



Published in final edited form as:

*Neurosci Lett.* 2018 September 14; 683: 111–118. doi:10.1016/j.neulet.2018.06.046.

## cGMP mediates short- and long-term modulation of excitability in a decision-making neuron in *Aplysia*

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### Abstract

In elementary neural circuits, changes in excitability can have a strong impact in the expression of a given behavior. One example is provided by B51, a neuron with decision-making properties in the feeding neural circuit of the mollusk *Aplysia*. The excitability of B51 is bidirectionally modulated by external and internal stimuli in a manner that is consistent with the corresponding induced changes in feeding behavior. For example, in operant reward learning, which up-regulates feeding, B51 excitability is increased *via* a cAMP-dependent mechanism. Conversely, following training protocols with aversive stimuli, which down-regulate feeding, B51 excitability is decreased. In this study, we tested the hypothesis that B51 decreased excitability may be mediated by another cyclic nucleotide, cGMP. Our results revealed that iontophoretic injection of cGMP was capable of inducing both short-term (45 min) and long-term (24 h) reduction of B51 excitability. We next investigated which biochemical trigger could increase cGMP cytosolic levels. The neurotransmitter nitric oxide was found to decrease B51 excitability through the activation of the soluble guanylyl cyclase. These findings indicate that a cGMP-dependent pathway modulates B51 excitability in a manner opposite of cAMP, indicating that distinct cyclic-nucleotide pathways bidirectionally regulate the excitability of a decision-making neuron.

### Keywords

*Aplysia*; Excitability; Neuronal plasticity; Second messengers

## 1. Introduction

In recent years, the role of plasticity of neuronal excitability has become increasingly apparent in diverse physiological and pathological areas, including: brain-metabolism axis [1], fragile X syndrome [2], depressive [3,4], and other neurological disorders [5], addiction

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Author contribution

A.G., J.F., M.L.W. and R.M. conceived the study and the developed the experimental design. A.G., J.F., 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.

Conflict of Interest

The authors have no actual or potential conflicts of interest.

[6], and learning and memory [7,8]. In circumstances of elementary computation within simple neural circuits, changes in excitability may be highly effective in influencing the expression of a given behavior [7–9]. One example is provided by neurons with decision-making features in which modifications in excitability are correlated with changes in behavior, such as neuron B51 in the mollusk *Aplysia californica* [10,11].

In *Aplysia*, the goal-oriented behavior of feeding is controlled by a well-characterized neural circuit, which generates the rhythmic output activity underlying the cycles of protraction, closure and retraction of the radula that constitute bites [12,13]. Within the feeding neural circuit, B51 is a neuron with *in vitro* decision-making features that contributes to the elicitation of ingestive buccal motor programs (BMPs), which are *in vitro* neurophysiological representations of bites/swallows [13–16]. B51 exhibits intrinsically-generated all-or-nothing bursts of action potentials, known as plateau potentials [14,17], which participate in the selection of ingestive BMPs [10,15]. B51 is capable of switching in an all-or-nothing fashion from an inactive sub-threshold state to an active plateau potential during the occurrence of ingestive BMPs [11,15,18]. Notably, artificial suprathreshold activation of B51 biases the feeding neural circuit to generate ingestive BMPs, whereas hyperpolarization of B51 prevents their occurrence [15].

Various external and internal stimuli modulate the expression of feeding [11] and, subsequently, induce modifications of B51 excitability that are consistent with the observed changes in feeding [11]. For example, an increase in B51 excitability has been associated with the increased number of ingestive BMPs *in vitro* and bites *in vivo* produced by operant reward learning [15,17,19]. Conversely, a decrease of B51 excitability has been observed following nonassociative training with aversive stimuli, which induces sensitization of defensive reflexes and reduces feeding [20]. A decrease of B51 excitability has been reported also following a food-satiation paradigm, which suppresses feeding for several days [18]. These findings highlight an interesting example of bidirectional modulation of excitability in a decision-making neuron, which can influence the outcome of a behaviorally-relevant neural circuit in opposite directions.

Previous work has identified cAMP as the second messenger underlying the increase of B51 excitability induced by operant reward learning [19,21]. In particular, iontophoretic injections of cAMP, mimicking short-term and long-term conditioning protocols, induced increases of B51 excitability lasting a few minutes and 24 h, respectively [19,21]. The goal of this study was to determine which second messenger would induce a decrease of B51 excitability. We focused on another cyclic nucleotide, cGMP, which is known to modulate neuronal excitability in numerous systems, including *Aplysia* [22,23]. In addition, cGMP signaling has been implicated in a form of learning that results in decreased feeding behavior in *Aplysia* [24,25], thus positing it as an interesting candidate to modulate B51 activity in a way that is consistent with feeding suppression.

## 2. Materials and methods

### 2.1. Animals

Adult specimen of *Aplysia californica* were obtained from South Coast Bio-Marine (San Pedro, CA), and were individually housed in two aquaria (Aquatic Enterprises Inc., WA) of continuously circulating 15 °C aquarium seawater (Instant Ocean) on a 12-h light/dark cycle. Animals were fed one strip of dried seaweed (3 × 19 cm; 0.5 g; Emerald Cove<sup>®</sup> Organic Pacific Nori; Great Eastern Sun, Asheville, NC) three times a week [26]. Animals were food deprived for 2–3 days before the beginning of an experiment [27]. Prior to each experiment, the general health and motivational state of an animal were tested by presenting it with a food stimulus. An animal was included in the study only if it generated bites in response to the food stimulus [27].

### 2.2. Dissection and ganglion preparation

*Aplysia* were anesthetized by injecting a volume of isotonic MgCl<sub>2</sub> (equivalent to 50% of the weight of the animal) through the foot [27,28]. The buccal ganglion was removed and pinned rostral-side up on the Sylgard-coated bottom of a Plexiglas recording chamber containing artificial seawater with a high concentration of divalent cations (high-divalent ASW), composed of 210 mM NaCl, 10 mM KCl, 145 mM MgCl<sub>2</sub>, 20 mM MgSO<sub>4</sub>, 33 mM CaCl<sub>2</sub>, and 10 mM HEPES, with the pH adjusted to 7.5 using NaOH [28]. Ganglia were subsequently desheathed to access the soma of neuron B51 [15,20].

