

Persistent Sodium Current Is a Nonsynaptic Substrate for Long-Term Associative Memory

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Summary

Although synaptic plasticity is widely regarded as the primary mechanism of memory [1], forms of nonsynaptic plasticity, such as increased somal or dendritic excitability or membrane potential depolarization, also have been implicated in learning in both vertebrate and invertebrate experimental systems [2–7]. Compared to synaptic plasticity, however, there is much less information available on the mechanisms of specific types of nonsynaptic plasticity involved in well-defined examples of behavioral memory. Recently, we have shown that learning-induced somal depolarization of an identified modulatory cell type (the cerebral giant cells, CGCs) of the snail *Lymnaea stagnalis* encodes information that enables the expression of long-term associative memory [8]. The *Lymnaea* CGCs therefore provide a highly suitable experimental system for investigating the ionic mechanisms of nonsynaptic plasticity that can be linked to behavioral learning. Based on a combined behavioral, electrophysiological, immunohistochemical, and computer simulation approach, here we show that an increase of a persistent sodium current of this neuron underlies its delayed and persistent depolarization after behavioral single-trial classical conditioning. Our findings provide new insights into how learning-induced membrane level changes are translated into a form of long-lasting neuronal plasticity already known to contribute to maintained adaptive modifications at the network and behavioral level [8].

Results

Learning-Induced Delayed Depolarization Is Concomitant with and Correlated to the Enhancement of the Persistent Sodium Current of the CGCs

Previous work already has shown that somal depolarization of the CGC is sufficient for the conditioned response to be triggered by the appropriate external stimulus [8], and here we investigated the learning-induced ionic mechanisms that support this depolarization. Here we compared the electrical properties as well as the low-threshold persistent sodium current of the CGCs ($I_{Na(P)}$) [9, 10] in groups of classically conditioned (CS/US paired), CS/US unpaired, and naive control animals (for technical details of the preparations, electrophysiological and classical conditioning experiments, and data analysis, see [Supplemental Experimental Procedures](#) available online). This current was targeted because, like similar types of persistent sodium currents in mammalian neurons [11–14], it makes an important contribution to the membrane potential of the CGC in naive animals [9, 10]. $I_{Na(P)}$ therefore appeared to be a suitable substrate for learning-induced changes resulting in a maintained depolarizing shift in the membrane potential [8].

Specifically, we tested the hypothesis that there is a link between $I_{Na(P)}$ and the previously described learning-induced delayed and persistent membrane potential (MP) depolarization of the CGCs. One set of electrophysiological experiments was performed in the first 12 hr after training (with preparations tested between 6 and 12 hr after the animals were subjected to the paired or unpaired protocol) and the second more than 24 hr after training (between 26 and 32 hr). The choice of test times was based on the previous observation that <12 hr after single-trial classical conditioning, the CGCs do not yet show the conditioning-induced depolarization that is present >24 hr after training [8].

The behavioral memory tests confirmed that the single-trial classical conditioning procedure was successful and resulted in long-term memory for the association between CS and US (see [Supplemental Results](#)). The electrophysiological tests performed on isolated CNS preparations also confirmed the previously published observation [8] that at >24 hr, but not <12 hr, after training, the CGC membrane potential in CS/US paired animals was significantly more depolarized compared to controls (see [Supplemental Results](#)).

After recording the membrane potential, each CGC was subjected to two different voltage-clamp protocols (for details, see [Supplemental Experimental Procedures](#)), one focusing on $I_{Na(P)}$, and a different one concentrating more on the much larger delayed rectifier potassium current, which has a much less negative activation threshold compared to $I_{Na(P)}$ [9]. The previously comprehensively characterized persistent sodium current $I_{Na(P)}$ activates at more negative potentials than any other current of the CGC, and it is the only inward current that remains noninactivated at the end of very long (~1 s) voltage steps [9, 10]. These characteristics of $I_{Na(P)}$ offered a unique opportunity to record this current in the –90 to –50 mV potential range without interference from other currents and therefore without the need to use specific channel

