pre-amplifier (IX2-700, Dagan Corporation, Minneapolis, MN). Fine-tipped glass microelectrodes were filled with 3 M potassium acetate (resistance 10-12 M Ω).

2.3.2.1. TSNs: One-electrode current-clamp was used for intracellular recordings from TSNs [9,37]. The somata of TSNs were identified based on their position in the VC cluster of the pleural ganglion, and the occurrence of action potentials in response to a single 3-ms stimulus delivered to nerve P9 [24,36]. Resting membrane potential (V_m) was recorded 5 min after impalement. Cells were included in the study only if they displayed a V_m of at least -35 mV [9,37]. TSNs were subsequently clamped at -45 mV [9,39] and their excitability was assessed as the number of spikes evoked by a 1-s, 2-nA depolarizing current pulse [9,24].

2.3.2.2. Neuron B51: The soma of neuron B51 was identified based on its relative size and position within the buccal ganglion and the occurrence of its characteristic intrinsically-generated all-or-nothing bursts of action potentials, known as plateau potentials [38,40]. If B51 did not fire spontaneously or in response to brief intracellular depolarizing pulses, then n.2,3 was briefly stimulated (1-2 seconds of 10 V, 0.5-ms pulses at 4 Hz), and the cell's response was used to aid in identification [6]. Two-electrode current-clamp was used for intracellular recordings from B51 [38]. The V_m was recorded 5 min after impalement. Cells were included in the study only if they displayed a V_m of at least -45 mV [6]. B51 was then current clamped at -60 mV, and the input resistance (R_m) and the excitability was measured. R_m was calculated from the voltage drop elicited by a 5-s, 5-nA hyperpolarizing current pulse [38]. The excitability of B51 was assessed by measuring the neuron's burst threshold, defined as the minimum amount of depolarizing current necessary to elicit a plateau potential outlasting the duration of the current pulse [38]. B51 burst threshold was determined by delivering a series of 5-s depolarizing current pulses, beginning at 5-nA intensity and increasing in intensity in 1-nA increments, at 10-s intervals [38]. Cells that did not exhibit a plateau potential by 25 nA were excluded from the study (5%) [9].

2.3.3. In vitro training protocols—We used two *in vitro* sensitization training protocols, single-trial and four-trial, inducing short-term (15 min) and long-term (24 h) changes in the excitability of TSNs and B51, respectively [9].

2.3.3.1. Single-trial in vitro training: Membrane properties (V_m , R_m and burst threshold for B51; V_m and number of action potentials for TSNs) were initially measured (pre-test; Fig. 4A). The current clamp was then removed and the ganglia were rested for 10 min before delivery of the single-trial training protocol. The single-trial training consisted of 10-s, 1 Hz, train of 10 500-ms, 60-Hz, 60-V impulses delivered simultaneously to the retained nerves P8 and P9 [9]. Controls consisted of untrained preparations that did not receive any electrical stimulation to P8/P9. Preparations were randomly assigned to receive training or serve as untrained controls, either in 0.37 mM L-NAME or vehicle. Cells were held throughout training. Fifteen min after training, membrane properties were measured again (post-test; Fig. 4A) to assess changes induced by training [9].

2.3.3.2. Four-trial *in vitro* training: Membrane properties of B51 and TSNs were initially measured as described above (pre-test; Fig. 5A), and then the electrodes were removed. Prior to removing the electrode from the recorded TSN, its location was marked by iontophoretically injecting 4-5 cells immediately surrounding it with Fast Green dye (10-nA hyperpolarizing current for approximately 30 seconds, or until the soma was dark green in color) [9,37,41] to aid in cell identification in 24-h post-test measurements. Images of pleural and buccal ganglia were also taken (MD800E microscope-mounted USB camera, AmScope, Irvine, CA), and the cells were annotated in Microsoft Paint to aid in the identification of B51 and TSN for 24-h post-test measurements of membrane properties. Once TSNs were labeled and images were taken (10-15 min), ASW was exchanged with either modified L-15 culture medium (L-15 medium (Sigma) supplemented with (in mM) NaCl 293.1, KCl 4.6, MgCl₂ 26, MgSO₄ 18.9, CaCl₂ 10.9, HEPES 30 and 0.10 g/l of streptomycin (Sigma) and 0.12 g/l of penicillin-G (Sigma), pH adjusted to 7.5 with NaOH [42]), or modified L-15 medium containing 0.37 mM L-NAME. To monitor the efficacy of subsequent training, one of the large neurons in the pleural ganglion adjacent to the medial edge of the VC cluster (that reliably responded to p8/p9 stimulation with fluctuations in the V_m)[43] was impaled and held throughout the training period. The *in vitro* training (or no training) protocol began 10 min after the medium exchange. The four-trial training consisted of four 10-s trains of P8/P9 electrical stimulation (each identical to that used for the single-trial training) spaced 30 min apart [23,37]. Controls consisted of untrained preparations that did not receive any electrical stimulation to P8/P9. Preparations were randomly assigned to either receive training or serve as untrained controls either in modified L15 medium, or in modified L15 medium containing 0.37 mM L-NAME. Once the training was concluded, the extracellular stimulating electrodes were gently removed, and the recording chamber was covered and stored at 15 °C for 23.5 h. On the next day, ganglia were rinsed in normal ASW or ASW containing 0.37 mM L-NAME and allowed to rest for 30 min. Post-test measurement of membrane properties commenced 24 h after training (Fig. 5A).