After desheathing, the high-divalent ASW was exchanged for normal ASW (composed of 450 mM NaCl, 10 mM KCl, 30 mM MgCl<sub>2</sub>, 20 mM MgSO<sub>4</sub>, 10 mM CaCl<sub>2</sub>, and 10 mM HEPES, with pH adjusted to 7.5 using NaOH; Mozzachiodi et al. [28]) and the preparation was 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. B51 identification and measurement of membrane properties

The soma of neuron B51 was identified based on its relative size and position (Fig. 1A1) and the occurrence of its characteristic intrinsically-generated plateau potentials [14,15,17]. Although standard two-electrode current-clamp technique was used for intracellular recording and stimulation in all experiments [15,19], different electrode configurations were employed. In the first two experiments, in which iontophoretic injection of cGMP was used, B51 was initially impaled with one electrode containing 3-M potassium acetate and one iontophoresis electrode loaded with either cGMP or vehicle (Figs. 1A1, Fig. 2A1) [19,21]. In all the remaining experiments, B51 was impaled with two electrodes containing 3-M potassium acetate [15].

Five min after impalement, a pre-test was conducted to measure resting membrane potential (V<sub>m</sub>), input resistance (R<sub>m</sub>) and excitability. Cells were included in the study only if they displayed a resting membrane potential of at least –45 mV [20]. After recording the V<sub>m</sub>, B51 was clamped at –60 mV. In experiments using an iontophoresis electrode, a subthreshold impulse of depolarizing current (5-s duration, 2-nA intensity) was used to

measure  $R_m$ , because injection of negative current would lead to cGMP iontophoresis into B51 [23]. In all other experiments,  $R_m$  was measured using a 5-s, 5-nA hyperpolarizing current impulse [15].

Excitability was assessed by measuring the threshold to elicit a plateau potential, defined as the minimum amount of depolarizing current necessary to elicit a burst of activity that outlasted the duration of a 5-s pulse of depolarizing current [15]. The burst threshold was measured by injecting 5-s depolarizing current pulses of incremental intensity at 10-s intervals, beginning at 5 nA, until the cell fired a plateau potential [15]. In cases in which B51 elicited a plateau potential at 5 nA, the neuron's response to 2 nA, 3 nA and 4 nA was also assessed to ensure that the burst threshold was not overestimated. Cells that did not exhibit a plateau potential by 25 nA were excluded from the study [27]. Post-tests were conducted identically to the pre-tests at different time points after injection/treatment protocols [19,27]. An increase of burst threshold would correspond to a decrease of excitability, whereas a decrease of burst threshold would correspond to an increase of excitability.

#### 2.4. Protocols for cGMP injection experiments

The first two experiments involved the iontophoretic injection of cGMP into the cytosol of B51. The iontophoresis electrode was loaded with either cGMP (30 mM in 1% NaHCO<sub>3</sub>) or vehicle (1% NaHCO<sub>3</sub>) [23]. We developed two iontophoretic injection protocols, single injection and four injections, to mimic *in-vitro* learning paradigms that use electrical nerve stimulations to induce short-term (15 min) and long-term (24 h) decrease of B51 excitability, respectively [27]. In both protocols, each injection consisted of 30 s of 20-nA hyperpolarizing current delivered through the iontophoresis electrode. Similar protocols, which previously delivered iontophoretic injection of cAMP, successfully increased B51 excitability [19,21]. Preparations were randomly assigned to receive either cGMP or vehicle injection.

In the single-injection experiment, one potassium acetate electrode and one iontophoresis electrode were used for the entire duration of the experiment (Fig. 1A1, A2). During the pre-test,  $V_m$ ,  $R_m$  and excitability were measured. Ten min after the end of the pre-test a single injection of either cGMP or vehicle was delivered. In a pilot experiment, a post-test was performed 5 min after the injection. In the subsequent experiment, post-tests were conducted 15 min, 30 min, 45 min, 1 h, after injection (Fig. 1A3).

In the four-injection experiment, one potassium acetate electrode and one iontophoresis electrode were used for the pre-test and treatment with cGMP/vehicle (Fig. 2A1). A pre-test was conducted as illustrated above for the single injection (Fig. 2A1). Ten min after the end of the pre-test, four consecutive injections of either cGMP or vehicle were delivered, spaced 30 min apart (Fig. 2A3). Ten min after the end of the injection protocol, electrodes were gently removed from B51 and ASW was exchanged with a modified L-15 culture medium. This medium consisted of L-15 medium (Sigma) supplemented with (in mM): NaCl 293.1, KC1 4.6, MgCl<sub>2</sub> 26, MgSO<sub>4</sub> 18.9, CaCl<sub>2</sub> 10.9, HEPES 30 and 0.10 g/l of streptomycin (Sigma) and 0.12 g/l of penicillin-G (Sigma), with the pH adjusted to 7.5 with NaOH [19,27]. The recording chamber was then covered and stored in an incubator at 15 °C for

23.5 h. On the next day, ganglia were rinsed in normal ASW and allowed to rest for 30 min [19,27]. Post-test measurements of membrane properties were conducted 24 h after the end of treatment using two potassium acetate electrodes (Fig. 2A2, A3).

## 2.5. Protocols for pharmacological inhibition/activation of the nitric oxide – cGMP signaling pathway

In the remaining experiments, we employed a series of chemicals to manipulate the nitric oxide (NO) – cGMP signaling pathway. We used 1H-(1,2,4) oxidazole (4,3-a) quinoxalin-1-one (ODQ) to inhibit the soluble guanylyl cyclase (sGC) [29]. ODQ was delivered *via* bolus application (final chamber concentration: 25  $\mu$ M in 0.1% DMSO [30]. Control preparations were treated with bolus application of DMSO (final chamber concentration: 0.1%). B51 membrane properties were measured prior to (pre-test) and 15 min after ODQ/DMSO application (post-test, Fig. 3A).

We used the NO donor *S*-nitroso-*N*-acetyl-penicillamine (SNAP) to trigger release of NO in the buccal ganglia [31]. SNAP was delivered *via* a bolus application (final chamber concentration: 100  $\mu$ M in 0.1% DMSO) [25]. Control preparations were treated with bolus application of DMSO (final chamber concentration: 0.1%). B51 membrane properties were measured prior to (pre-test) and after SNAP/DMSO application (post-test, Fig. 4A). To ensure that we captured the effects of released NO, post-tests were conducted 3 min after SNAP application.