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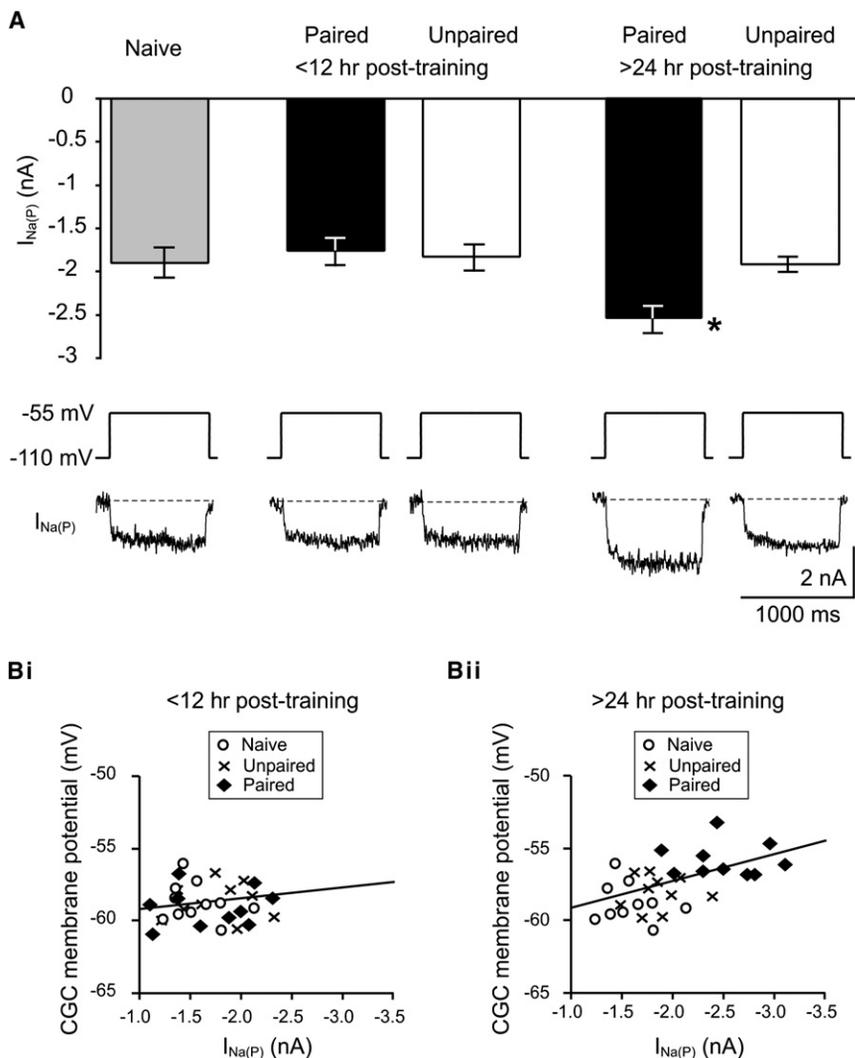


Figure 1. Concomitant and Correlated Increase in $I_{Na(P)}$ and CGC Membrane Potential Depolarization after Classical Conditioning

(A) Mean steady-state $I_{Na(P)}$ amplitudes (\pm SE) in CGCs in preparations from naive animals and from CS/US paired and unpaired animals at <12 hr and >24 hr after training ($n = 10$ preparations in each group). The current responses compared here were evoked by the -110 mV to -55 mV voltage step of the first type of multiple-step protocol used on each cell (see Supplemental Experimental Procedures). Examples of current traces are shown under the corresponding bar diagrams.

(B) Scatterplots with regression lines of measured membrane potential against $I_{Na(P)}$ (evoked by a -110 mV to -55 mV voltage step) from the same experiments that yielded the data shown in (A).

in the experiments (Figure 1Bi), in the post-24 hr tests, a significant positive correlation was found between these two variables (Figure 1Bii; Pearson's correlation test, $R^2 = 0.21$, $p < 0.01$). In the post-24 hr tests, the majority of the values from the CS/US paired group (Figure 1Bii, diamonds) were clustered in the larger $I_{Na(P)}$ /more depolarized membrane potential region, and the majority of the naive and CS/US unpaired control data (Figure 1Bii, circles and crosses, respectively) was clustered in the smaller $I_{Na(P)}$ /less depolarized region (Figure 1Di), indicating that the main contributing factor to the correlation emerging >24 hr after training was the learning-induced parallel changes in both membrane potential and $I_{Na(P)}$.

blockers to isolate it from other currents. As a first step of our analysis, we therefore compared $I_{Na(P)}$ in CS/US paired, unpaired control, and naive groups by measuring the steady-state persistent inward current evoked by the 800 ms depolarizing step from -110 mV to -55 mV. In each electrophysiological experiment, we recorded both the left and the right CGC, and the averaged data from both CGCs were used for statistical comparisons.

