Activity-Independent Homeostasis in Rhythmically Active Neurons

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Summary

The shal gene encodes the transient potassium current (I_A) in neurons of the lobster stomatogastric ganglion. Overexpression of Shal by RNA injection into neurons produces a large increase in I_A, but surprisingly little change in the neuron’s firing properties. Accompanying the increase in I_A is a dramatic and linearly correlated increase in the hyperpolarization-activated inward current (I_h). The enhanced I_h electrophysiologically compensates for the increased I_A, since pharmacological blockade of I_h uncovers the physiological effects of the increased I_A. Expression of a non-functional mutant Shal also induces a large increase in I_h, demonstrating a novel activity-independent coupling between the Shal protein and I_h enhancement. Since I_A and I_h influence neuronal activity in opposite directions, our results suggest a selective coregulation of these channels as a mechanism for constraining cell activity within appropriate physiological parameters.

Introduction

Many neurons, as functional units in networks generating complex behaviors, must develop and maintain a stable physiological identity despite continuously changing inputs both during development and in the adult. Each neuron’s intrinsic excitable properties and preferred activity range are defined by the pattern of specific ion channels, receptors, and enzymes that it expresses. One important mechanism for developing and maintaining stable neuronal function is activity-dependent homeostasis: both vertebrate and invertebrate neurons can restore normal firing patterns after imposed changes in firing activity induced, for example, by loss of normal synaptic inputs or prolonged pharmacological block of activity. Such activity-dependent responses involve slow compensatory changes in the balance of ionic currents expressed in the neuron (LeMasson et al., 1993; Turrijiano, 1999; Turrijiano et al., 1994, 1995, 1998; Desai et al., 1999; Golowasch et al., 1999; Spitzer, 1999; Galante et al., 2001). However, since the trigger for the homeostatic response is a change in activity, the neuron must at least temporarily fire in an abnormal fashion.

Here we report a novel activity-independent homeostatic mechanism that could function in concert with activity-dependent mechanisms to maintain neuronal firing patterns within their normal ranges. We demonstrate that a neuron can sense and adapt to overexpression of one ion channel type in the absence of significant changes in physiological activity. This is accomplished by increasing the amplitude of a second current with some opposing physiological effects, providing a balancing electrophysiological compensation for the induced current.

We set out to study the role of the shal gene, a member of the shaker family of voltage-dependent K⁺ channel genes, in rhythmically active pyloric dilator (PD) neurons in the stomatogastric ganglion (STG) of the spiny lobster, Panulirus interruptus. These neurons are members of the pacemaker kernel of the pyloric network, a well-understood rhythmic motor pattern generator (Johnson and Hooper, 1992; Ayali and Harris-Warrick, 1999). The PD neurons fire rhythmic bursts of action potentials superimposed on slow membrane potential oscillations. The relatively stereotyped oscillatory properties of these neurons are determined by the complement of ion channels they express and their synaptic inputs (Getting, 1989; Selverston and Moulins, 1985). The fast transient potassium current (I_h) helps to shape PD neuron firing properties, including the maximal spike frequency during a burst and the rate of postinhibitory rebound (Tierney and Harris-Warrick, 1992). Single-cell RT-PCR and immunocytochemistry have shown that the shal gene encodes I_h in pyloric neurons within the STG (Baro et al., 1997, 2000, 2001; Baro and Harris-Warrick, 1998). To further study the role of I_h, we artificially increased I_h amplitude by microinjecting shal RNA into the PD neurons. Despite large increases in I_h amplitude, the firing properties of the neurons were essentially unchanged. Surprisingly, the increased I_h is compensated by a corresponding endogenous upregulation of the hyperpolarization-activated inward current, I_A. Our findings suggest a novel level of regulation for specific ion channels that promotes stability in the activity of individual neurons as well as the entire network.

Results

Significant Increase in I_h 72 hr after Microinjection of shal-GFP RNA

To visualize protein expression after shal RNA injection, the sequence for green fluorescent protein (GFP) was ligated near the C-terminal end of the shal clone. GFP ligation had no effect on the biophysical properties of the Shal current when expressed in Xenopus oocytes: the amplitude of the current, voltage dependence of activation and inactivation, and rates of inactivation were all unaffected (n = 6). To be sure that appending GFP had no effect, we carried out parallel measurements with both shal-GFP and shal RNA injections into the PD neurons for all the experiments described below, using an anti-Shal antibody to visualize successful Shal expression after the physiological measurements. No sta-
Subtle Effects of Shal Overexpression on Rhythmic Activity and Intrinsic Firing Properties

In preparation for our experiments studying the physiological consequences of manipulating shal expression, we established an organotypic culture system of the STG with maximal physiological stability. Culturing STG neurons with altered modulatory inputs can modify the complement of active ionic currents (Turrigiano et al., 1994). To avoid this, we cultured the STG with the appropriate motor nerves and associated commissural and esophageal ganglia attached (Thoby-Brisson and Simmers, 1998; see Experimental Procedures). Baseline pyloric activity remained stable for at least 3 days in culture with this approach; the only parameter that was significantly affected by culturing was the number of spikes.
per burst, which decreased by 33% after 72 hr in culture (Figure 1D).

