HHS Public Access

Author manuscript

Nat Neurosci. Author manuscript; available in PMC 2016 August 29.

Published in final edited form as:

Nat Neurosci. 2008 October ; 11(10): 1146–1148. doi:10.1038/nn.2184.

Changes in neuronal excitability serve as a mechanism of long-term memory for operant conditioning

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Abstract

Learning can lead to changes in the intrinsic excitability of neurons. However, it is unclear to what extent these changes persist and what role they play in the expression of memory. Here, we report that *in vitro* analogues of operant conditioning produce a long-term (24 h) increase in the excitability of an identified neuron (B51) critical for the expression of feeding in *Aplysia*. This increase in excitability, which is cAMP dependent, contributes to the associative modification of the feeding circuitry, providing a mechanism for long-term memory storage.

Although a widely held view is that learning leads to changes in synaptic efficacy[1], an increasing body of evidence suggests that learning can also lead to changes in the intrinsic excitability of neurons[2]. However, it is unclear to what extent the changes in excitability persist, and their role in the expression of memory is still elusive[2]. In this study, we examined the extent to which changes in intrinsic excitability can contribute to the storage of long-term memory (LTM) of operant conditioning[3].

Feeding behavior in *Aplysia* can be modified by operant conditioning both *in vivo*[4] and *in vitro* [4–7]. Contingent reinforcement of bites leads to the increased expression of feeding behavior both immediately and 24 h after training[4]. Moreover, *in vitro* operant conditioning induces short-term (1 h) changes in the expression of the patterned motor activity, which is the neural correlate of feeding behavior generated by the feeding central pattern generator (CPG)[5] located in the buccal ganglion. The changes in the output of the CPG are associated with a short-term increase in the intrinsic excitability of neuron B51[6,7], which is a key element of the feeding CPG[6–9] (Supplementary Methods). Here, we examined whether *in vitro* operant conditioning produces long-term changes in the CPG, whether these changes are associated with long-term modifications of the intrinsic excitability of B51 and whether a long-term change in excitability contributes to the expression of LTM.

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Note: Supplementary information is available on the Nature Neuroscience website.

Competing interests statement: The authors declare that they have no competing financial interests.

To examine whether an *in vitro* analogue of operant conditioning could express LTM, buccal ganglia from naïve animals were trained *in vitro* for 15 min with contingent, yoke or control protocols. Electrical stimulation of E n.2 was used as reinforcement[4,5] (Fig. 1a). In the contingent group, the reinforcement was contingent on the occurrence of an ingestion-type buccal motor program[5] (iBMP; Fig. 1a), whereas in the yoke group, the reinforcement was not contingent on the occurrence of a BMP. The control group received no reinforcement (Supplementary Methods and Supplementary Fig. 1). During a 10-min observation time 24 h after training, the numbers of iBMPs in the contingent (2.2±0.8 iBMPs, n=13), yoke (0.3±0.2 iBMPs, n=13) and control (0.2±0.1 iBMPs, n=13) groups were significantly different (H_2=10.31, P<0.05; Fig. 1b). *Post hoc* analysis revealed that the number of iBMPs was significantly greater in the contingent group as compared to the yoke group (q=3.48, P<0.05) and to the control group (q=4.77, P<0.05). In contrast, no significant difference was detected between the yoke and the control groups (q=0.42, P≥0.05). The analysis of the number of egestive BMPs (eBMPs), which are neural correlates of rejection behavior[5], measured 24 after training, revealed no significant difference among the contingent (3.9±1.1 eBMPs, n=13), the yoke (3.4±1.0 eBMPs, n=13) and the control groups (2.2±0.4 eBMPs, n=13; H_2=0.56, P=0.756). These results indicate that the activity of the CPG associated with feeding behavior can be selectively modulated in a long-term manner by an *in vitro* operant procedure.