At >24 hr, but not at <12 hr, after training, the $I_{Na(P)}$ of the CGCs in CS/US paired animals was significantly enhanced compared to both CS/US unpaired and naive animals (Figure 1A; ANOVA, $F[2,29] = 7.7$; $p < 0.002$; Tukey's $p < 0.001$; examples of $I_{Na(P)}$ recordings from each group are shown below the graphs). The same analysis also showed that, similarly to the feeding response scores and CGC membrane potential, $I_{Na(P)}$ was not different between the CS/US unpaired and naive control groups (Tukey's, $p > 0.05$).

Together, the <12 hr and >24 hr post-training CGC membrane potential and $I_{Na(P)}$ measurements showed that in CS/US paired animals, the learning-induced membrane potential depolarization was concomitant with an enhanced $I_{Na(P)}$, i.e., both were absent <12 hr but present >24 hr after training. At the same time, whereas in the pre-12 hr experiments no significant correlation was found between the membrane potential and $I_{Na(P)}$ data from the CGCs in all the preparations tested

To assess the consistency of the learning-induced change in the net persistent inward current over its whole activation range, we plotted I-V curves with all the command potential levels used in first voltage-clamp protocol and calculated the integrals of these curves. In the experiments performed <12 hr after training, we did not find an overall significant difference between the CS/US paired, unpaired, and naive groups (Figures 2Ai and 2C). However, as with the results of analysis of currents evoked by a step to -55 mV, there was a significant overall difference among the three groups in the I-V curve integral calculated from the current measurement data >24 hr after training (ANOVA, $F[2,29] = 15.4$, $p < 0.0001$), with this value being significantly larger (Tukey's, $p < 0.05$) in the CS/US paired group than in the unpaired or naive control group (Figures 2Bi and 2C).

We performed a second type of analysis after calculating the persistent sodium conductance ($g_{Na(P)}$) values for the -70 mV to -50 mV activation voltage range for the naive group and the paired and unpaired groups from both the <12 hr and >24 hr post-training tests (Figures 2Aii and 2Bii). A one-sample t test of the ratios of the mean paired to naive and unpaired to naive conductance values revealed that this ratio was only significantly higher than 1 in the paired group tested >24 hr after training ($t = 4.0$, $df = 4$, $p < 0.016$, Figure 2Dii).