Since modest (10%–25%) changes in $I_h$ significantly change the firing properties of STG neurons (Tierney and Harris-Warrick, 1992; Kloppenburg et al., 1999), we expected that overexpression of shal or shal-GFP would also alter the firing properties of individual PD neurons. Surprisingly, we found only subtle changes in PD electrophysiological activity after Shal or Shal-GFP expression. (Figure 1C). When we quantified the parameters of PD activity in the pyloric rhythm 72 hr after injection, the cycle frequency, oscillation amplitude, minimal membrane potential, slope of the rising phase of the oscillation, and spikes per burst, all factors that are affected by $I_h$ amplitude (Tierney and Harris-Warrick, 1992), were not significantly changed after shal expression (Figure 1D).

We recorded the PD neuron activity at 8 hr intervals from the time of injection to the final recordings at 72 hr. At no time did the shal-injected PD neuron show any significant change in activity compared to the controls, or compared to that seen in the acutely isolated neurons (n = 6). Thus, injection of shal RNA does not appear to alter the firing properties in the actively cycling preparation at any time after injection.

One possible explanation was that synaptic inputs from the actively cycling network masked more subtle changes in PD firing properties following the increase in $I_h$. To test this, we isolated PD neurons from all detectable synaptic input by a combination of 5,6-carboxyfluorescein photoactivation of electrotonically coupled neurons and blockade of descending inputs with tetrodotoxin applied in a pool around the innervating nerve (see Experimental Procedures), and we examined their posthyperpolarization rebound properties. The isolated neurons, in current clamp, were held at −50mV and then hyperpolarized to various levels before being depolarized just above action potential threshold (Figure 2). The latency to the first action potential following the hyperpolarizing step and the first interspike interval (ISI, time between first and second spikes) are positively correlated to $I_h$ amplitude (Harris-Warrick et al., 1995a; Tierney and Harris-Warrick, 1992), yet both parameters showed only small changes after Shal expression following large hyperpolarizations. For example, after a step to −100mV, we found a significant 40% increase in the first ISI (64 ± 6 ms in control to 89 ± 11 ms in Shal-expressing neurons; p < 0.05; n = 10) and a 28% increase in the first spike latency (Figure 2B; 170 ± 80 ms in control to 218 ± 72 ms in Shal-expressing neurons; p = 0.06; n = 10). Both effects are expected with an increase in $I_h$ amplitude (Tierney and Harris-Warrick, 1992), but were unexpectedly small, since dopamine, which inhibits PD neurons primarily by an increase of only 10% in $I_h$ amplitude, produced similar increases in first ISI and first spike latency (Kloppenburg et al., 1999).

**Increase in $I_h$ Is Accompanied by an Increase in $I_h^*$**

In the isolated Shal-expressing PD neurons, we also noted a significant increase in the amplitude of the depolarizing voltage sag during the constant hyperpolarizing current step (Figure 2A). This sag voltage increased in the Shal-expressing neurons by 100% after a 500 ms step to −100mV (2.2 ± 1.1 to 4.4 ± 1.7mV; p < 0.01; Figure 2B) and by 180% at −120mV (4.1 ± 3.5 to 11.3 ± 5.2mV; p < 0.01; n = 10). The sag voltage is produced by the hyperpolarization-activated inward current, $I_h$, which participates in postinhibitory rebound, oscillations, bistability, and increasing spike frequency (Kiehn and Harris-Warrick, 1992; Harris-Warrick et al., 1995a; McCormick and Pape, 1990; Accili et al., 2002). Consistent with the increased sag voltage, PD neurons which expressed Shal and consequently had an increased $I_h$ (Figure 3A) showed a dramatic increase in $I_h$ amplitude when measured by voltage clamp (Figure 3B). The increased current was slowly activated during long hyperpolarizing voltage steps from −40mV and was completely blocked by 10 mM Cs⁺ (Figure 3C; n = 4), characteristic features of $I_h$. We measured the amplitude of $I_h$ as the difference between the value reached at the end of the instantaneous initial step and the value reached at the end of the voltage step. During a voltage step to −90mV (Figure 3B), there was a 541% increase in $I_h$ amplitude in Shal-GFP-expressing neurons (27 ± 17 nA versus 4.1 ± 2.3 nA in control neurons, n = 14 each; p < 0.05). The enhanced current had somewhat slower activation and deactivation parameters and a slightly hyperpolarized voltage activation curve compared to the endogenous $I_h$; we do not know the molecular basis for this difference (J.N. MacLean et al., unpublished data). As a consequence, the $I_h$ enhancement increased with the size of the hyperpolarizing voltage step; however, even at physiological voltages (−50 to −60mV) there was a 200%–
300% increase in $I_h$ (Figure 3D). This may be an underestimation of the increase in $I_h$ at physiological voltages, due to the difficulty of clamping $I_h$ that may be localized in the electrically remote neuropil. Identical results were seen after injecting shal RNA lacking the GFP tag ($n = 5$). The Shal-expressing neurons also had an increase in the instantaneous current jump at the beginning of the hyperpolarizing voltage step (Figures 3B and 3C; see also Figure 6B). This current was significantly reduced by Cs$^+$ (Figure 3C); during a voltage step to $-90 \text{ mV}$, 10 mM Cs$^+$ reduced the instantaneous current amplitude by 45%, from $29 \pm 8 \text{ nA}$ to $20 \pm 5 \text{ nA}$ ($p < 0.05$; $n = 4$), not significantly different from the instantaneous current in control neurons ($16 \pm 10 \text{ nA}$; $p = 0.7$), which was itself not sensitive to Cs$^+$ ($n = 3$; see also Kiehn and Harris-Warrick, 1992). In addition, the extrapolated reversal potential of the Cs$^+$-sensitive current was the same as that for $I_h$. This suggests that the enhanced instantaneous current in Shal-overexpressing PD neurons arises from an increase in $I_h$ that is active at the holding potential.