We next determined whether the long-term effects of operant conditioning on the activity of the CPG were accompanied by long-term changes in the membrane properties of B51. These experiments only included two training protocols (contingent and yoke), because the previous experiment found no significant difference between the yoke and the control groups. Twenty four hours after training, the resting membrane potential (RMP), the input resistance (R_{in}) and the burst threshold (BT) [i.e., the minimum amount of injected current necessary to elicit an all-or-nothing sustained several-second burst of spikes (plateau potential)[8] in B51] were measured (Supplementary Methods). Operant conditioning led to a significant increase in R_{in} and a significant decrease in the BT of B51 (R_{in}: Contingent, 2.5±0.2 MΩ, n=10; Yoke, 1.9±0.1 MΩ, n=10; P<0.05; BT: Contingent, 7.9±0.6 nA, n=9; Yoke, 10.9±1.1 nA, n=9; P<0.05; Fig. 2a–d). No differences were observed in RMP (Contingent, −57.9±1.1 mV, n=10; Yoke, −56.2±2.1 mV, n=10; P=0.52). These results indicate that long-term changes in R_{in} and BT are produced by operant conditioning. Moreover, they indicate that in B51 the phenotype of expression (i.e., changes in R_{in} and BT, without changes in RMP) of both short- and long-term memory are the same.

The long-term changes in excitability of B51 could be due to intrinsic changes in B51 or due to changes in the properties of other presynaptic neurons that are electrically coupled to B51 or that produce tonic synaptic modulation of B51. To address this issue, we examined whether long-term changes in B51 could be produced by a single-cell analogue of operant conditioning consisting of an isolated B51 neuron in culture[4]. This analogue induces an increase in the excitability of B51 immediately after training similar to that produced by *in vivo* and *in vitro* training protocols[4,6,7]. For the long-term study, we followed the same protocol as established previously[4] (Supplementary Methods). The training phase was 10 min during which plateau potentials were contingently reinforced with a brief iontophoretic puff of dopamine, which mediates the reinforcement[4], onto B51 immediately after the

plateau potential (contingent reinforcement group). An unpaired control was also performed where the dopamine delivery was delayed for 40 s after each plateau potential. The membrane properties of B51 were measured prior to (pre-test) and 24 h after (test) training. Contingent training led to a significant increase in the R_{in} and a significant decrease in the BT of B51 24 h after training (R_{in}: Contingent Reinforcement, 41.3±3.3% of pre-test, n=4; Unpaired Control, −9±3.4% of pre-test, n=4; P<0.05; BT: Contingent Reinforcement, −63.4±4.7% of pre-test, n=4; Unpaired Control, 11.8±7.8% of pre-test, n=4; P<0.05; Fig. 2e–h). The long-term changes in the biophysical properties (i.e., R_{in} and BT) produced by the single-cell analogue were identical to those produced by contingent reinforcement of iBMPs in the ganglia.

B51 receives rhythmic synaptic input from other elements of the CPG. This input sometimes fires the cell and other times it does not. Supra-threshold input is associated with the expression of a plateau potential in B51, which is critical for the expression of an iBMP[6,7,9] (e.g., Fig. 1a). Therefore, an increase in R_{in} and decrease in BT would increase the likelihood for the activation of B51 by synaptic input from other elements of the CPG. Indeed, the number of CPG-generated plateau potentials expressed by B51 during a 10-min observation time was increased significantly 24 h after training (Contingent, 5.1±1.4 plateau potentials, n=13; Yoke, 2.1±0.7 plateau potentials, n=13; P<0.05; Fig. 2i,j). However, the long-term changes in the output of the CPG (Figs. 1b and 2i,j) could be due to changes in other elements of the circuit and consequent changes in synaptic input to B51. To address this issue, we attempted to selectively induce long-term changes in R_{in} and BT of B51 and examine the consequences of these changes on the CPG-induced activity of B51. cAMP has been shown in numerous studies to induce long-term changes in neuronal properties[10]. In addition, adenylyl cyclase appears to serve as a molecular site of associative convergence in B51 during operant conditioning, which leads to the activation of PKA and phosphorylation of the transcription factor CREB1[11]. This pathway resembles the signaling cascades underlying long-term synaptic plasticity and LTM in other model systems, including vertebrates[12]. Furthermore, cAMP injection into cultured B51 neurons induces a rapid increase in the excitability similar to that produced in the short term by the single-cell analogue of operant conditioning[11].