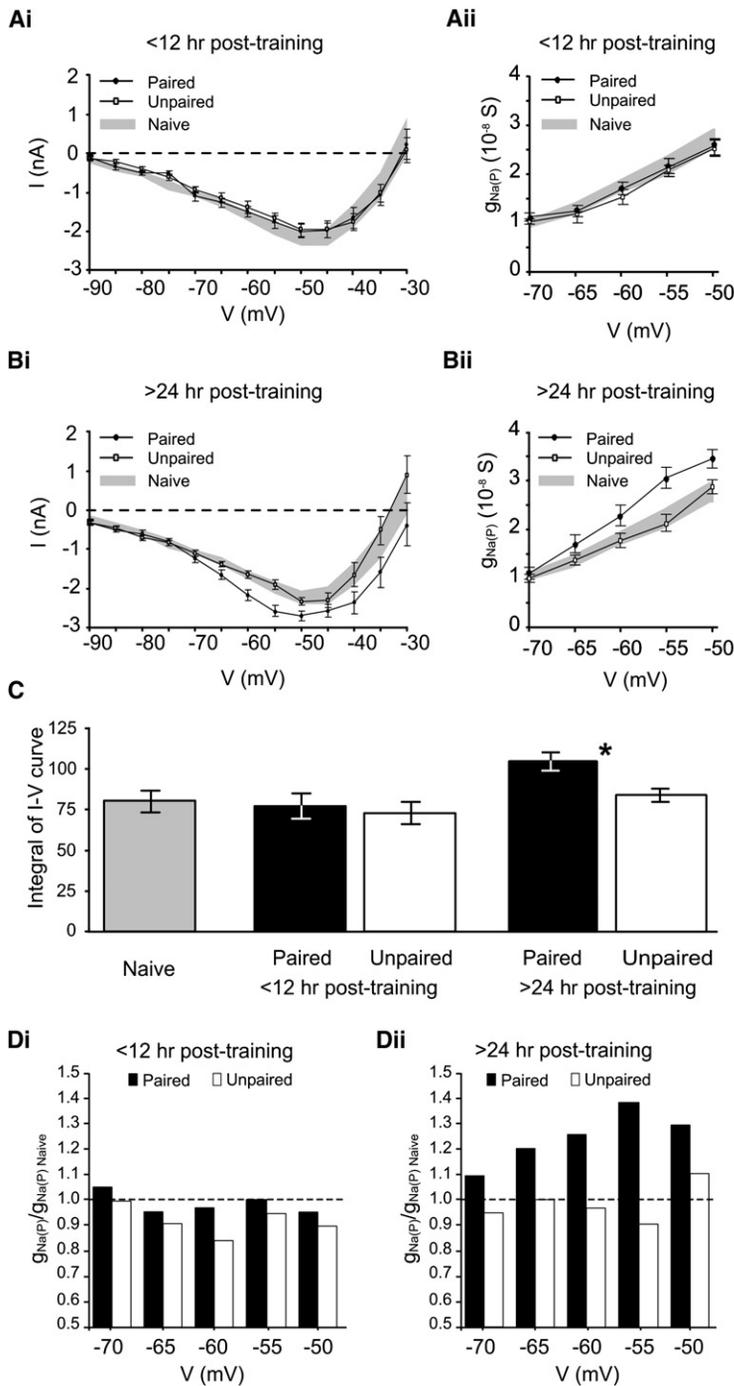


Figure 2. Delayed Learning-Induced Changes in $I_{Na(P)}$ and $g_{Na(P)}$ of the CGCs in a Broad Range of Activation Voltages

(A and B) (part i) Current-voltage (I-V) relationships of the steady-state persistent inward current recorded in CGCs from CS/US paired and unpaired control animals at <12 hr and >24 hr after training and from naive animals (gray band represents the range of mean \pm SE values in the latter group). (part ii) Calculated persistent sodium conductance ($g_{Na(P)}$) values in CGCs from CS/US paired and unpaired control animals at <12 hr and >24 hr after training and from naive animals.

(C) Means of the integrals of the I-V curves (\pm SE) calculated for the CS/US paired and unpaired control groups tested <12 hr after training and from the naive group.

(D) Ratios of paired and unpaired CGC $g_{Na(P)}$ to the naive CGC $g_{Na(P)}$ values calculated at the same voltage steps at <12 hr and >24 hr after training.

We performed an additional experiment to rule out the possibility that the learning-induced depolarization of the membrane potential resulted from the emergence of a new persistent current type not carried by sodium ion, rather than an increase in $I_{Na(P)}$. If this were the case, one still would expect to see a difference in the CGC membrane potential between paired and control preparations and a persistent inward current in CGCs in paired preparations, even in the absence of external sodium. In normal saline, the CGCs were significantly more depolarized in paired preparations ($n = 14$) versus each of the controls (naive, $n = 9$; unpaired, $n = 11$) (Figures 3A and 3Bi). However, when normal saline was replaced with a sodium-free one (Figure 3A), the CGC membrane potential stabilized at the same level, at ~ -80 mV, in all three groups (Figure 3Bii). Voltage-clamp tests in sodium-free saline with a 800 ms long voltage step from -110 mV to -55 mV detected no persistent inward current in the CGCs from either the paired or the control groups (Figure 3C), confirming that the increase in the size of the persistent inward current in CGCs from the paired group is indeed due to an enhanced persistent sodium conductance.