We used *N*<sup>G</sup>-Nitro-L-arginine-methyl ester hydrochloride (L-NAME) to inhibit the NO synthase [29]. L-NAME was delivered *via* a bolus application (final chamber concentration: 0.37 mM in ASW) [31]. Control preparations were treated with bolus application of ASW. B51 membrane properties were measured prior to (pre-test) and 3 min after L-NAME/ASW application (post-test, Fig. 4A).

In the final experiment, ganglia were initially incubated in either ODQ (final chamber concentration: 25  $\mu$ M in 0.1% DMSO) or DMSO (final chamber concentration: 0.1% DMSO) for 30 min before pre-test measurements were taken (Fig. 5A). SNAP was then delivered *via* bolus application and post-test measurements were taken 3 min after treatment (Fig. 5A).

## 2.6. Data analysis

For each membrane property of each neuron recorded, the percent change was calculated as  $[(\text{post-pre/pre}) \times 100]$  to assess modifications due to treatments [15,19,28]. We were not able to collect every measurement from B51 at each of the time points, due to the inability to maintain recordings until the end of the experiment.

For the experiment in which the effects of the single cGMP/vehicle injection on B51 membrane properties were analyzed at multiple time points (Fig. 1A3), data were normally distributed with equal variance. Therefore, we used a repeated-measure one-way ANOVA to compare changes in each membrane property between cGMP and vehicle groups at different time points [32]. The Student-Newman-Keuls pairwise *post-hoc* comparisons (*q*) was then used to isolate the sources of significance. In all the remaining experiments, data were not

normally distributed and, therefore, the comparisons between treated and control groups were conducted using the Mann-Whitney U test [33]. 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. Single cGMP injection induces short-term decrease of B51 excitability

We initially investigated whether cGMP injection altered B51 membrane properties. To ensure that we captured cGMP-dependent cellular changes, we first measured B51 membrane properties 5 min after a single injection of cGMP/vehicle. B51 excitability was significantly decreased in cGMP-injected cells (change in burst threshold:  $24.57 \pm 11.54\%$  of pre-test;  $n = 8$ ), compared to the vehicle-injected cells (change in burst threshold:  $7.65 \pm 3.05\%$  of pre-test;  $n = 10$ ;  $p < 0.05$ ;  $U = 16.00$ ). The cGMP-dependent decrease of B51 excitability was not accompanied by significant changes in either  $V_m$  (change in  $V_m$ , cGMP:  $7.22 \pm 1.84\%$  of pre-test;  $n = 8$ ; vehicle:  $-4.70 \pm 1.17\%$  of pre-test;  $n = 10$ ;  $p = 0.31$ ;  $U = 28.00$ ), or  $R_m$  (change in  $R_m$ , cGMP:  $-4.10 \pm 8.22\%$  of pre-test;  $n = 7$ ; vehicle:  $4.65 \pm 3.78\%$  of pre-test;  $n = 9$ ;  $p = 0.17$ ;  $U = 18.00$ ).

In the next experiment, we analyzed the time course of the decreased excitability induced by a single injection of cGMP (Fig. 1A3). Analysis of B51 excitability revealed an overall statistical significance among the different time points ( $F_{7,103} = 5.75$ ;  $p < 0.05$ ). *Post-hoc* analysis highlights that B51 excitability was significantly decreased in cGMP-injected cells (Fig. 1B1, C), compared to the vehicle-injected cells (Fig. 1B2, C) at the following time points: 15 min ( $p < 0.05$ ;  $q = 4.30$ ), 30 min ( $p < 0.05$ ;  $q = 4.20$ ) and 45-min ( $p < 0.05$ ;  $q = 4.82$ ). B51 excitability was no longer significantly different between cGMP-injected cells and vehicle-injected cells at 1 h after the injection ( $p = 0.05$ ;  $q = 1.89$ ). In contrast to excitability, neither  $V_m$  ( $F_{7,102} = 0.71$ ;  $p = 0.67$ ) nor  $R_m$  ( $F_{7,85} = 0.90$ ;  $p = 0.51$ ) were significantly altered by cGMP injection at any of the time points examined.

#### 3.2. Four cGMP injections induce long-term decrease of B51 excitability

We next analyzed the long-term (24 h) effects of cGMP injection on B51 membrane properties. Four cGMP injections induced a significant decrease of B51 excitability 24 h after the treatment (Fig. 2B1, C), compared to vehicle-injected cells ( $p < 0.05$ ;  $U = 16.00$ ; Fig. 2B2, C). Conversely, significant differences were not observed between cGMP-injected and vehicle-injected cells for either  $V_m$  ( $p = 0.15$ ;  $U = 27.00$ ) or  $R_m$  ( $p = 0.82$ ;  $U = 37.00$ ) 24 h after treatment.

#### 3.3. Inhibition of sGC activity increases B51 excitability

To confirm the role of cGMP signaling in the modulation of B51 excitability, we investigated the effects of blocking the sGC enzyme with ODQ [29,30]. Application of ODQ induced a significant increase of B51 excitability 15 min after treatment (Fig. 3B1, 3C), compared to application of DMSO ( $p < 0.05$ ;  $U = 6.00$ ; Fig. 3B2, C). ODQ did not alter either  $V_m$  ( $p = 0.51$ ;  $U = 32.00$ ) or  $R_m$  ( $p = 0.08$ ;  $U = 14.00$ ) 15 min after application.