The increase in $I_h$ was temporally linked with the increase in $I_A$ over time after shal RNA injection, as no delay between the increase in $I_A$ and $I_h$ was observed; both currents began to increase at 48–60 hr and grew in parallel. As a consequence, the increase in $I_A$ shows a steep positive correlation with the increase in $I_h$ (Figure 3E). If we compare Shal-expressing and control PD pairs from the same ganglion (to reduce variation between individual animals), the percent increase of $I_h$ in the Shal-expressing neurons shows a strong positive relationship with the percent increase in $I_A$ ($R = 0.764$ for a linear relationship; $p < 0.05$; $n = 8$ pairs). This strongly suggests a causal relationship between the increases in $I_A$ and $I_h$.

**Specificity of the Effect: Other Currents Are Unmodified Following shal Expression**

Upregulation of $I_h$ could counterbalance an induced increase in $I_A$, since both currents are active at voltages below spike threshold, are of opposite sign, and have opposite effects on postinhibitory rebound and spike frequency (Harris-Warrick et al., 1995b). To determine whether there was a specific link between the increases in $I_A$ and $I_h$, or whether injection of shal RNA induces a nonspecific upregulation of other ionic currents, we measured three other currents in control and Shal-GFP-expressing PDs: the voltage-dependent calcium current $I_{Ca}$, the calcium-dependent potassium current $I_{K(Ca)}$, and the noninactivating voltage-activated potassium current $I_{K(V)}$. We made these comparisons using pairs of PD neurons from the same ganglion to reduce interindividual variability.

$I_{Ca}$ was of particular interest, as the level of intracellular calcium has been proposed as the monitor for activity-dependent homeostatic processes (Turrigiano et al., 1994). Following expression of shal-GFP, as monitored by fluorescence, there was no significant change in $I_{Ca}$ from the control PD neurons. During a step to $-10 \text{ mV}$, the current was $9 \pm 4 \text{ nA}$ in control and $8 \pm 5 \text{ nA}$ in Shal-GFP-expressing PDs ($n = 5$ pairs; $p = 0.4$; Figure 4Ai). These currents often showed a delayed secondary inward peak reflecting currents generated in distal regions of the cell that were not under adequate voltage control (B.R. Johnson et al., 2001, Soc. Neurosci., abstract). We limited our analysis to the first peak of cur-
Figure 4. Specificity of the Shal-Evoked Increase in $I_h$

(A-C) Shal expression does not modify other currents. Left: representative currents (average of 10 steps to the indicated voltage) of $I_{Ca}$ (Ai), $I_{K(Ca)}$ (Bi), and $I_{K(V)}$ (Ci). In each case, the upper current is from the shal-GFP-injected neuron, while the lower current is from the control neuron in the same ganglion. Right: current-voltage relationships of $I_{Ca}$ (Aii), $I_{K(Ca)}$ (Bii), and $I_{K(V)}$ (Cii).

(D and E) Injection of shaker RNA does not enhance $I_h$. A confocal image of anti-Shaker labeling in a shaker injected PD neuron (D). $I_h$ measurements at $-90\text{mV}$ in a PD neuron pair, one of which was injected with shaker RNA (E).

Current. The mean current/voltage relationship of $I_{Ca}$ was unmodified by Shal expression (Figure 4Ai).

Following blockade of $I_{Ca}$ with 4-aminopyridine (4-AP), the remaining outward current consisted of a calcium-dependent ($I_{K(Ca)}$) and a calcium-independent component ($I_{K(V)}$). $I_{K(Ca)}$ was isolated after blockade of $I_{K(Ca)}$ with 600 μM extracellular CdCl$_2$ (Kloppenburg et al., 1999). No difference in $I_{K(Ca)}$ was observed between control and experimental PDs at any voltage (n = 6 pairs). During a voltage step to $+20\text{mV}$, the $I_{K(Ca)}$ amplitude was $44 \pm 4 \text{nA}$ in control and $43 \pm 3 \text{nA}$ in Shal-GFP-expressing PDs (p = 0.5) (Figure 4Bii). The mean current/voltage relationship of $I_{K(Ca)}$ was unmodified by expression of Shal (Figure 4Bii). $I_{K(Ca)}$ was isolated from the 4-AP-resistant total outward currents by digitally subtracting the $I_{K(V)}$ subsequently measured in the same cell in the presence of Cd$^{2+}$. Once again, there was no measurable difference in $I_{K(Ca)}$ between PD neuron pairs at any voltage (n = 6 pairs). In response to a voltage step to $+20\text{mV}$, $I_{K(Ca)}$ amplitude was $360 \pm 8 \text{nA}$ in control and $331 \pm 6 \text{nA}$ in Shal-expressing PDs (p = 0.2; Figure 4Cii). The mean current-voltage relationship of $I_{K(Ca)}$ was unmodified by Shal expression (Figure 4Cii). These results, combined with the linear relationship between increases in $I_{Ca}$ and $I_h$, are consistent with a specific linkage between $I_{Ca}$ and $I_h$, the remaining outward current is selectively targeted to the axons of STG neurons, outside the ganglion and beyond the range of detection using voltage clamp (Baro et al. 2000). Following injection of shaker RNA, we demonstrated successful ex-
pression of the Shaker gene using immunocytochemical labeling of the Shaker protein (Figure 4D). We had previously found that Shaker expression does not lead to an increase in \( I_h \) amplitude as measured by voltage clamp from the soma (data not shown). The intensity of Shaker labeling in the soma far surpassed that observed for the native channel (Baro et al. 2000). However, there was no increase in \( I_h \) amplitude at any voltage tested. For example, in response to a voltage step to \(-90\) mV (Figure 4E), following expression of Shaker protein, the measured current amplitude of \( I_h \) was \( 4.3 \pm 2.3 \) nA (\( n = 5 \), including two pairs), which is not different from \( I_h \) measured in control PDs (\( 4.1 \pm 2.3 \) nA, \( p = 0.5 \)). This suggests that the link between shal and \( I_h \) may be specific to this protein.