We first determined whether cAMP was necessary for the long-term changes in excitability, by blocking cAMP signaling with Rp-cAMP bath applied on cultured B51 neurons during the single-cell analogue of operant conditioning (Supplementary Methods). Contingent training in the presence of Rp-cAMP blocked the contingent-dependent increase in the R_{in} and the decrease in the BT of B51 when tested 24 h after training (R_{in}: −8.5±7.2% of pre-test, n=4; P<0.05; Fig. 2g; BT: 4.2±7.1% of pre-test, n=4; P<0.05; Fig. 2h). Next, we injected cAMP into B51 in the ganglia and examined whether such injections were sufficient to produce long-term changes in excitability. cAMP or vehicle was injected into the soma of B51 during a 10-min period (Supplementary Methods and Supplementary Fig. 2). The RMP, R_{in} and BT of B51 were measured prior to (pre-test) and 24 h after (test) injection. Although the injection itself appeared to produce some run down of the cell, cAMP-injected cells showed a significant long-term increase in R_{in} compared to the vehicle-injected group (cAMP, 15.7±5.5% of pre-test, n=10; Vehicle, −14.5±5.0% of pre-test, n=12, P<0.05; Fig. 3a), and a significant long-term decrease in B51 BT compared to the vehicle-injected group

(cAMP, −5.2±9.4% of pre-test, n=8; Vehicle, 35.7±16.6% of pre-test, n=9, P<0.05; Fig. 3b). No change was observed in the RMP (cAMP, 4.5±2.5% of pre-test, n=11; Vehicle, 3.0±2.1% of pre-test, n=12, P=0.46; Fig. 3c). These results indicate that cAMP is both necessary and sufficient for the long-term plasticity in B51. We also examined the consequences of the selective increase in R_{in} and decrease in BT of a single B51 neuron on the CPG activity. We counted the number of CPG-elicited plateau potentials during a 10-min observation time 24 h after injecting B51 with either cAMP or vehicle. Remarkably, the number of plateau potentials was significantly greater in B51 neurons injected with cAMP (4.0±1.0 plateau potentials, n=8) as compared to those injected with the vehicle (1.0±0.5 plateau potentials, n=9, P<0.05; Fig. 3d,e). These findings indicate that the cAMP-induced increase in R_{in} and decrease in BT were sufficient to increase the recruitment of B51 by CPG-generated synaptic input and thereby contributed to the generation of more iBMPs and to the LTM for operant conditioning. One possible explanation for the long-term effect of cAMP on B51 excitability is that the PKA-dependent phosphorylation of CREB1[12] could regulate genes encoding for either voltage-dependent channel proteins or accessory proteins that regulate membrane channels. Although further experimentation is required, these findings suggest that mechanisms of memory storage based on changes in excitability can share some common molecular pathways with those that rely on changes in synaptic strength[12].

Numerous studies found that several forms of learning produce changes in excitability[2]. In *Aplysia,* the excitability of sensory neurons is enhanced following both sensitization[13,14] and classical conditioning[15]. In the case of sensitization, biophysical correlates were found in the sensory neurons[13] that could be mimicked by cAMP[11], but their impact on the behavior was not assessed. One of the unique aspects of the present study was the ability to mimic the learning-induced changes in excitability at the single-cell level in B51 by injecting the neuron with cAMP and measure these effects on the CPG activity. Remarkably, the cAMP-induced long-term increase of B51 excitability also augmented the likelihood of B51 to become active, which in turn, increased the likelihood of the CPG to elicit ingestive motor patterns. Because of the all-or-nothing nature of the plateau potential in B51, a change in its excitability can directly increase the likelihood for the elicitation of a plateau potential, thus biasing the CPG towards generating a greater number of iBMPs and, in turn, a greater number of bites, as occur following operant conditioning[4,5]. In conclusion, these findings provide compelling evidence that contingent-dependent changes in excitability at the level of a neuron critical for the expression of the behavior can represent a relevant mechanism of LTM storage by which operant conditioning alters the expression of that behavior.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Institute of Mental Health Grant MH 58321. We thank Dr. Marcy Wainwright for help with the illustrations.