### 3.4. B51 excitability is modulated by NO signaling

In the next series of experiments, we began to characterize which neurotransmitter could lead to a cytosolic increase of cGMP, and to the subsequent decrease of B51 excitability. Because in numerous model systems this role is played by NO [34], we analyzed its contribution in modulating B51 excitability by: 1) artificially increasing NO levels in the ganglia with SNAP (Fig. 4A) [31] and 2) inhibiting the activity of the NO synthase with L-NAME (Fig. 4A) [29]. Application of SNAP significantly decreased B51 excitability 3 min after treatment (Fig. 4B1, C), compared to application of DMSO ( $p < 0.05$ ;  $U = 6.00$ ; Fig. 4B2, C), without altering either  $V_m$  ( $p = 0.27$ ;  $U = 10.00$ ) or  $R_m$  ( $p = 1.00$ ;  $U = 17.00$ ). Conversely, application of L-NAME significantly increased B51 excitability 3 min after treatment (Fig. 4D1, E), compared to application of ASW ( $p < 0.05$ ;  $U = 13.00$ ; Fig. 4D2, E), without altering either  $V_m$  ( $p = 0.86$ ;  $U = 52.00$ ) or  $R_m$  ( $p = 0.31$ ;  $U = 40.00$ ).

### 3.5. The NO-dependent decrease of B51 excitability is mediated by the sGC

In the final experiment, we investigated whether the NO-induced decrease of B51 excitability occurred through the activation of the sGC. In preparations incubated with ODQ, SNAP application failed to decrease B51 excitability (Fig. 5B1, C), as occurred in preparations in which SNAP was applied following incubation with DMSO (Fig. 5B2, C;  $p < 0.05$ ;  $U = 10.00$ ). No significant changes were found in either  $V_m$  ( $p = 0.40$ ;  $U = 20.00$ ) or  $R_m$  ( $p = 0.69$ ;  $U = 24.00$ ).

## 4. Discussion

In this study, we analyzed the contribution of the second messenger cGMP to the decreased excitability of B51, a decision-making neuron in the *Aplysia* feeding neural circuit. Our results indicate that cGMP decreases B51 excitability through an increase of the neuron's plateau potential threshold. In particular, a single cGMP injection leads to a rapid decrease of B51 excitability, which lasts for up to 45 min (Fig. 1C). Four consecutive cGMP injections induced a more persistent decrease of B51 excitability that lasted for at least 24 h (Fig. 2C). The use of ODQ revealed that B51 excitability is physiologically modulated by the activity of the sGC (Fig. 3C). The inhibition of sGC increased B51 excitability, suggesting that a basal tonic activity of the cyclase controls the neuron's excitability. Together with the iontophoretic injection results, these findings indicate that a cGMP signal regulates B51 excitability.

We next investigated which neurotransmitter might elevate the cytosolic levels of cGMP and, subsequently, decrease B51 excitability. The cGMP signaling cascade is activated by the neurotransmitter NO in numerous systems, including: hippocampal neurons [35], olfactory neurons [36], dorsal-root ganglia neurons [37] as well as *Aplysia* sensory neurons [23]. Furthermore, NO signalling has been shown to regulate *Aplysia* feeding [38]. In particular, *in vivo* manipulations of the NO pathway modulate the feeding response, with the NO synthase inhibitor L-NAME increasing feeding and the NO donor SNAP reducing feeding [31]. The *in vitro* effects of SNAP and L-NAME on B51 paralleled those *in vivo*: SNAP decreased excitability, whereas L-NAME increased excitability (Figs. 4C, E). The up and down regulation exerted by L-NAME and SNAP on B51 excitability indicates that the

basal levels of NO tonically control the neuron's activity. The final experiment of this study demonstrated that the SNAP-induced decrease of B51 excitability was blocked by ODQ (Fig. 5C), thus indicating that NO modulates B51 excitability through the activation of the sGC. Overall, these results are consistent with the previously reported regulation of feeding behavior by the NO-cGMP pathway [31,38], and suggest that NO modulates feeding, at least in part, by regulating B51 excitability.

Neither the short-term nor the long-term cGMP-induced decrease of B51 excitability were accompanied by modifications of Vm or Rm. This evidence indicates that the plasticity is intrinsic to B51 [17,19,21,33]. The lack of changes in the neuron's resting membrane properties also suggests that cGMP may modulate voltage-gated conductances responsible for the genesis of the plateau potential in B51. Recent findings have implicated voltage-gated Na<sup>+</sup> channels as the intrinsic mechanism contributing to the decrease of B51 excitability induced by sensitization training [39]. Therefore, it is plausible that a cGMP-mediated signalling cascade may reduce B51 excitability by acting on the properties of voltage-gated Na<sup>+</sup> channels, a modulatory mechanism that has been previously reported in the olfactory receptor cells of the newt *Cynops pyrrhogaster* [36].

Combined with previous findings, our results provide an interesting model of bidirectional modulation in which B51 excitability is regulated by different cyclic-nucleotide pathways. In particular, a cAMP-mediated pathway leads to an increase of B51 excitability [19,21]. This pathway is activated in operant reward learning, which up-regulates feeding and increases B51 excitability [15,17,21]. The cAMP-mediated pathway is triggered in B51 by the convergence of Ca<sup>2+</sup> entry during the plateau potential and dopamine released onto B51 during contingent reinforcement [11,21]. Our current results reveal that B51 excitability is modulated, in an opposite manner, by cGMP, which can be elevated in the cytosol through the NO-dependent activation of the sGC.

Previous evidence of bidirectional modulation of excitability has been provided in both vertebrates and invertebrates. In rodents, opposite changes in excitability have been reported in pyramidal neurons of the baso-lateral amygdala following different forms of learning, such as positive and negative olfactory conditioning [40]. In the leech *Hirudo medicinalis*, the excitability of the S interneurons, which participate in the defensive whole-body shortening, is increased following sensitization training and is decreased following habituation training [41]. These examples illustrate that bidirectional changes in behaviorally-relevant neurons contribute to the encoding of stimuli with opposite values, such as appetitive vs. aversive, or aversive vs. neutral. In the case of B51, the present findings, combined with previous observations, delineate a scenario in which opposite changes in B51 excitability are sustained by distinct cyclic nucleotides mediated cascades.

Examples of both convergence and divergence of second-messenger cascades modulating neuronal excitability can be found in the *Aplysia* nervous system. For instance, in the sensory neurons that mediate defensive withdrawal reflexes [42], cyclic-nucleotide signals show converging effects in which different triggers, such as noxious stimuli *via* cAMP [42], and injury *via* cGMP [23], both increase neuronal excitability. Similarly, distinct neuromodulators, such as serotonin *via* cAMP, and NO *via* cGMP, lead to an increase in the



excitability of the modulatory metacerebral cells [43]. Now, this study, together with previous research [19,21], provides evidence of divergence of excitability modulation by second-messenger cascades within B51, a neuron with decision-making characteristics. Due to the decision-making nature of B51 [10,18], and the great flexibility exhibited by feeding to changes in external and internal state [11,38], the bidirectional modulation of its excitability by distinct second-messenger cascades may represent a key mechanism to regulate the output of the feeding neural circuit in opposite directions.