A patch-clamp-based analysis of the source of the increase in $I_{Na(P)}$ (i.e., increased conductance of single channels and/or an increased number of channels) would not have been feasible in the intact nervous system, and comparing isolated CGCs from trained and control animals was beyond the scope of the present work. We, however, performed immunohistochemical experiments by using an $Na_v1.9$ sodium channel antibody to establish whether there was

The activation voltage of the delayed rectifier potassium current I_K of the CGC is -50 mV [9]. With the experiments performed in normal saline, it was theoretically possible that in the command potential range positive to -50 mV, a learning-induced decrease in this outward current may have contributed to an increase in the net persistent inward current. However, our tests using the second voltage-clamp protocol (holding potential, -60 mV; stepping to levels from -50 mV to $+30$ mV in 10 mV increments) found no significant learning-induced changes in I_K across the whole test voltage range (Figure S1), which confirmed that the learning-induced increase in the net inward current over its entire activation range was due to the enhanced persistent inward current.

any sign of training-induced increased sodium channel density >24 hr after training. The persistent sodium current carried by this vertebrate channel type is very similar to the persistent sodium current we recorded from the CGC [10]. This similarity indicated possible structural conservation, which was supported by results of standard method control and specificity tests in western blot experiments with both *Lymnaea* and rat CNS homogenates and also in immunohistochemical sections from the *Lymnaea* CNS (Supplemental Experimental Procedures and Figure S2). By using immunohistochemistry, we found significantly increased specific staining in the CGCs in the CS/US paired group versus the CS/US unpaired and naive control groups (Figure 3D), indicating that an increase in the

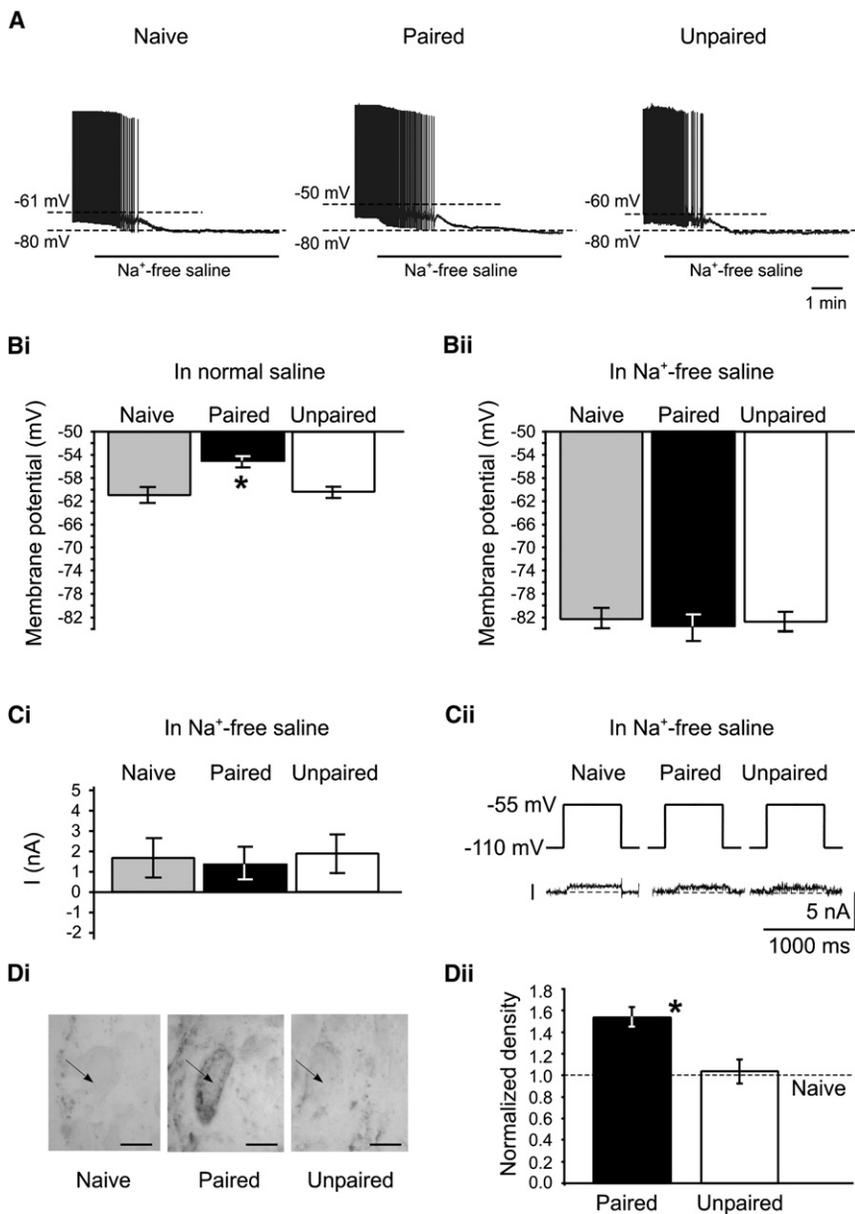


Figure 3. $I_{Na(P)}$ Channels Are the Substrates for Long-Term Memory >24 hr after Single-Trial Classical Conditioning

(A–C) In sodium-free saline, there is no difference in the membrane potential or persistent inward currents of CGCs from trained and control animals.