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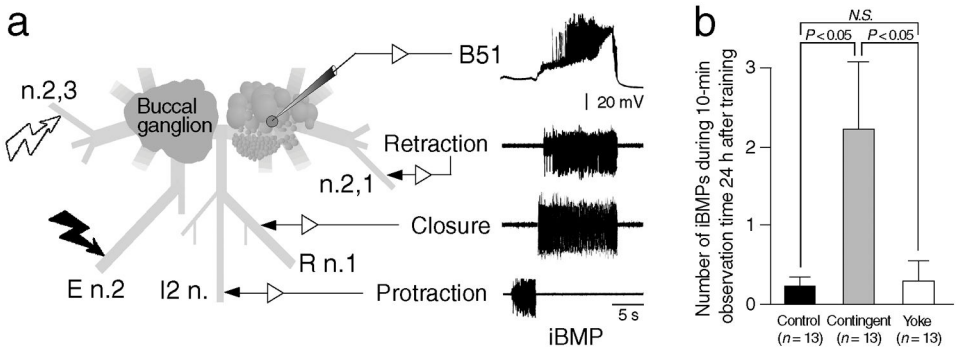

Figure 1.

In vitro operant conditioning of fictive feeding behavior produced long-term changes in the activity of the CPG. (**a**) Isolated buccal ganglia were trained *in vitro* with an operant protocol in which the reinforcement consisted of electrical stimulations of the E n.2 nerve (black arrow). Fictive feeding behavior (iBMP) was recorded extracellularly from the peripheral nerves controlling protraction, closure and retraction of the radula. Intracellular recordings were used to monitor the recruitment of B51 during iBMPs. Although only extracellular nerve recordings were used in this first series of experiments, an example of intracellular recording from B51 is shown in Fig. 1a simultaneously with the expression of an iBMP to illustrate the relationship between B51 bursting activity and the occurrence of an iBMP. Note the prolonged plateau potential of B51 that is in phase with the radula closure activity occuring during retraction. These features of B51 indicate that plateau potentials can be used as a reliable readout for an iBMP. Tonic low-frequency electrical stimulation of nerve n.2,3 (white arrow) was used to activate the CPG during training and testing. (**b**) 24 h after training, the contingent group expressed a significantly greater number of iBMPs as compared to the yoke and control groups. In this and subsequent figures, data are displayed as means ± s.e.m. The Kruskal-Wallis (*H*) test was used followed by *post hoc* pairwise comparisons with the Student-Newman-Keuls test (*q*).