Future investigations will examine which external triggers physiologically activate the NO-cGMP pathway in B51. Previous studies revealed that the same NO-cGMP pathway that modulates feeding and B51 excitability is also activated during a form of learning in which *Aplysia* learns that food is not edible and downregulates the expression of bites accordingly [24,25]. The involvement of the NO-cGMP pathway might extend to other forms of learning in which feeding is reduced. For example, sensitization training suppresses feeding and decreases B51 excitability [20,26]. That activation of the NO-cGMP pathway induces changes in B51 excitability that are analogous to those induced by *in vivo* and *in vitro* sensitization training [20,27], suggesting that this signalling cascade might also contribute to the behavioral and cellular modifications induced by sensitizing stimuli. Finally, we recently reported that, *in Aplysia*, food satiation suppresses feeding for several days and decreases B51 excitability [18]. It is plausible to hypothesize that the NO-cGMP pathway might also play a role in this other process by which feeding and B51 excitability are down regulated by the animal's internal state.

## Acknowledgments

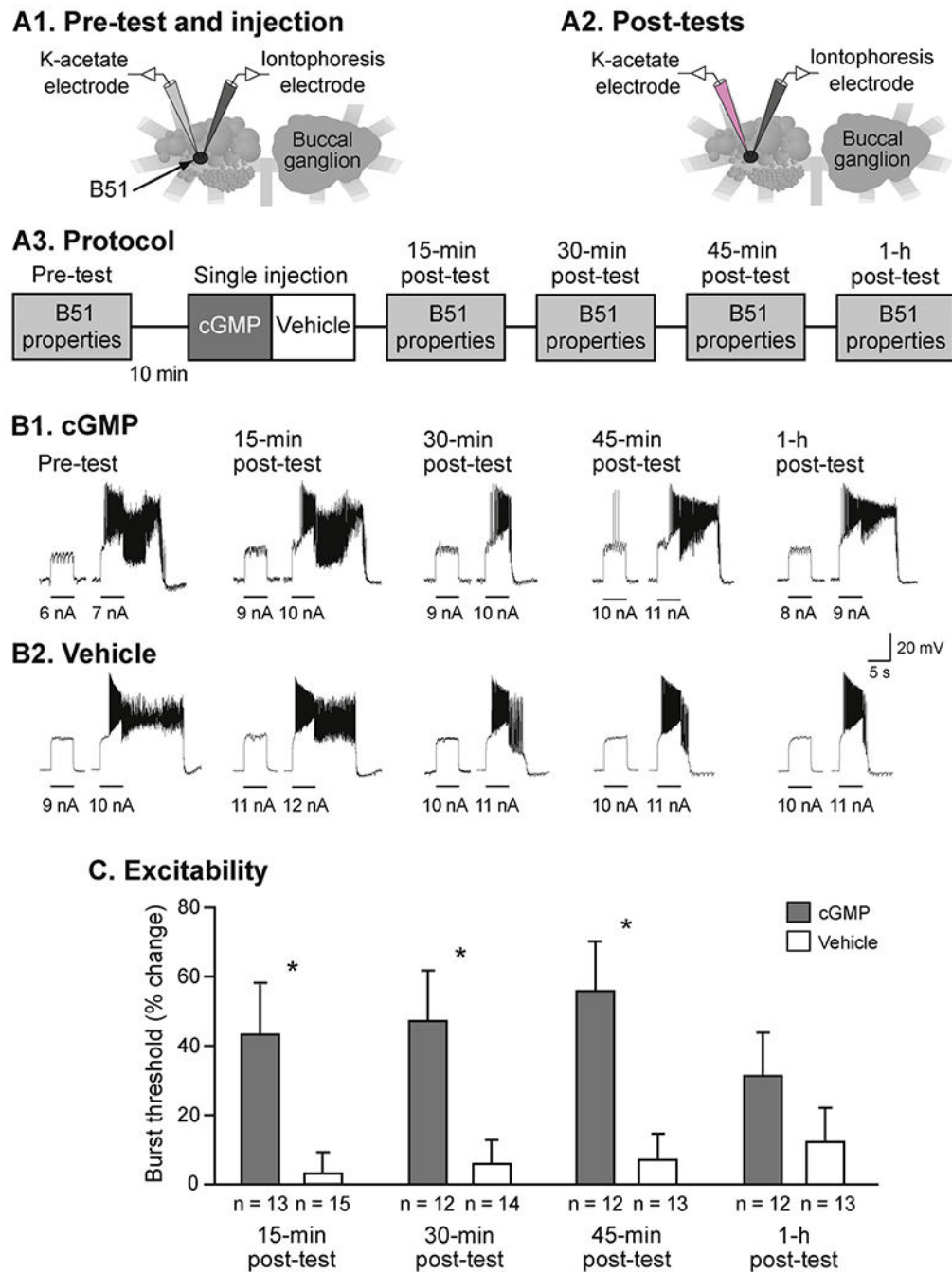
This work was supported by NIH-NIGMS grant SC3GM111188 to R.M. The funding source had no involvements in preparation of the article, collection, analysis and interpretation of data, writing of the report, and in the decision to submit the article for publication.