2.4. SNAP treatment

In this experiment, ganglia were prepared as described in section 2.3.1., with the exception that the high-divalent ASW was replaced with ASW for all preparations. Membrane properties of TSNs and B51 were measured as described in section 2.3.2 above (pre-test; Fig. 3A). Five min after the measurements were completed, preparations were randomly assigned to receive treatment with either SNAP or vehicle (DMSO). SNAP was delivered via a bolus application (final chamber concentration: 100 µM in 0.1% DMSO) [19]. Control preparations were treated with bolus application of DMSO (final chamber concentration: 0.1%) [19]. Post-test measurements were conducted 15 min after treatment (Fig. 3A).

2.5. 5-HT treatment

Only the pleural-pedal ganglia were used in this experiment [44]. 5-HT (Alfa Aesar, Ward Hill, MA) was dissolved in ASW to make a 50-mM stock. Stock solutions were made fresh daily and stored at 4 °C. The stock solution was diluted to 50 mM in ASW prior to being bath applied to the ganglia [9]. The experimental design was identical to the one used for the *in vitro* single-trial training, with 5-HT treatment replacing the training [9]. Ganglia were initially incubated in either L-NAME (final chamber concentration: 0.37 mM in ASW) or

ASW (vehicle control) for 30 min before pre-test measurements were taken (Fig. 6A). Membrane properties of TSNs were measured as described above (pre-test; Fig. 6A). Five min after the measurements were completed, preparations were randomly assigned to receive one of the following bolus treatments: 5-HT in ASW (final chamber concentration: 50 μ M), ASW, 5-HT in L-NAME (final chamber concentrations, 5-HT: 50 μ M, L-NAME: 0.37 mM) and L-NAME (final chamber concentration: 0.37 mM). Treatment lasted five min. At the end of this period, 5-HT was washed out of with 7.5 mL of either ASW or 0.37 mM L-NAME in ASW. Ten min after the end of the washout (i.e., 15 min after the beginning of treatment with 5-HT or ASW), membrane properties were measured again (post-test) to assess changes induced by treatment (Post-test measurements were conducted 15 min after treatment (Fig. 6A).

2.6. Data analysis

In vivo, the change in TSWR duration was calculated as [post-test TSWR duration]/[pre-test TSWR duration] [5,23]. Sensitization manifested as a change in TSWR duration > 1 [5,23]. The change in feeding behavior was analyzed as differences in bites, calculated as bites during post-test minus bites during pre-test [5,45]. Feeding suppression manifested as a difference in bites < 0 [5,46].

In vitro, for each membrane property of each neuron recorded, the percent change was calculated as [(post-pre/pre) $\times 100$] to assess modifications due to training and/or treatment [9,19]. A change in TSN number or spikes $> 100\%$ indicates increased TSN excitability, whereas a change in B51 burst threshold $> 100\%$ signifies decreased B51 excitability [9,19]. We were not able to collect every measurement from TSNs and B51 in each preparation. This discrepancy was caused primarily by: 1) the inability to find TSNs and B51 in every preparation, 2) the inability to maintain recordings until the end of the experiment.