**Pharmacological Reduction of \( I_h \) Reveals the Electrophysiological Effect of Increased \( I_h \) in shal-Injected Neurons**

We hypothesize that the Shal-expressing neurons do not change their firing properties because the compensatory increase in \( I_h \) counteracts the effects of our induced increase in \( I_h \). To test this hypothesis, we applied a submaximal dose of Cs\(^+\) (5 mM) to synaptically isolated PD pairs in the same ganglion, blocking \( I_h \) by approximately 70\% (\( n = 4 \) pairs). In both the control and Shal-expressing PD neurons, the sag voltage in current clamp was significantly reduced by 5 mM Cs\(^+\) (Figure 5A). Addition of Cs\(^+\) to control PDs evoked a 23\% increase in the latency to the first spike following hyperpolarization to \(-120\) mV, (117 ± 28 versus 144 ± 35 ms). However, in the PD neurons expressing Shal-GFP, Cs\(^+\) evoked a significantly larger increase (59\%) in the first spike latency (146 ± 23 versus 237 ± 78 ms following hyperpolarization to \(-120\) mV; Figure 5B). Enhancement of the Cs\(^+\) effect on first spike latency in Shal-expressing neurons was significant at all prestep voltages tested (\( p < 0.05 \)). Similarly, the first interspike interval was also more sensitive to \( I_h \) blockade in the Shal-GFP-expressing neurons than in the controls. Following application of Cs\(^+\) to control neurons, the first ISI increased by 40\% (34 ± 4 versus 46 ± 2 ms following a step to \(-120\) mV), but in paired Shal-GFP-expressing neurons, the first ISI increased by 79\% (43 ± 1 versus 78 ± 4 ms; Figure 5B). This difference was also significant at all prestep voltages tested (\( p < 0.025 \)). Thus, by pharmacologically reducing \( I_h \), we revealed the physiological effect of the shal-evoked increase in \( I_h \) on the neuronal firing properties. These data indicate that the neuronal increase in \( I_h \) does indeed counteract the physiological effect of the artificial increase in \( I_h \).

**Expression of a Nonfunctioning Shal Protein**

**Results in an Increase in \( I_h \)**

We emphasize that the shal-evoked increase in \( I_h \) and the compensatory increase in \( I_h \) occurred during a period when there were no significant changes in PD rhythmic activity. To verify that this compensatory increase in \( I_h \) was truly activity independent, we generated an inactive shal-GFP mutant. We mutated the potassium channel signature GYG sequence (aa 372–374) in the pore region of the Shal channel to AFA (Figure 6Aii), resulting in a protein that, when expressed in *Xenopus* oocytes, did not generate a functional channel (data not shown). When expressed in PD neurons (as monitored by GFP fluorescence, Figure 6Ai), this protein did not modify \( I_h \) amplitude compared to controls (at \(+30\) mV, 383 ± 9 versus 379 ± 29 nA; \( p = 0.4 \); \( n = 5 \); Figures 6Bi and Cii). Despite this, injection of the mutant RNA evoked a 375\% ± 29\% increase in \( I_h \) amplitude during a voltage step to \(-90\) mV (15 ± 5 versus 4 ± 2 nA in control neurons; \( p < 0.005 \); \( n = 5 \) pairs; Figures 6Bi and Cii). This increase in \( I_h \) following expression of the mutant Shal was not significantly different from the \( I_h \) increase following expression of normal Shal (\( p = 0.4 \)).

As expected for an increase in \( I_h \) that is uncompensated by an increase in \( I_h \), these neurons displayed marked changes in their rhythmic activity relative to control PD neurons in the same ganglion (Figure 7A). They were more depolarized (\(-46.0 \pm 8.2 \) versus

![Figure 5. Blockade of \( I_h \) Uncovers the Effect of Enhanced \( I_h \) in shal-Expressing PD Neurons](image-url)
There was no change in the oscillation cycle frequency, cially elevating the transient K neurons (n/H11005/H11006/H11006 activity of the mutant shal due to the increase in I_h that was not compensated by is a negligible change in activity of the oscillating PD Activity-dependent homeostasis is thought to be medi-ated by changes in gene transcription (Thoby-Brisson as capable of elevating I_h levels as the normal Shal RNA shal-GFP does not alter the properties of I_A and thus cannotinteractions (1.4 since this is dominated by other neurons and network in a cell, we showed that there is a compensatory upreg-
sate for this unbalanced increase in I_h. IA. Further indication that the mechanism is activity-inde-
neuron did not activate additional currents to compen-
sor; p < 0.05) compared to control (B) IA (Bi) and Ih (Bii) in the control (gray) and mutant Shal-GFP (black)
expressing neurons. (C) Mean amplitude of I_h (±30mV, [Cii]) and I_h (±90mV, [Ciii]) after expression of mutant Shal compared to control neurons (n = 5 pairs).