(A) Examples of voltage traces of CGCs from naive and CS/US paired and unpaired preparations being washed into sodium-free saline. The CGC membrane potential in all three different preparations hyperpolarized to ~ -80 mV, even though in normal saline the CGC from the CS/US paired preparation was depolarized by ~ 10 mV compared to the other two preparations.

(B) Statistical comparisons of CGC membrane potential data (means \pm SE) between the three groups in normal and sodium-free saline, respectively. ANOVA: (Bi) $F[2, 32] = 6.9, p < 0.04$; Tukey's, $p < 0.05$ (paired versus unpaired and naive). (Bii) $F[2, 29] = 0.9, p = 0.4$ (n.s.).

(Ci) A statistical comparison of steady-state currents (means \pm SE) evoked by 800 ms voltage steps from -110 mV to -55 mV in CGCs in preparations from the three experimental groups in sodium-free saline. ANOVA: $F[2, 22] = 0.9, p = 0.4$ (n.s.).

(Cii) Examples of the small persistent outward (most likely potassium) currents recorded in most CGCs in preparations from all three groups. (D) The density of Na_v1.9 channel-like proteins is increased in the CGC >24 hr after single-trial classical food-reward conditioning.

(Di) An example of increased immunostaining in CGC cell bodies (arrowed) from CS/US paired versus CS/US unpaired and naive animals. Scale bars represent 50 μ m.

(Dii) A statistical comparison of the density of immunostaining in CGCs (means \pm SE) from CS/US paired and unpaired animals ($*p < 0.006$, unpaired t test, $df = 10, t = 3.50$). Integrated density values obtained in the two experimental groups were normalized to the values measured in CGCs in sections from the naive group mounted on the same slide. These results correlate well with the findings from the current measurement experiments (see Figure 1A).

number of Na_v1.9-like sodium channels is a factor contributing to the enhanced persistent sodium conductance and current.

The Training-Induced Increase in $I_{Na(P)}$ Fully Accounts for the Depolarization of the CGC Membrane Potential in Conditioned Animals

Our current experiments demonstrated a learning-induced concomitant and correlated increase in $I_{Na(P)}$ and membrane potential depolarization in the CGCs. Previous work has shown that injection of cAMP into the soma both enhances $I_{Na(P)}$ and depolarizes the CGC membrane potential [10], whereas removal of sodium from the external medium strongly hyperpolarizes it [9, 10]. These three observations together indicated a possible causal link between the learning-induced increase in $I_{Na(P)}$ and depolarization of the membrane potential. To test this potential causality, we utilized the predictive power of a recently constructed Hodgkin-Huxley-type model of the electrical properties of the CGC soma membrane [15, 16]. This computational model, which is based on voltage-clamp

data from CGCs from naive animals obtained in a previous set of experiments [10], replicates accurately the spontaneous tonic activity and shape of action potentials of the CGCs (Figures S3A and S3B) and provides accurate descriptions of the effects of removing known ionic conductances, including $g_{Na(P)}$ (Figure S3C), on membrane potential, spike shape, and frequency [16]. We therefore hypothesized that the same model might also provide useful quantitative information about the effects of learning-induced increases in $g_{Na(P)}$ on the membrane potential, independently of the findings of the voltage-clamp experiments in CGCs from trained and naive animals.