When four groups were used, the statistical tests employed were selected based on the normality of distribution of a given set of data. Data that were normally distributed were analyzed with parametric statistics using a One-way ANOVA (F), followed by the Student-Newman-Keuls pairwise *post-hoc* comparisons (q) to isolate the sources of significance [47]. Data that were not normally distributed were analyzed with nonparametric statistics using either the Kruskal-Wallis test (H), followed by the nonparametric version of the Student-Newman-Keuls pairwise *post-hoc* comparisons (q), if the groups had equal sample sizes [25,42], or the Dunn's pairwise *post-hoc* comparisons (Q) if the groups had non-equal sample sizes [47]. When two groups were used, comparisons between conducted using the Mann-Whitney U test [9,45]. In all experiments, data were reported as mean $\pm$ SEM and were analyzed using the statistical package of SigmaPlot 11.0 (Jandel Scientific, San Rafael, CA). Statistical significance was set at $p < 0.05$.

3. Results

3.1. NO is necessary for short-term feeding suppression, but not for short-term sensitization

To investigate the necessity of NO signaling for short-term sensitization and feeding suppression, we used the single-trial training protocol (Fig. 1A1,1B1) [5]. Four groups of animals were compared: trained injected with vehicle (T-V), untrained injected with vehicle (UT-V), trained injected with L-NAME (T-L) and untrained injected with L-NAME (UT-L). Analysis of the TSWR revealed an overall statistical significance among the four groups ($H_3 = 11.99$; $P < 0.05$; Fig. 1A2). *Post-hoc* analysis with the Student-Newman-Keuls test revealed that the T-V group was significantly different from the UT-V group ($q = 4.02$; $p < 0.05$) and that the T-L group was significantly different from the UT-L group ($q = 5.57$; $p < 0.05$). However, the T-V group was not significantly different from the T-L group ($q = 1.24$; $p > 0.05$), thus indicating that L-NAME did not prevent the expression of short-term sensitization.

Analysis of feeding revealed an overall statistical significance among the four groups ($F_{3,48} = 4.98$; $p < 0.05$; Fig. 1B2). *Post-hoc* analysis with the Student-Newman-Keuls test revealed that the T-V group was significantly different from both the UT-V ($q = 5.00$; $p < 0.05$) and the T-L groups ($q = 4.26$; $p < 0.05$), and also that the T-L group was not significantly different from the UT-L group ($q = 0.96$; $p > 0.05$), thus indicating that L-NAME prevented the expression of short-term feeding suppression.

3.2. NO is necessary for both long-term sensitization and long-term feeding suppression

We next investigated the necessity of NO for long-term sensitization and feeding suppression, using the four-trial training protocol (Fig. 2A) [5]. Four groups of animals were compared: trained injected with vehicle (T-V), untrained injected with vehicle (UT-V), trained injected with L-NAME (T-L) and untrained injected with L-NAME (UT-L). Analysis of the TSWR revealed an overall statistical significance among the four groups ($H_3 = 12.89$; $P < 0.05$; Fig. 2B). *Post-hoc* analysis with the Dunn's test revealed that the T-V group was significantly different from both the UT-V ($Q = 2.83$; $p < 0.05$) and the T-L groups ($Q = 2.72$; $p < 0.05$), and also that the T-L group was not significantly different from the UT-L group ($Q = 0.03$; $p > 0.05$), thus indicating that L-NAME prevented the expression of long-term sensitization.

Analysis of feeding revealed an overall statistical significance among the four groups ($F_{3,54} = 5.81$; $p < 0.05$; Fig. 2C). *Post-hoc* analysis with the Student-Newman-Keuls test revealed that the T-V group was significantly different from both the UT-V ($q = 5.28$; $p < 0.05$) and the T-L groups ($q = 4.05$; $p < 0.05$), and also that the T-L group was not significantly different from the UT-L group ($q = 0.41$; $p > 0.05$), thus indicating that L-NAME prevented the expression of long-term feeding suppression.

3.3. NO release induces concurrent increased TSN excitability and decreased B51 excitability

The goal of this experiments was to investigate whether controlled release of NO via bath application of 100 μ M SNAP (Fig. 3A) could induce excitability changes in TSNs and B51 analogous to those caused by *in vitro* sensitization training. SNAP induced a significant increase of TSN excitability 15 min after treatment, when compared to preparations treated with DMSO ($p < 0.05$; $U = 17.00$; Fig. 3B1, 3B2). The SNAP-induced increase of TSN excitability was not accompanied by significant changes in Vm between SNAP- and DMSO-treated preparations ($p = 0.37$; $U = 36.00$). SNAP also induced a concurrent significant decrease of B51 excitability 15 min after treatment, when compared to preparations treated with DMSO ($p < 0.05$; $U = 0.00$; Fig. 3C1, 3C2). The SNAP-induced decrease of B51 excitability was not accompanied by significant changes in either Vm ($p = 0.72$; $U = 28.00$) or Rm ($p = 0.07$; $U = 7.00$) between SNAP- and DMSO-treated preparations.