-52.4 ± 8.3mV at the trough of the oscillation; p < 0.01), had larger amplitude oscillations (10.5 ± 5.4 versus 8.0 ± 4.9mV; p < 0.01), had a steeper slope of the rise phase (0.046 ± 0.001 versus 0.028 ± 0.001mV/ms; p < 0.01), and exhibited more spikes per burst (6.8 ± 1.7 versus 5.0 ± 1.0; p < 0.01) and larger amplitude spikes (8.7 ± 2.6 versus 7.5 ± 2.6mV; p < 0.05) compared to control neurons (n = 5 pairs for all measurements; Figure 7B). There was no change in the oscillation cycle frequency, since this is dominated by other neurons and network interactions (1.4 ± 0.4 versus 1.4 ± 0.5 Hz). Thus, the activity of the mutant shal-injected neurons changed due to the increase in I_h that was not compensated by an increase in I_h. This experiment also suggests that the neurons’ ability to compensate for induced changes in ionic currents is limited; after I_h had been elevated in response to expression of the mutant Shal protein, the neuron did not activate additional currents to compensate for this unbalanced increase in I_h.

The Increase in I_h Is Not Affected by a Transcription Blocker
Activity-dependent homeostasis is thought to be mediated by changes in gene transcription (Thoby-Brisson and Simmers, 2000). To test whether activity-indepen-
dent homeostasis also requires changes in transcription, we studied the effect of the RNA synthesis inhibitor actinomycin-D (ACD) (Pedreira et al., 1996; Reich and Goldberg, 1964), which effectively blocks transcription in crustaceans, including spiny lobsters, at concentrations of 20–100 µM (Eads and Hand, 1999; Pedreira et al., 1996; Thoby-Brisson and Simmers, 2000). ACD (100 µM) was applied to four PD pairs throughout the 72 hr in culture. Rhythmic activity was unmodified by the presence of ACD in the bath (data not shown; Thoby-Brisson and Simmers, 2000). Since translation is unmodified by ACD, there was a large increase in I_h amplitude following injection of shal-GFP RNA. For example, during a step to +30mV, the shal-GFP-injected PD neurons exhibited a 72% increase in I_h (636 ± 42 versus 370 ± 41 nA; p < 0.05). Despite the presence of ACD, we observed an accompanying increase in I_h, comparable to that observed in the absence of ACD at all measured potentials. A 725% increase in I_h was observed at −90mV (−33 ± 21 versus −4 ± 3 nA; p < 0.001); this increase is not significantly different from that seen in the absence of ACD (p = 0.6). These results suggest that activity-independent homeostasis occurs in the presence of ACD at concentrations that abolish activity-dependent homeostasis (Thoby-Brisson and Simmers, 2000) and that the compensatory increase in I_h is regulated by posttranscriptional mechanisms.

Discussion

While most of the research on neuronal plasticity fo-cuses on understanding how neurons’ activity can be changed (for example, with learning), an equally impor-tant issue is how neurons develop a unique set of prop-erties and then maintain them within a specific range in the face of continually changing inputs. Many neuron types use activity-dependent homeostatic mechanisms, where induced changes in activity trigger compensatory changes in the ratios of voltage-dependent ion channels and receptors (LeMasson et al., 1993; Turrigiano, 1999; Turrigiano et al., 1994, 1995, 1998; Desai et al., 1999; Golowasch et al., 1999; Spitzer, 1999). However, for these activity-dependent mechanisms to be called into play, the neurons must fire outside their normal range for a period of time.

Here, we have demonstrated a novel activity-independent mechanism for homeostasis in neurons. After artifi-cially elevating the transient K⁺ current (I_h) expressed in a cell, we showed that there is a compensatory upreg-u-lation of an endogenous pacemaker current (I_h). During the 3 days required for the expression of Shal, there is a negligible change in activity of the oscillating PD neurons. Even when isolated from normal synaptic inter-actions and given nonphysiological steps of current in-jection, the neuron shows only modest changes in post-inhibitory rebound and spike frequency, far smaller than would be expected from the magnitude of increase in I_h. Further indication that the mechanism is activity-inde-pendent comes from our use of a mutant shal RNA, encoding a nonfunctioning potassium channel. This does not alter the properties of I_h and thus cannot change the activity of the neuron, but was nevertheless as capable of elevating I_h levels as the normal Shal protein.
Specificity of the shal RNA Effect
Several laboratories have reported the nonspecific enhancement of a hyperpolarization-activated inward current, $I_h$, in Xenopus oocytes after injection of RNA encoding a number of membrane-bound proteins, including minK/IsK, the shaker potassium channel, the BIR9 nonconducting member of the inward rectifier potassium channel family, the dopamine D2 receptor (Tzounopoulos et al., 1995), phospholemman (Moorman et al., 1992), and the NB protein of the influenza B virus (Shimbo et al., 1995). However, this nonspecific response is distinct from that seen in our experiments with shal injections into neurons. First, unlike the Xenopus $I_h$ current, which is activated only at nonphysiological spike voltages (below ~130mV) (Kuruma et al., 2000), the $I_h$ enhanced by shal RNA injection into PD neurons becomes active at physiologically significant subthreshold voltages. Second, activation of $I_h$ does not serve any obvious function in the oocytes, nor does it compensate for the proteins that were expressed. In the PD neurons, $I_h$ is an inward current that can compete with the shal-evoked outward $I_a$ at subthreshold voltages, and it actively contributes to maintaining the normal firing properties of the shal-injected PD neurons (see below). Its amplitude shows a strong linear correlation with the increased level of $I_h$ in the injected neurons, suggesting that it is specifically enhanced by the increase in Shal protein. Third, shal expression does not alter other ionic currents that we tested, including $I_{CaL}$, $I_{KCa}$, and $I_{K(V)}$. Thus, increases in Shal protein appear to selectively enhance $I_h$ as part of a homeostatic compensatory mechanism. Finally, we successfully overexpressed in PD neurons the lobster shaker gene, which encodes an $I_h$ selectively targeted to the axons of STG neurons. The presence of the Shaker channel protein did not produce an increase in $I_h$. Expression of GFP alone also did not alter $I_h$. These results suggest that the link between Shal and $I_h$ is not simply an artifact of channel RNA injection, and thus may be specific. It is, of course, possible that increased expression of other membrane proteins may also enhance $I_h$; even so, it would not detract from the physiological relevance of the link between shal and $I_h$.