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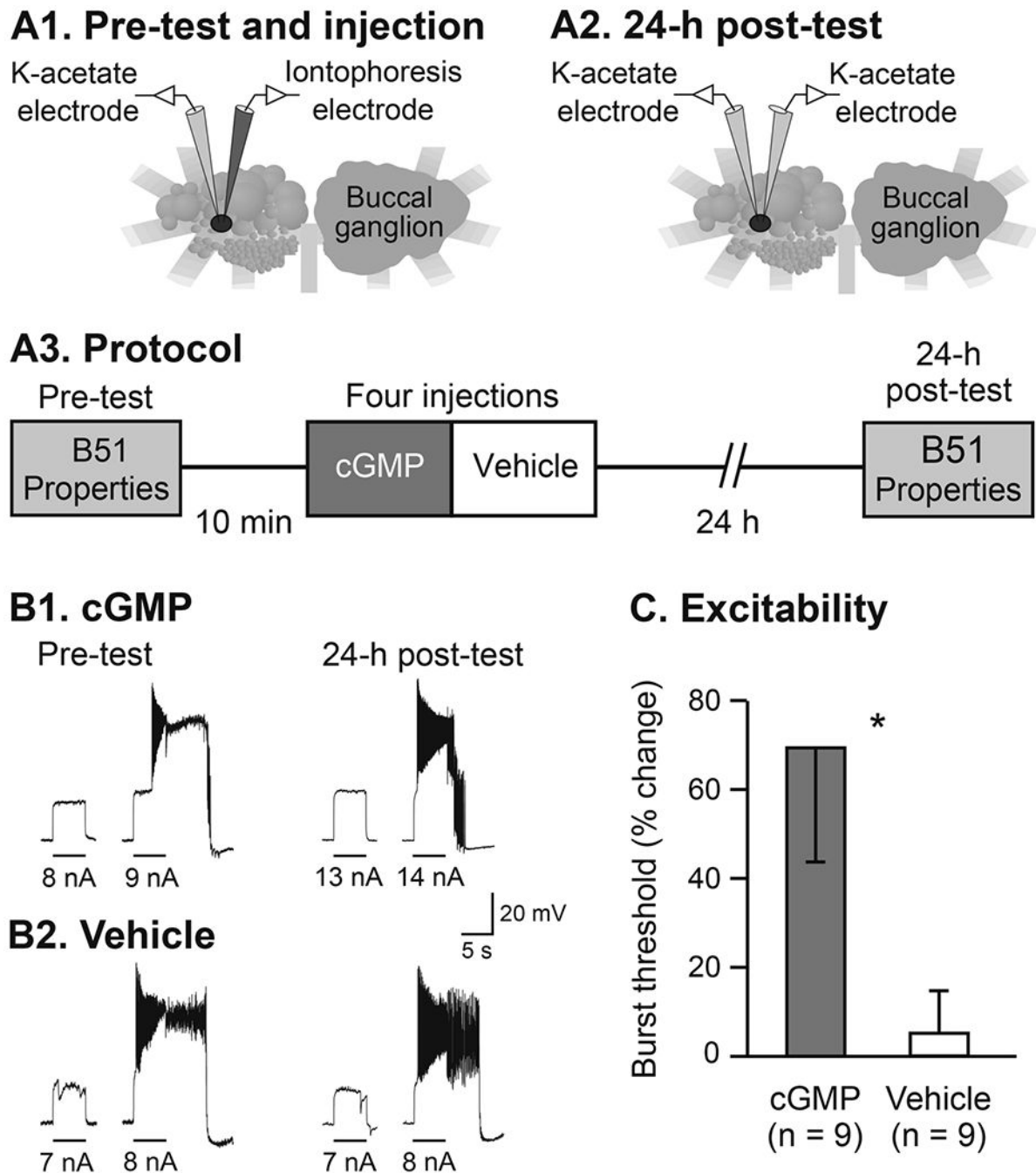
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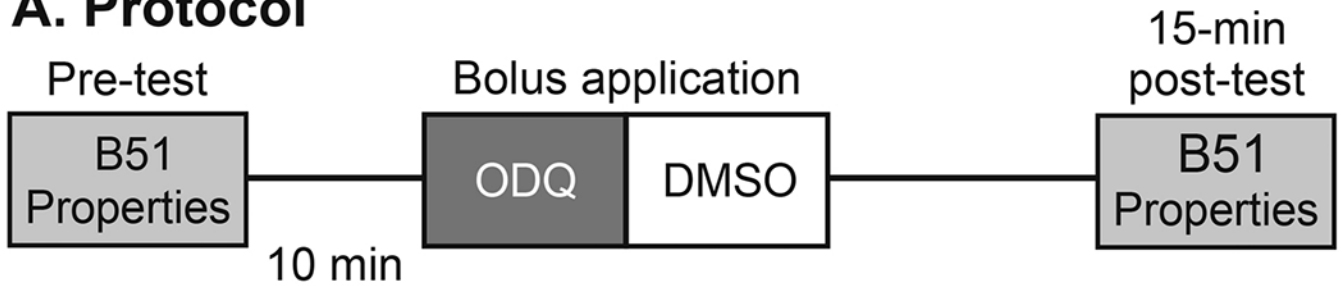
**Fig. 1.** Time course of the effects of single injection of cGMP on B51 excitability. (A) Placements of recording and iontophoretic electrodes in B51 during pre-test (A1) and post-tests (A2), and timeline of B51 measurements prior to and after the single-injection (A3). (B) Sample traces of B51 burst threshold from cGMP-injected (B1) and vehicle-injected cells (B2). (C) Summary data illustrate that the single cGMP injection significantly increased B51 burst threshold for up to 45 min after treatment. In this and in the following figure, statistical significance is indicated by an asterisk.



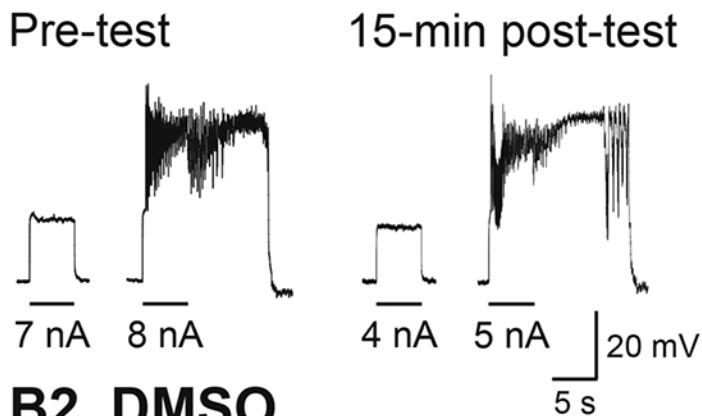
**Fig. 2.**

A four-cGMP injection protocol induced a decrease of B51 excitability 24 h after treatment. (A) Placement of recording and iontophoretic electrodes in B51 during pre-test (A1) and 24-h post-test (A2), and timeline of B51 measurements prior to and 24 h after the four-injections (A3). (B) Sample traces of B51 burst threshold from cGMP-injected (B1) and vehicle-injected cells (B2). (C) Summary data illustrate that four cGMP injections significantly increased B51 burst threshold 24 h after treatment.