3.4. NO contributes to training-induced short-term decreased B51 excitability, but only marginally to short-term increased TSN excitability

In this experiment, we used the *in vitro* analog [9] to investigate the contribution of NO to training-induced short-term increased TSN excitability and decreased B51 excitability. We used the single-trial *in vitro* training protocol (Fig. 4A) [9] and compared the following groups of preparations: trained incubated with vehicle (i.e., ASW, T-V), untrained incubated with vehicle (UT-V), trained incubated with L-NAME (T-L) and untrained incubated with L-NAME (UT-L). Analysis of TSN excitability revealed an overall statistical significance among the four groups ($H_3 = 12.68$; $p < 0.05$; Fig. 4B1-B4, 4C). *Post-hoc* analysis with the Student-Newman-Keuls test revealed that increased TSN excitability was observed in both T-V and T-L preparations (T-V vs. UT-V; $q = 4.36$, $p < 0.05$; T-L vs. UT-L; $q = 4.40$, $p < 0.05$). However, although increased TSN excitability was expressed in T-L preparations, the amount of excitability change was significantly smaller (about 30%) in T-L than in T-V preparations (T-L vs. T-V; $q = 3.70$; $p < 0.05$), thus revealing a small, but significant effect of L-NAME on short-term increased TSN excitability. Analysis of TSN Vm revealed no overall statistical significance among the four groups ($H_3 = 1.67$; $p = 0.64$).

L-NAME appeared to have a stronger effect on the short-term decreased B51 excitability. Analysis of B51 excitability revealed an overall statistical significance among the four groups ($H_3 = 14.11$; $p < 0.05$; Fig. 4D1-D4, 4E). *Post-hoc* analysis with the Student-Newman-Keuls test revealed that the T-V group was significantly different from both the UT-V ($q = 3.42$; $p < 0.05$) and the T-L groups ($q = 2.81$; $p < 0.05$), and also that the T-L group was not significantly different from the UT-L group ($q = 0.72$; $p > 0.05$), thus indicating that L-NAME prevented the short-term decreased B51 excitability. Analysis of B51 resting membrane properties revealed no overall statistical significance among the four groups for either Vm ($H_3 = 6.44$; $p = 0.09$) or Rm ($H_3 = 2.29$; $p = 0.51$).

3.5. NO is necessary for training-induced long-term decreased B51 excitability, but not for long-term increased TSN excitability

We next used the *in vitro* analog [9] to investigate the contribution of NO to training-induced long-term increased TSN excitability and decreased B51 excitability. We used the four-trial

in vitro training protocol (Fig. 5A) [9] and compared the following groups of preparations: trained incubated with vehicle (i.e., ASW, T-V), untrained incubated with vehicle (UT-V), trained incubated with L-NAME (T-L) and untrained incubated with L-NAME (UT-L). Analysis of TSN excitability revealed an overall statistical significance among the four groups ($H_3 = 10.66$; $p < 0.05$; Fig. 5B1-B4, 5C). *Post-hoc* analysis with the Student-Newman-Keuls test revealed that increased TSN excitability was observed in both T-V and T-L preparations (T-V vs. UT-V; $q = 4.66$; $p < 0.05$; T-L vs. UT-L; $q = 3.74$, $p < 0.05$). In addition, the increased TSN excitability was not significantly different between the T-V and T-L groups ($q = 2.09$; $p > 0.05$), thus indicating that L-NAME did not block the expression of long-term increased TSN excitability. Analysis of TSN Vm revealed no overall statistical significance among the four groups ($H_3 = 5.1$; $p = 0.17$).

Analysis of B51 excitability revealed an overall statistical significance among the four groups ($H_3 = 12.88$; $p < 0.05$; Fig. 5D1-D4, 5E). *Post-hoc* analysis with the Student-Newman-Keuls test revealed that the T-V group was significantly different from both the UT-V ($q = 4.88$; $p < 0.05$) and the T-L groups ($q = 5.02$; $p < 0.05$), indicating that L-NAME blocked the expression of long-term decreased B51 excitability. Interestingly, B51 excitability was significantly increased, instead of decreased in the T-L group when compared to the UT-L group ($q = 4.12$; $p < 0.05$), indicating that L-NAME not only prevented the expected long-term decreased excitability, but converted it into a longterm increased excitability. Analysis of B51 resting membrane properties revealed no overall statistical significance among the four groups for either Vm ($H_3 = 4.76$; $p = 0.19$) or Rm ($H_3 = 4.95$; $p = 0.18$).