Physiological Mechanisms of Neuronal Homeostasis
This activity-independent homeostasis coregulates two currents that are active in the critical voltage range below spike threshold; some of their physiological effects are opposing, and their ratio has been previously demonstrated to regulate neuronal spike and pacemaker activity (Angstadt and Calabrese, 1989; Harris-Warrick et al., 1995b). We previously showed in pyloric neurons that the rate of postinhibitory rebound and the initial spike frequency after inhibition are coregulated by $I_h$ and $I_a$ (Harris-Warrick et al., 1995b); pharmacologically induced decreases in $I_h$ led to more rapid postinhibitory rebound and shorter first ISIs, while pharmacologically induced decreases in $I_a$ had the opposite effect. In the shal-expressing neurons, both $I_h$ and $I_a$ are significantly increased, but in such a ratio that their balance of activity is retained, and the cell shows no change in firing activity. We were able to disturb this balance in either direction. Partial blockade of $I_h$ with Cs$^+$ uncovered an exaggerated delay in postinhibitory rebound and an increase in ISI that is caused by the shal-evoked increase in $I_a$. In contrast, injection of the nonfunctional shal mutant RNA evoked a large increase in $I_h$ that was not balanced by an increase in $I_a$; this led to opposite effects on firing properties compared to those evoked by Cs$^+$. We obtained varying levels of expression of $I_h$ in different PD neurons, and these were accompanied by the appropriate (and linearly correlated) enhancement of $I_h$ to maintain the firing properties of the neurons within their normal range of activity. This suggests that different levels of $I_h$ and $I_a$ expression can be tolerated in the PD neurons while still maintaining an essentially normal pattern of firing, as long as the functional balance between the two is preserved (Figure 1D).

Golowasch et al. (1999) demonstrated that there can be as much as a 2- to 4-fold variability in the density of
five voltage-dependent currents in an identified pyloric neuron between animals; nonetheless, the firing properties of the neuron show a stereotyped pattern of activity that does not change significantly from animal to animal. Goldman et al. (2001) modeled these data and identified directions in the parameter space, representing coordinated changes in sets of ionic conductances, where the basic pattern of neuronal activity did not change. This suggests that specific ratios of currents, rather than set levels of expression of individual currents, critically determine cell activity. The coordinated changes in \( I_A \) and \( I_h \) that we observed are an example of how rather dramatic changes in the levels of two currents will not alter the firing properties of the neuron so long as their ratio remains in the correct range. This implies that the relation between \( I_A \) and \( I_h \) may represent an important pairing of currents for maintaining PD stability. We do not know whether this regulation is bidirectional, that is, whether artificial increases in \( I_h \) would also lead to compensatory increases in \( I_A \). Resolution of this issue will require cloning and expression of the lobster gene encoding \( I_h \).

Possible Mechanisms of Activity-Independent Homeostasis

Our results raise interesting issues about the possible molecular mechanisms underlying this regulatory interaction. First, the increase in \( I_h \) appears to be regulated at a step after transcription, as it is not affected by ACD at concentrations that block transcription in crustaceans by over 90% (Eads and Hand, 1998; Thoby-Brisson and Simmers, 2000). Second, the increase in \( I_h \) amplitude relies on translation of \( shal \) RNA, because when the injected \( shal \) or \( shal-GFP \) RNA was not successfully translated, there was no change in \( I_h \). Further, although all neurons were injected with approximately the same amount of \( shal \) RNA, there is a linear relationship between the varying amount of \( I_h \) (which is likely related to the amount of protein made) and \( I_h \) (Figure 3E). Third, the increase in \( I_h \) is not dependent on any actual increase in functional \( I_h \), since equivalent increases in \( I_h \) were seen after injection of the mutant \( shal \) RNA that did not alter the level of \( I_h \). Thus, it appears that the increase in \( I_h \) only requires the presence of increased Shal protein in the cell.