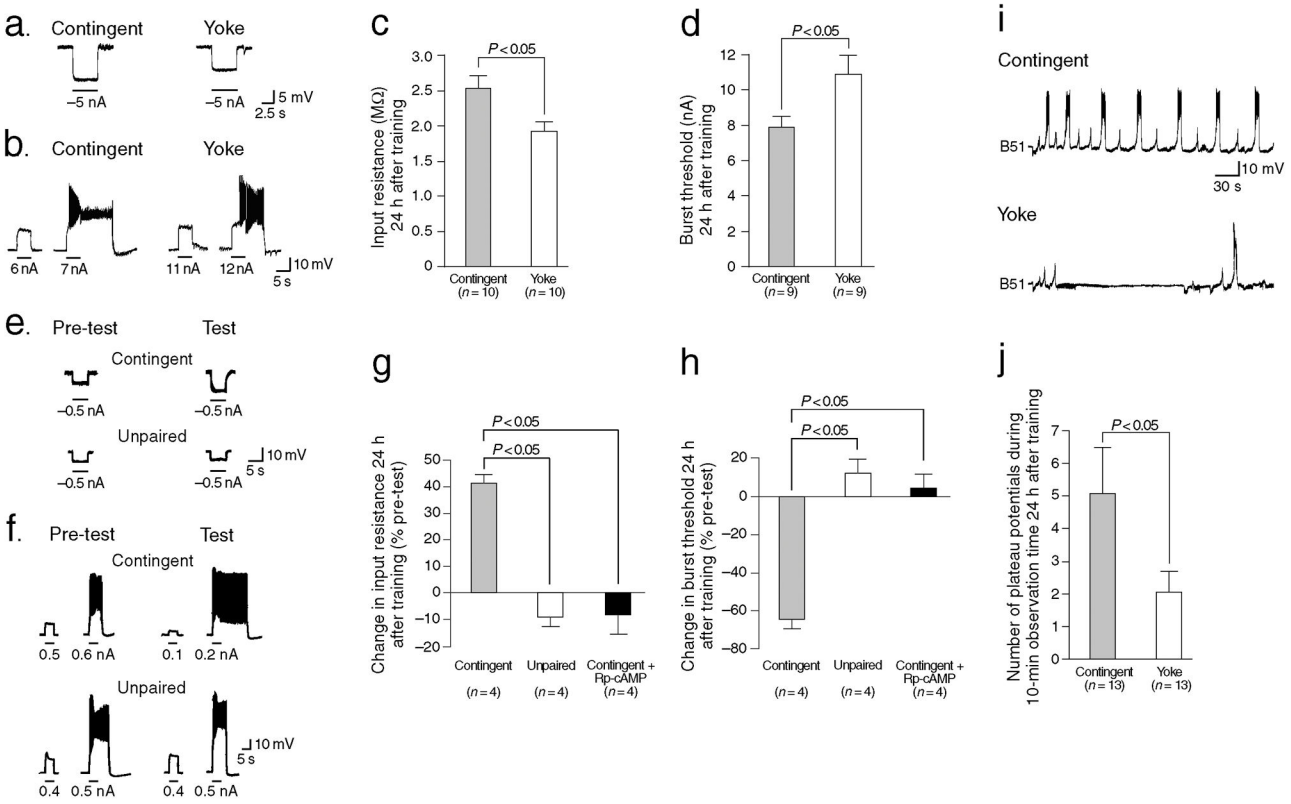

Figure 2.

Long-term changes in B51 membrane properties produced by the *in vitro* and single-cell analogues of operant conditioning. (**a–d**) The *in vitro* analogue produced long-term changes in R_{in} (**a**) and BT (**b**) 24 h after training. (**a**) R_{in} was greater in a contingent preparation (left) compared to a yoke preparation (right), as indicated by the larger deflection in the membrane potential. (**b**) For BT, less current (7 nA) was sufficient to generate a plateau potential from a contingent preparation (left) compared to a yoke preparation (12 nA, right). (**c**,**d**) Summary data. R_{in} (**c**) was significantly greater and BT (**d**) was significantly lower in the contingent group compared to the yoke group. (**e–h**) The single-cell analogue also produced long-term changes that were blocked by Rp-cAMP. R_{in} (**e**) and BT (**f**) before (pre-test) and 24 h after contingent and unpaired training (test). Summary data. R_{in} (**g**) was significantly increased and BT (**h**) was significantly decreased in the contingent group compared to the unpaired control. These changes were both blocked by Rp-cAMP (**g**,**h**). (**i,j**) The *in vitro* analogue produced long-term changes in the number of CPG-elicited plateau potentials. (**i**) Plateau potentials 24 h after training. (**j**) Summary data. The number of plateau potentials was significantly greater in the contingent group compared to the yoke group. Data in panel **g** and **h** were analyzed with Kruskal-Wallis tests followed by Student-Newman-Keuls pairwise comparisons. Data in panels **c, d** and **j,** as well as those in Fig. 3, were analyzed using two-tailed Mann-Whitney *U* tests.