3.6. NO is not necessary for the 5-HT induced increased TSN excitability

The goal of this experiment was to determine whether NO might contribute to the 5-HT induced TSN increased excitability. We used *in vitro* application of 50 μ M 5-HT, which induces increased TSN excitability 15 min after treatment (Fig. 6A) [9,48]. Four groups of preparations were used: incubated with ASW and treated with 5-HT in (5-HT), incubated with ASW and treated with ASW (ASW), incubated with L-NAME and treated with 5-HT (5-HT in L-NAME), and incubated in L-NAME and treated with L-NAME (L-NAME). Analysis of TSN excitability revealed an overall statistical significance among the four groups ($H_3 = 16.90$; $p < 0.05$; Fig. 6B1-B4, 6C). *Post-hoc* analysis with the Student-Newman-Keuls test revealed that increased TSN excitability was observed in preparations treated with either 5-HT (5-HT vs. ASW; $q = 5.02$; $p < 0.05$) or 5-HT with L-NAME (5-HT in L-NAME vs. L-NAME; $q = 5.74$; $p < 0.05$). However, the increased TSN excitability was not significantly different between the 5-HT and 5-HT in L-NAME groups ($q = 1.81$; $p > 0.05$), thus indicating that L-NAME did not block the expression of the 5-HT induced increased TSN excitability. Analysis of TSN Vm revealed no overall statistical significance among the four groups ($H_3 = 5.52$; $p = 0.14$).

4. Discussion

This study provides compelling evidence that NO signaling contributes to the behavioral and cellular modifications induced by sensitizing stimuli in *Aplysia*. The main focus of this

study was to characterize the modulatory signal(s) responsible for feeding suppression and decreased B51 excitability. We identified NO as a key modulator of both feeding suppression and decreased B51 excitability. Interestingly, we also discovered that NO is necessary for long-term sensitization, revealing a novel role of NO in this form of nonassociative learning.

4.1. NO contributes to both short-term and long-term feeding suppression

Our results show that NO signaling plays a substantial role in both short-term and long-term feeding suppression (Figs. 1B2, 2C), suggesting that multiple modulatory signals (i.e., 5-HT and NO) are concurrently triggered by the exposure to sensitizing stimuli. These findings indicate that NO acts as a modulator necessary for the induction of feeding suppression in different temporal domains, regardless of the training protocol. A similar role of NO signaling in learning-induced plasticity of feeding behavior has been previously reported in *Aplysia*. L-NAME blocks both 30-min and 24-h memory of an associative paradigm in which *Aplysia* learns that food is inedible, indicating that NO contributes to both short-term and long-term forms of this memory [22,29]. Combined with these results, our findings indicate that, in *Aplysia*, a common NO-dependent induction mechanism unifies nonassociative and associative learning paradigms that downregulate feeding behavior. In contrast, a different induction signaling cascade mediated by dopamine underlies forms of appetitive associative learning (i.e., classical and operant conditioning) in which feeding behavior is upregulated [49].

4.2. NO contributes to long-term sensitization, but not to short-term sensitization

The focus of this study was to characterize the role of NO as modulatory signal mediating the training-induced feeding suppression and the underlying decreased B51 excitability. However, in order to provide a comprehensive view of the effects of NO beyond the feeding neural circuit, we also analyzed a possible contribution of NO to sensitization. Our findings revealed that NO contributes to sensitization, although the contribution is restricted to long-term sensitization (Figs. 1A2, 2B). The persistence of short-term sensitization in the presence of L-NAME supports the long-standing view of 5-HT as the neurotransmitter sustaining sensitization in *Aplysia* [2,50]. Nevertheless, the lack of long-term memory in trained animals treated with L-NAME suggests that NO also contributes to the expression of long-term sensitization. A role for NO signaling in sensitization is, indeed, not completely novel in *Aplysia*: NO is known to mediate a form of nociceptive long-term sensitization caused by peripheral injury [15,20].