Several mechanisms could explain how \( I_h \) increases in response to an increase in Shal protein. One possibility is that increased Shal protein could trigger a post-translational modification (such as phosphorylation) to activate existing but inactive \( I_h \) channels. Another possibility is that increased Shal proteins could enhance membrane insertion of a preexisting depot of \( I_h \) channels. We consider both of these possibilities to be unlikely due to the magnitude of the increase in \( I_h \), which can be up to 20-fold (Figure 3D). A third possibility is that the coordination occurs during translation, and that the RNAs encoding \( shal \) and \( I_h \) are cotranslated in some fixed ratio. Recent research has demonstrated that RNA trafficking can be mediated via RNA granules, dense structures containing multiple species of mRNA, ribosomes, and the Staufen protein, which are found in the somatodendritic compartment (Blichenberg et al., 1999; Wanner et al., 1997). RNAs with similar trafficking pathways are often coassembled into the same granules (Carson et al., 2001). Importantly, RNA granules can incorporate exogenously introduced RNA (Ainger et al., 1993; Rook et al., 2000). It has been suggested that coassembly of multiple RNAs into the same granules acts as a mechanism to coordinate gene expression (Moulard et al., 2001). Thus, it is possible that \( shal \) RNA localizes to RNA granules containing \( I_h \) channel RNA and that the two proteins are cotranslated. A fourth possibility is that \( I_A \) and \( I_h \) channels are coassembled at some stage preceding membrane insertion. The long delay between \( shal \) RNA injection and an increase in current may reflect \( I_h \) channels waiting for translation of \( I_h \) channels before both are inserted together into the membrane. Indirect support for this possibility comes from the failure of Shaker overexpression to increase \( I_h \). Previous experiments have demonstrated that Shaker overexpression does not result in an increase in \( I_h \) amplitude measured in the soma, so upregulation of \( I_h \) may require insertion of the coupled channel protein. An equally likely explanation for this result, however, is a direct and specific link between Shal and \( I_h \) proteins. A final possibility is that increased Shal protein reduces the steady-state degradation of \( I_h \) channel protein. These possible mechanisms are not mutually exclusive.

Implications for Neuronal Function

While the functional role of activity-independent homeostasis in normal function is not known, several possibilities can be envisioned. During development, neurons often achieve adult firing patterns while they are small, and undergo dramatic increases in volume and surface area while maintaining the same electrophysiological properties. A mechanism for coordinating the expression of ionic currents could help to maintain this physiological homeostasis. During normal activity, the neuron is exposed to a continually changing pattern of modulatory input, which could change gene expression in ways that would eventually move its firing pattern out of the normal range; an activity-independent homeostatic mechanism could combine with activity-dependent mechanisms to actively constrain activity within the normal range, promoting stability of information transfer. It is possible that activity-dependent and activity-independent mechanisms may be also activated in disease states, or in response to chronic drug use, in attempts to retain relatively normal neuronal activity under changed conditions. In all of these examples, plasticity in ion channel expression serves the purpose of maintaining stability in neuronal firing properties and preventing runaway changes that could be deleterious.

Experimental Procedures

Preparation

California spiny lobsters (Panulirus interruptus; Don Tomlinson Commercial Fishing, San Diego, CA) were anesthetized by cooling on ice for 30 min. The stomatogastric ganglion (STG), with its motor nerves and associated commissural and esophageal ganglia, was dissected and pinned in a UV-sterilized Sylgard-coated dish. PD somata were impaled with glass microelectrodes (10–25 MΩ; 3 M KCl) and identified by a 1:1 correspondence of action potentials recorded intracellularly in the soma and extracellularly from the PD motor nerve (Kloppenburg et al., 1999).
Organotypic Culture of the STG
Following RNA injection, the ganglia, motor nerves, and associated commissural and eosophageal ganglia were placed into filter-sterilized, oxygenated L-15 media and incubated at 16°C. L15 media contained Leibovitz’s L15 media with L-glutamine (GIBCO), penicillin (2500 unit/l)-streptomycin (2.5 mg/l) (Sigma), 250 µg/l Fungizone (GIBCO), and additional salts to raise the osmolarity to that of normal lobster saline. The medium was changed every 24 hr. In four preparations, a final concentration of 100 µM actinomycin-D (Sigma), 0.1% DMSO, was applied to the ganglia every 24 hr.

Construction of Shal-GFP Fusion Protein
The cdna fragments coding for the full length of green fluorescent protein (GFP; GIBCO) were amplified by polymerase chain reaction using the primers 5’-ACTACTA CTCCGTCTAGAGGAGGAAGGGG GAGAAGCTTGTAG-3’ and 5’-ACTACTAC TTCAATACAGCAGGCTGTTGA CAGCTGCC-3’, digested by EcoN1, and subcloned at the EcoN1 cutting site of the XbaI terminal of shal-e15 (between amino acids 649 and 650), a splice form of lobster shal (Baro et al., 2001), in BlueScript vector.

Microinjection of Neurons
Following physiological identification, pyloric neurons were injected with RNA using pressure pulses (40 psi, 10–90 ms duration, 0.2 Hz, for 2–10 min) with a home-built pressure injector. Microelectrodes were broken to a tip diameter of 1–2 µm and had resistances of about 1 MΩ when filled with 3 M KCl. The tips were filled with 0.2–1.0 M microscope shal or shal-GFP RNA with 0.04%–0.07% fast green in sterile distilled water. Control neurons were injected with the fast green solution alone or with RNA encoding GFP; both control injections had no detectable effects on the control neurons.