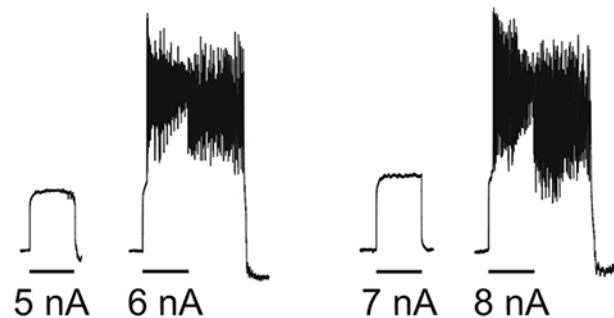
## A. Protocol



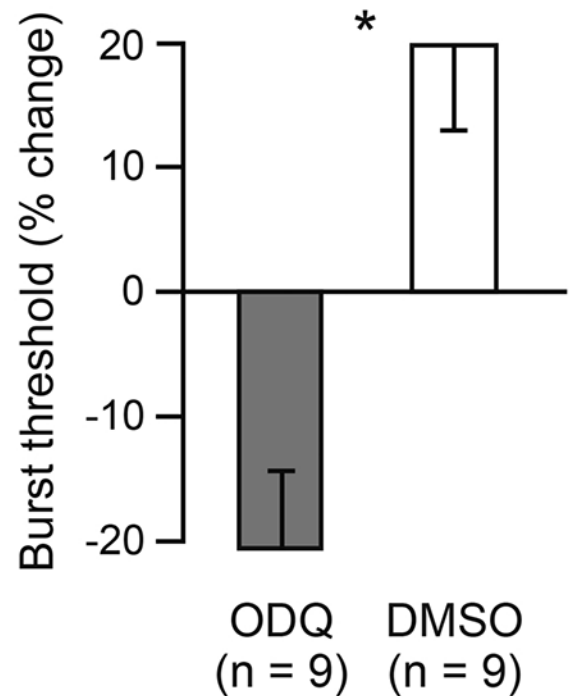
## B1. ODQ



## B2. DMSO

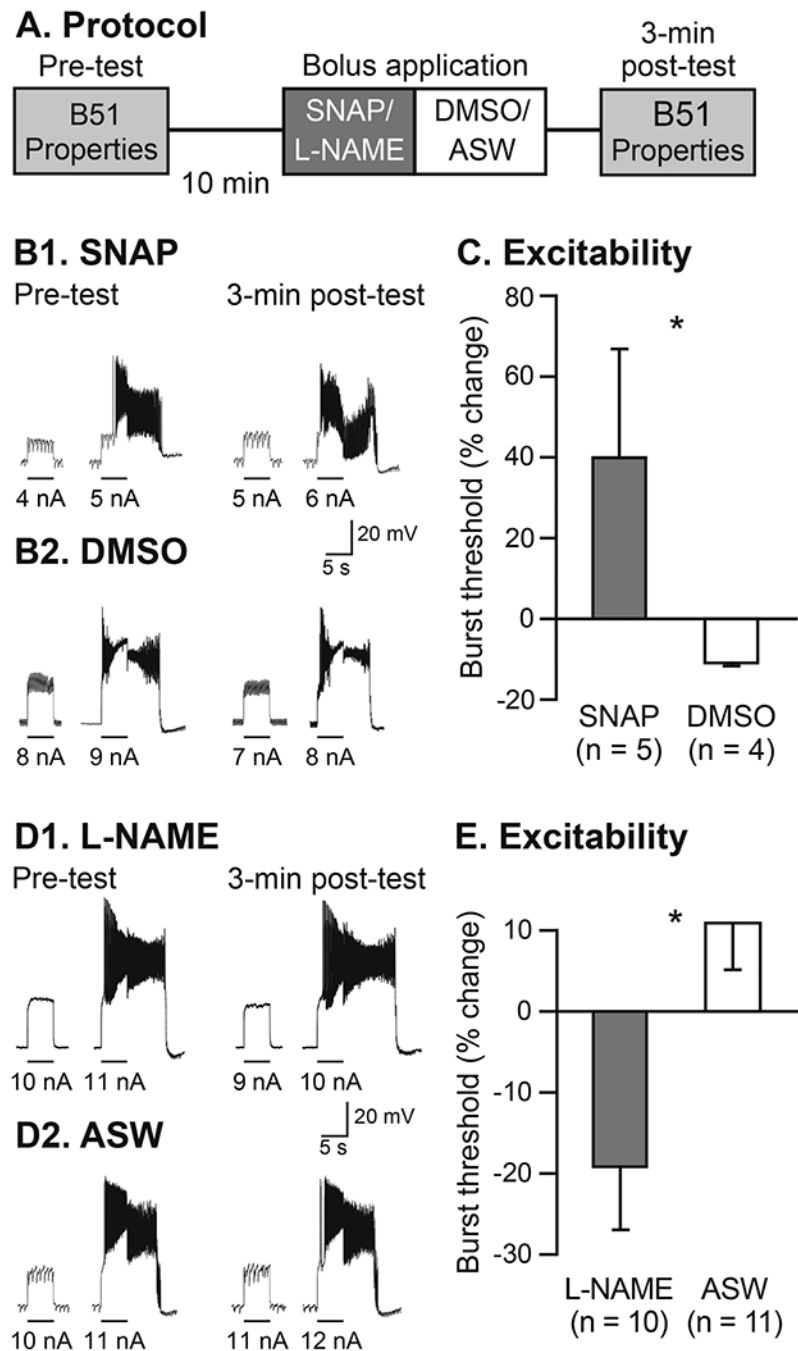


## C. Excitability



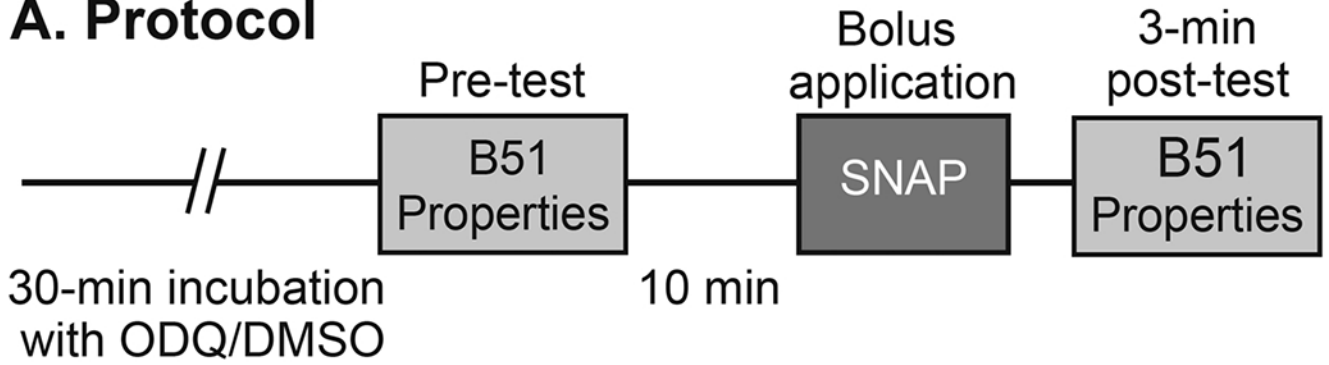
**Fig. 3.**

Inhibition of sGC activity increased B51 excitability. (A) Protocol of B51 measurements prior to and 15 min after ODQ/DMSO treatment. (B) Sample traces of B51 burst threshold from ODQ-treated (B1) and DMSO-treated cells (B2). (C) Summary data illustrate that ODQ significantly decreased B51 burst threshold 15 min after treatment.

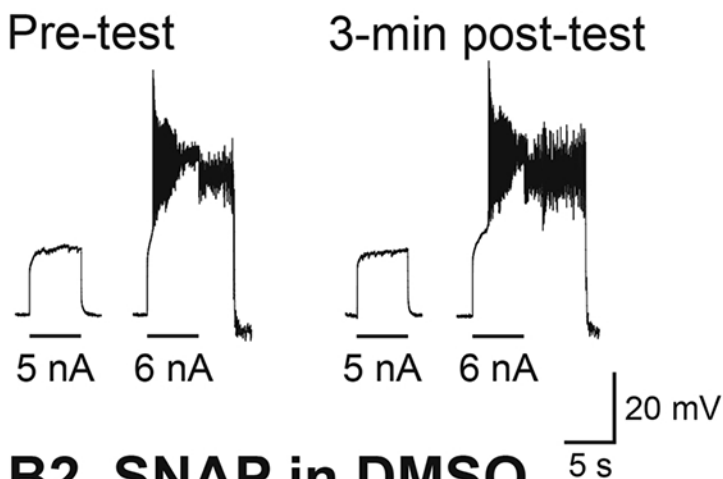


**Fig. 4.** B51 excitability is modulated by NO signaling. (A) Protocol of B51 measurements prior to and 3 min after treatments with either SNAP/DMSO or L-NAME/ASW. (B) Sample traces of B51 burst threshold from SNAP-treated (B1) and DMSO-treated cells (B2). (C) Summary data