An unresolved question is in which way 5-HT and NO interact during training to induce the formation of long-term sensitization. In *Aplysia*, classical conditioning of the siphon withdrawal reflex, which depends on 5-HT release during tail shocks [51], also requires NO to induce the pairing-specific changes in the siphon neural circuit [16]. In this preparation, the unpaired training, which consists of sensitizing tail shocks spaced from siphon taps, is affected by blocking the NOS [16], with an even more pronounced effect when the unpaired group is treated with the NO scavenger oxymyoglobin [16]. Consequently, it is possible that NO might work synergistically with 5-HT to amplify the activity of the protein-kinase A (PKA) via cAMP activation [16,52], which is critical for long-term memory formation [2,8].

Modulation of PKA by NO has been previously demonstrated in both the honeybee *Apis mellifera* [53] and the cricket *Gryllus bimaculatus* [54], where the activation of PKA, required for long-term olfactory memory formation, is triggered by a NO/cGMP pathway.

It has also been reported that NO can modulate the activity of various monoaminergic neurotransmitters, including acetylcholine and 5-HT, in vertebrates and invertebrates [55]. For example, inhibition of the NO pathway interferes with normal 5-HT modulatory function in behaviors such as aggression in mice [56] and scototaxis [57] and reaction to novelty [58] in zebrafish. In *Aplysia*, NO modulates neurotransmission at cholinergic synapses in the buccal ganglion [59]. In the form of nociceptive sensitization caused by peripheral injury, 5-HT and NO also seem to work synergistically [43], but the nature of their interaction remains unclear.

Another possible role for NO in long-term sensitization is that it might function as the retrograde messenger that has been postulated to contribute to long-term synaptic facilitation in *Aplysia* [60,61]. In both vertebrates and invertebrates, retrograde messengers, including NO, have been proposed to link presynaptic changes with the postsynaptic modification necessary to sustain forms of long-lasting synaptic plasticity [60–62]. In this model, by blocking NOS, L-NAME may disrupt the link between presynaptic and postsynaptic changes, block long-term facilitation and, subsequently, may prevent the expression of long-term sensitization. This hypothesis may also explain the contribution of NO selectively to long-term sensitization and not to short-term sensitization, which is known to depend primarily on presynaptic mechanisms rather than a synergistic contribution of presynaptic and postsynaptic mechanisms [60,61].

4.3. NO mimics sensitization training in inducing increased TSN excitability and decreased B51 excitability

Earlier work provided support for the modulatory role of NO in the *Aplysia* nervous system by localizing NOS immunoreactivity in numerous neurons distributed throughout the buccal, cerebral and pleural-pedal ganglia [59,63]. In addition, previous evidence indicates that both TSNs and B51 contain NO-signaling pathways capable of altering these neurons' excitability in manners similar to what is observed following sensitization training [15,19]. Our results indicate that replacing *in vitro* training with bath application of the NO donor SNAP [17,19] induced concurrent short-term increased TSN excitability (Fig. 3B2) and decreased B51 excitability (Fig. 3C2), thus revealing the presence of a NO pathway that can be potentially recruited by sensitizing stimuli.

4.4. NO contributes to both short-term and long-term decreased B51 excitability

The experiments conducted in the *in vitro* analog in the presence of L-NAME confirmed the above hypothesis that NO-dependent signaling, recruited by sensitizing stimuli, alters B51 excitability (Figs. 4E, 5E). These findings parallel the effects of L-NAME on feeding suppression and indicate that NO contributes to the induction of training-induced feeding suppression and the underlying decreased B51 excitability. It is likely that NO activates a downstream cGMP-dependent second-messenger cascade leading to an increase of B51 excitability. Patterns of cytosolic cGMP injection have been previously shown to induce

short-term and long-term decrease of B51 excitability, depending on the injection protocol [19]. Conversely, another cyclic nucleotide, cAMP, increases B51 excitability in operant conditioning [42,64]. These results suggest a scenario in which the excitability of decision-making neuron B51 can be bidirectionally regulated by different learning paradigms altering feeding behavior in opposite direction: operant conditioning via a dopamine/neuronal activity – cAMP pathway [49,64], sensitization training via a NO-cGMP pathway.

L-NAME not only prevented training-induced decrease in B51 excitability, but it caused a significant increase of excitability in the T-L group, as compared to the UT-L group (Fig. 5E). B51 excitability has been shown to depend on the levels of NO and small guanylyl cyclase (sGC) activity, with NO and sGC exerting a tonic downregulation of the neuron's basal excitability [19]. Therefore, it is possible that the combination of prolonged incubation with L-NAME and P8/9 nerve stimulation may cause a reversal of B51 excitability, resulting in the significant increased excitability measured 24 h after training.