Voltage-Clamp Recordings
\(I_{\text{LH}}\), \(I_{\text{KCa}}\), \(I_{\text{KNa}}\), and \(I_{\text{GSH}}\) were isolated with a combination of pharmacological block, voltage inactivation, and digit current subtraction protocols as previously described (Kloppenburg et al., 2002). Briefly, neurons were voltage clamped using an Axocклап 28 amplifier and aClamp software (Axon Instruments) in lobster saline containing tetrodotoxin (TTX, 0.1 µM) and picrotoxin (PTX, 5 µM) to block most synaptic inputs. For \(I_{\text{LH}}\) and \(I_{\text{KCa}}\) CdCl (600 µM) was also added to eliminate calcium and calcium-dependent currents. \(I_{\text{LH}}\) was measured by a digital subtraction method where the leak-subtracted currents evoked by depolarizing steps from −50 mV (where \(I_{\text{LH}}\) is nearly completely inactivated) were subtracted from the leak-subtracted currents evoked by similar steps with a 500 ms prestep to −120 mV to remove inactivation. For \(I_{\text{LH}}\), the neurons were held at −40 mV (where \(I_{\text{LH}}\) is inactive) and then hyperpolarized with 10 s steps at 10 mV increments from −60 to −120 mV. These currents were not leak subtracted.

For measurement of \(I_{\text{KCa}}\) and \(I_{\text{KNa}}\), TTX, PTX, CaCl (5 or 10 mM), and 4-aminopyridine (4 mM) were used to isolate the current. The remaining current was then the sum of \(I_{\text{LH}}\) and \(I_{\text{GSH}}\) and \(I_{\text{KNa}}\). After \(I_{\text{LH}}\) was blocked indirectly with CdCl (600 µM), the remaining component was \(I_{\text{GSH}}\). This was then digitally subtracted from the summed current to yield \(I_{\text{KCa}}\).

For measurement of \(I_{\text{GSH}}\), the preparation was superfused with saline containing TTX, PTX, Ca²⁺, 4-AP, and 100 mM TEA-Cl (NaCl was reduced to maintain osmolarity). TEA and Ca²⁺ were additionally iontophoresed into neurons to greatly reduce outward currents.

Analysis of Rhythmic Activity
The minimal membrane potential was measured as the most hyperpolarized potential in the trough of the rhythmic activity. The amplitude of the oscillation was the difference between the most depolarized potential of the slow wave oscillation (under the spikes) and the minimal membrane potential. The slope of the rise phase was calculated from a straight line from the minimal membrane potential to the threshold point of the first spike. The frequency was the inverse of the time between the most hyperpolarized potential of two adjacent oscillations. All measures were based on average measures of at least 20 cycles.

Current Clamp Recordings of Synaptically Isolated Neurons
Neurons were isolated from detectable synaptic input by a combination of a vaseline pool containing 10⁻⁷ M TTX on the sole input nerve, 5,6-carboxyfluorescein photoinactivation of presynaptic neurons (the anterior burster and ventricular dilator neurons), and pharmacological blockade of glutamatergic synaptic inputs with 5 µM PTX applied to the ganglion.

Construction of Shal Pore Mutant Protein
The QuickChange kit (Stratagene) was used to obtain G[376,378]A and Y[377,379]F in shal-GFP. The pair of mutagenic oligonucleotide primers (Nucleic Acid Facility, Cornell University) used to introduce the desired mutations was 5’-CATATGACGACTTAAGCTGCGCC GACATGGTCCACCAC-3’ and 5’-GTGGGGAACCATGTCGGCGAAT GCTAACGGCGTCATTG-3’.

Xenopus Oocyte Expression
Xenopus oocyte expression studies were performed as previously described (Baro et al., 1998). Briefly, capped RNA was transcribed from linearized shal or shal-GFP DNA clones with a T3 Messenge machine kit (Ambion) using T3 RNA polymerase. A Sutter Instrument micropipette (model NA-1; San Rafael, CA) was used to inject ~100 nl of cRNA (concentration ~50 ng/µl) into Xenopus oocytes, which were isolated and maintained according to Quic et al. (1992). Recordings were made by two-electrode voltage clamp 2–3 days later.

Immunocytochemistry
After voltage clamp, the STG was fixed in 3.2% paraformaldehyde in 1× phosphate buffered saline (PBS) overnight at 4°C. The fix was washed out with eight changes of PBST (PBS + 0.3% Triton X-100) over 2–8 hr. The tissue was incubated for 48 hr in a rabbit anti-Shaker primary antibody (Baro et al., 2000; 1:2000 1× PBST plus 5% normal goat serum (NGS)). The primary antibody was washed out with 1× PBST for 2hr. The secondary antibody was preabsorbed with lobster brain ganglia to reduce nonspecific binding. The STG was then incubated overnight with the preabsorbed donkey anti-rabbit-Cy3 secondary antibody (Jackson Immunoresearch Laboratories; 1:400 dilution in 1× PBST plus 5% NGS). The secondary antibody was washed out with 1× PBS for 2–8 hr. All incubations were performed at 4°C with constant shaking. The STG was mounted on a polylysine coated coverslip, run through an ethanol dehydration series (30%, 50%, 70%, 2× 95%; 5 min each), and cleared in xylene (2×, 5 min each). The STG was mounted on a slide with DPX mounting media (Fluka) for 24 hr. The slides were visualized with a Biorad 600 confocal microscope.

Statistics
Student’s t tests and analysis of variance were performed using Excel software (Microsoft, Redmond, WA). Pearson correlation was performed using SPSS. Values are given as mean ± standard deviation.

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