illustrate that SNAP significantly increased B51 burst threshold 3 min after treatment. (D) Sample traces of B51 burst threshold from L-NAME-treated (D1) and ASW-treated cells (D2). (E) Summary data illustrate that L-NAME significantly decreased B51 burst threshold 3 min after treatment.

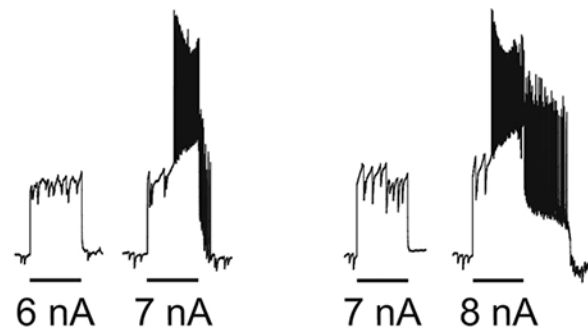
## A. Protocol



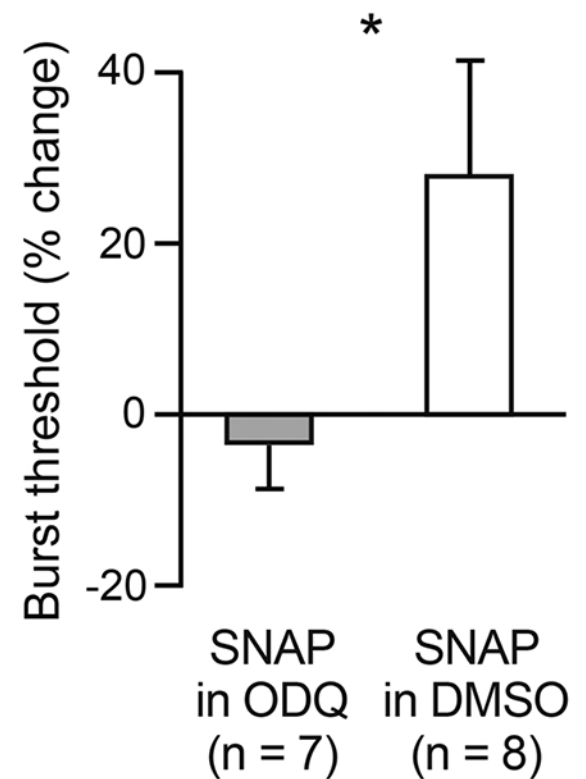
## B1. SNAP in ODQ



## B2. SNAP in DMSO



## C. Excitability



**Fig. 5.**

The NO-dependent decrease of B51 excitability is mediated by the sGC. (A) Protocol of incubation with ODQ/DMSO and subsequent measurements of B51 properties prior to and 3 min after SNAP treatment. (B) Sample traces of B51 burst threshold from SNAP-treated cells incubated with ODQ (B1) and SNAP-treated cells incubated with DMSO (B2). (C) Summary data illustrate that ODQ incubation prevented the SNAP-induced increase of B51 threshold.