4.5. NO marginally contributes to short-term increased TSN excitability, but plays no role in long-term increased TSN excitability.

The experiments conducted in the *in vitro* analog in the presence of L-NAME revealed that NO contributes only partially to short-term increased excitability of TSNs (Fig. 4C), but not at all to long-term increased excitability (Fig. 5C). In the short-term, the increased TSN excitability was still observed in trained preparations treated with L-NAME, with no effect of L-NAME on TSN resting properties. However, the amount of excitability increase observed in trained preparations treated with L-NAME was significantly less (about 30%) than the one measured in trained preparations treated with vehicle (Fig. 4C).

This result indicates that there is a NO-dependent component contributing, at least in part, to the increased TSN excitability. 5-HT is known to mediate the increased TSN excitability and can substitute for nerve stimulation in inducing this form of plasticity [2,9,44]. Therefore, our findings reveal a possible interaction between NO and 5-HT in the modulation of TSN excitability by *in vitro* training. The persistence of the 5-HT-induced short-term increased TSN excitability in the presence of L-NAME (Fig. 6C) rules out a downstream role of NO in 5-HT modulation of TSN excitability and suggests for an upstream regulatory role of 5-HT function instead, as has been previously demonstrated in vertebrates [55,56].

Our results show that L-NAME partially blocks TSN increased excitability *in vitro* without preventing the occurrence of short-term sensitization. How can this apparent discrepancy be resolved? Together with the increased TSN excitability, used in this study as *in vitro* readout of learning-induced plasticity, additional cellular correlates, such as synaptic facilitation of the sensory-motor synapse [2] and changes at the level of interneurons [65] sustain sensitization of withdrawal reflexes in *Aplysia*. It is, therefore, possible that the reduced increased TSN excitability observed in L-NAME may have minimal impact on the sensitized TSWR.

Unexpectedly, L-NAME did not block the long-term increase of TSN excitability induced by the four-trial *in vitro* training. Although there was a trend for increased TSN excitability in the T-L group to be lower than the T-V group, it was not statistically significant (Fig. 5C).

Long-term synaptic facilitation of sensorimotor synapses and long term increased TSN excitability are co-expressed in the form of long-term sensitization induced by the training used in this study [24]. The increased TSN excitability induced by SNAP application drove our original decision to employ this form of plasticity as *in vitro* correlate of sensitization. However, the persistence of long-term increased TSN excitability in experimental conditions in which NOS inhibition prevents the expression of long-term sensitization suggests that mechanisms sustaining long-term sensitization, other than long-term increase of TSN excitability, may depend on NO activity. It is plausible that long-term synaptic facilitation [8,24,37,66] might be NO dependent and its blockade by L-NAME may cause the observed lack of long-term sensitization. Finally, it must be acknowledged that the ganglionic configuration of the *in vitro* analog used in this study may not express some of the correlates sustaining long-term sensitization. For example, in long-term sensitization in *Aplysia*, 5-HT functions not only as a neurotransmitter, but also as a neurohormone, which is released in a protein-synthesis dependent manner into the hemolymph following sensitizing stimuli and remains elevated in hemolymph for up to 24 h [31]. This systemic, humoral role of 5-HT is not captured *in vitro* in the ganglionic preparation. In this case, NO may contribute to up-regulate serotonergic synapses to generate the persistent release of 5-HT triggered by long-term sensitization training.

5. Conclusions

This study provides evidence that, in *Aplysia*, the same aversive stimulus leads to the activation of multiple modulatory signals, which cause distinct behavioral and cellular modifications. Behaviorally, we have identified NO as a mediator of training-induced suppression of feeding and B51 decreased excitability. In addition, our findings revealed an unexpected role of the NO pathway in long-term sensitization, suggesting that both 5-HT and NO are necessary for the formation of long-term memory for sensitization. These results indicate that although NO function is compartmentalized within the feeding neuronal circuit [6,9], both NO and 5-HT contribute to the induction of long-term sensitization, suggesting a possible interaction between the two pathways.

At the cellular level, NO appears to be both necessary and sufficient to induce the training-induced decreased excitability of B51 that is correlated with feeding suppression. However, although NO was also found necessary, at least in part, for training-induced short-term increased TSN excitability, it was not necessary for the induction of long-term increased excitability. Because sensitization in *Aplysia* is sustained primarily by concurrent synaptic facilitation of the sensorimotor synapse and increased excitability of the sensory neurons [2,67], the effects of L-NAME on short-term and long-term TSN excitability may guide the design of future experiments to dissect the individual contributions of synaptic and intrinsic plasticity to the storage of short-term and long-term memory.