$I_{Na(P)}$ difference values taken from the CS/US paired and naive groups were entered into the model without the modeler being aware of the origin of the current data or the measured CGC membrane potential values in the different groups, and predictions were made concerning the changes in membrane potential caused by changes in the maximal persistent sodium conductance $g(\max)_{Na(P)}$ (see Supplemental Experimental

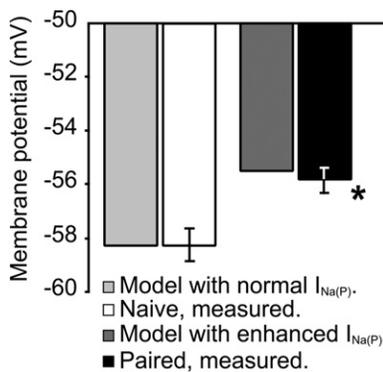


Figure 4. Computational Modeling of Learning-Induced Electrical Changes in the CGC

Enhancement of the maximal persistent sodium conductance $g(\max)_{Na(P)}$ (and therefore $I_{Na(P)}$) replicates the depolarizing effect of classical conditioning on membrane potential. The means (\pm SE) of the measured membrane potential data of real CGCs from the naive and CS/US paired groups ($n = 10$ preparations each) are shown together with the computed membrane potential value when $g(\max)_{Na(P)}$ was increased by 38.9% in a computational model of the naive CGC (for more technical details, see Supplemental Experimental Procedures).

Procedures for more detail). The model showed that if $g(\max)_{Na(P)}$ (and therefore $I_{Na(P)}$) is increased by 38.9% (the observed mean percentage difference in $I_{Na(P)}$ between the CS/US paired and naive group; Figure 1), this will depolarize the CGC soma membrane from -58.3 mV (the mean value of the naive group's CGC membrane potential) to -55.6 mV (Figure 4). The mean of the measured membrane potential values in the CS/US paired group was -55.8 mV, and this remarkably close match between the calculated and measured changes in MP (Figure 4) provides evidence that the learning-induced increase in $I_{Na(P)}$ is not only concomitant with and correlated to but also causal to the learning-induced changes in MP.

Discussion

The main experimental finding of this work is that the learning-induced somal membrane potential depolarization of a molluscan modulatory neuron involved in long-term memory [8] occurs concomitantly with and is correlated to an increase in the persistent sodium current of the same cell [9, 10] >24 hr after training. These findings were also corroborated by immunohistochemical experiments that showed a selective increase in the density of $Na_v1.9$ sodium channel-like proteins in the CGC soma membrane. A likely causal link between the learning-induced increase in $I_{Na(P)}$ and membrane potential depolarization was explored by computer simulation, which showed that the appropriate enhancement of $I_{Na(P)}$ in a computational model of the naive CGC can mimic the effect of classical conditioning on the somal membrane potential of the real neuron. Thus, for the first time we linked a learning-induced change in a persistent sodium current to nonsynaptic plasticity in an identified neuron involved in associative memory. This is therefore an example of a specific type of nonsynaptic plasticity with information now available on both its underlying ionic mechanisms and involvement in long-term network and behavioral plasticity [8].

Previous work examining the ionic mechanisms of learning-induced nonsynaptic plasticity found that the main types of currents that contribute to increased intrinsic excitability are

carried by calcium and potassium ions or through hyperpolarization-activated cationic channels [7]. We found no training-induced decrease in the delayed rectifier type potassium current, which in theory could have contributed to membrane potential depolarization. The previously identified calcium currents of the CGC [9] inactivate too rapidly to have any maintained effect on the membrane potential. Our previous analyses found no evidence for the types of change in neuronal excitability that could indicate changes in calcium currents or the A-type potassium current [8] or for the presence of a hyperpolarization-activated current [9]. Thus, it seems that unlike other types of nonsynaptic plasticity, training-induced somal depolarization is uniquely based on a change in a persistent sodium current.

A persistent sodium current also exists in the mammalian hippocampus [17, 18], but potential links between $I_{Na(P)}$ and known examples of learning-induced nonsynaptic plasticity [2, 5, 7] in this key brain area for associative learning have not been explored yet. In the light of our present findings, it would be interesting to investigate how learning-induced changes in $I_{Na(P)}$ might contribute to already known and yet-to-be-discovered forms of associative nonsynaptic plasticity in the mammalian brain.

At systems level, previously only pathological forms of plasticity, such as hyperalgesia, neuropathic pain, and epilepsy have been linked to an increase in $I_{Na(P)}$ [19–22], so our work is the first to show a role for this type of current in an important nonpathological form of plasticity, long-term memory after associative learning.

Supplemental Data

Supplemental Data include Supplemental Results, Supplemental Experimental Procedures, and three figures and are available at <http://www.current-biology.com/cgi/content/full/18/16/1221/DC1/>.

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