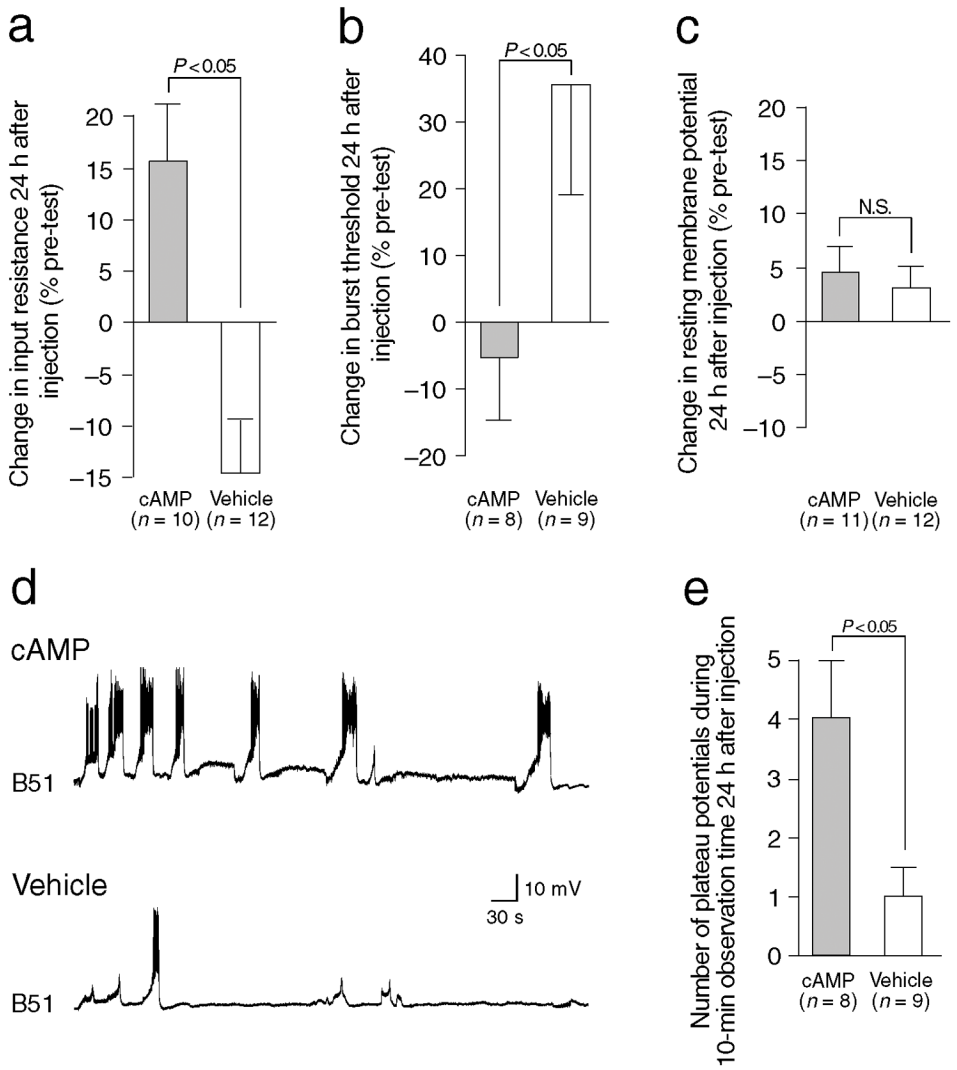

Figure 3.

cAMP injection induced long-term changes in B51 similar to those produced by *in vitro* operant conditioning and led to an increase in the expression of iBMPs. (**a**) Summary data illustrate that cAMP induced a significant increase in R_{in} compared to the decrease observed in the vehicle-injected group. (**b**) cAMP also induced a significant decrease in the BT of B51 compared to the increase observed in the vehicle-injected group. (**c**) Summary data illustrate that the change in RMP did not differ between the cAMP- and the vehicle-injected groups. (**d**) Representative intracellular recordings from B51 illustrating the number of CPG-elicited plateau potentials measured during a 10-min observation time 24 h after injection of either cAMP or vehicle. (**e**) Summary data illustrate that, 24 h after injection, the number of CPG-elicited plateau potentials was significantly greater in the cAMP-injected group as compared to the vehicle-injected group.