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Highlights

- In *Aplysia*, aversive stimuli are known to induce feeding suppression and sensitization
- We found that NO is necessary for feeding suppression and underlying neuronal excitability changes
- NO also contributes, at least in part, to 5-HT dependent sensitization and underlying neuronal excitability changes
- Findings suggest multiple modulators (5-HT and NO) contribute to the widespread changes induced by aversive stimuli

Fig 1.

NO does not contribute to the expression of sensitization, but it is necessary for the expression of feeding suppression following single-trial *in vivo* training. The TSWR (A1) and feeding (B1) were measured prior to, and 15 min after training. Single-trial training is indicated by a lightning bolt. The NOS inhibitor L-NAME was injected 1 h prior to the beginning of training (A1, B1). Short-term sensitization was observed in both T-L animals and T-V animals (A2). Conversely, short-term feeding suppression was prevented in T-L animals, but it was observed in T-V animals (B2). In this and in the following figures, values are expressed as mean $\pm$ SEM. Statistical significance was set at $p < 0.05$. In this and in the following figures, shared capitalized letters denote statistical similarity among groups.

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Fig 2.

NO is necessary for the expression of both long-term sensitization and feeding suppression following four-trial *in vivo* training. The TSWR and feeding were measured prior to and 24 h after training (A). Four-trial training is indicated by four lightning bolts. L-NAME was injected 1 h prior to the beginning of training (A). Long-term sensitization was prevented in T-L animals, but it was observed in T-V animals (B). Similarly, long-term feeding suppression was prevented in T-L animals, but it was observed in T-V animals (C).

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Fig 3.

NO release via SNAP increases TSN excitability and decreases B51 excitability. (A) Protocol of TSN and B51 measurements prior to and 15 min after treatments with either SNAP or DMSO. (B1) Sample traces of TSN firing from SNAP-treated and DMSO-treated preparations. (B2) Summary data illustrate that SNAP significantly increased TSN excitability 15 min after treatment. (C1) Sample traces of B51 burst threshold from SNAP-treated and DMSO-treated preparations. (C2) Summary data illustrate that SNAP

significantly decreased B51 excitability 15 min after treatment. In this figure, statistical significance is indicated by an asterisk.

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Fig 4.

NO contributes to training-induced short-term decreased B51 excitability, but only marginally to short-term increased TSN excitability. (A) Protocol of incubation with L-NAME/ASW and subsequent measurements of TSN and B51 properties prior to and 15 min after the single-trial *in vitro* training (lightning bolt). (B) Sample traces of TSN firing from T-V (B1), UT-V (B2), T-L (B3) and UT-L (B4) preparations. (C) Summary data illustrate that L-NAME partially blocked the short-term increased TSN excitability. (D) Sample traces of B51 burst threshold from T-V (D1), UT-V (D2), T-L (D3) and UT-L (D4) preparations.

(E) Summary data illustrate that L-NAME blocked the short-term decreased TSN excitability.

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Fig 5.

NO is necessary for training-induced long-term decreased B51 excitability, but not for long-term increased TSN excitability. (A) Protocol of incubation with L-NAME/ASW and subsequent measurements of TSN and B51 properties prior to and 24 h after the four-trial *in vitro* training (lightning bolts). (B) Sample traces of TSN firing from T-V (B1), UT-V (B2), T-L (B3) and UT-L (B4) preparations. (C) Summary data illustrate that L-NAME did not block the long-term increased TSN excitability. (D) Sample traces of B51 burst threshold

from T-V (D1), UT-V (D2), T-L (D3) and UT-L (D4) preparations. (E) Summary data illustrate that L-NAME blocked the long-term decreased TSN excitability.
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Fig 6.

NO is not necessary for the 5-HT induced increased TSN excitability. (A) Protocol of incubation with L-NAME/ASW and subsequent measurements of TSN and B51 properties prior to and 15 min after bolus treatment. (B) Sample traces of TSN firing from 5-HT (B1), ASW (B2), 5-HT in L-NAME (B3) and L-NAME (B4) preparations. (C) Summary data illustrate that L-NAME did not block the 5-HT induced increased TSN excitability. Farruggella et al., 2018