Synaptic Augmentation Contributes to Environment-Driven Regulation of the *Aplysia* Siphon-Withdrawal Reflex

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This research shows that short-term synaptic plasticity can play a critical role in shaping the behavioral response to environmental change. In *Aplysia*, exposure to turbulent environments produces a stable reduction in the duration of the siphon-withdrawal reflex (SWR) and the responsiveness of siphon motor neurons. Recovery takes >1 min after a brief (10 sec–5 min) exposure but <1 min after a long (10 min) exposure. Here we demonstrate that (1) in-turbulence and post-turbulence phases of regulation depend on different cellular processes and (2) the post-turbulence phase of regulation is mediated by augmentation (AUG), an activity-dependent form of short-term synaptic plasticity. In reduced preparations (tail, siphon, and CNS), we show that treatment with $100 \mu\text{M}$ D-tubocurarine has no effect on in-turbulence regulation but blocks up to 90% of post-turbulence regulation, indicating that these phases of regulation are mediated by distinct cellular process. We then show that (1) turbulence induces activity in L30 inhibitory interneurons, (2) this activation produces AUG that lasts 1 min after a brief exposure to turbulence, and (3) manipulations that attenuate L30 AUG also attenuate regulation after brief turbulence. We also found that long (10 min) exposures to turbulence do not produce a post-turbulence phase of regulation because L30 activity declines over the course of a long turbulence exposure, leading to the decay of AUG before turbulence offset. Our results demonstrate a specific behavioral function of AUG and show how interactions between cellular processes can confer temporal sensitivity in the network regulation of behavior.

Key words: inhibition; adaptation; interneuron; network; short-term enhancement; plasticity

Introduction

A difficult challenge in neuroscience is linking experience-dependent changes in behavior to specific processes operating in the nervous system. One problem is that there is rarely a 1:1 relationship between behavioral and neural phenomena. For example, a single behavioral process can reflect the operation of multiple cellular processes operating in parallel throughout a neural system (Frost et al., 1988; Krasne and Teshiba, 1995). An important research goal, then, is to understand the principles of network operation that integrate diverse cellular processes during the regulation of behavior.

The siphon withdrawal reflex (SWR) in *Aplysia* is useful for exploring the links between neural and behavioral regulation. The SWR can be regulated by tactile and chemical stimuli (Stopfer et al., 1993) and can also be regulated by changes in the ambient environment, such as an exposure to water turbulence (Fischer et al., 2000). Turbulent environments produce two phases of SWR regulation. During turbulence, the threshold for eliciting an SWR increases, with a concomitant decrease in SWR duration. This phase of regulation is stable throughout a 10 min turbulence exposure (Fischer et al., 2000). After turbulence, SWR responsiveness recovers with a time course of recovery that is inversely related to exposure duration (Calin-Jageman and

Fischer, 2003). After brief (10 sec–5 min) turbulence, there is a persistent inhibition of SWR duration that lasts >1 min. After long (10 min) exposures, however, SWR duration recovers rapidly, in <1 min. Thus, exposure duration effects post-turbulence but not in-turbulence regulation, suggesting that these two phases of regulation are mediated by distinct cellular processes.

To explore the cellular basis of environmental regulation of the SWR, we examine here the contribution of two types of cellular processes that could decrease SWR responsiveness: (1) cellular adaptation processes such as spike-frequency adaptation and sensory adaptation and (2) augmentation (AUG) at the inhibitory synapses of L30 interneurons. Cellular adaptation processes are prominent in the SWR circuit (Lieb and Frost, 1997) and can function to reduce circuit excitability. AUG is an activity-dependent form of short-term synaptic plasticity that enhances synaptic efficacy and can last several minutes (Magleby and Zengel, 1975). In the SWR circuit, L30 interneurons express AUG in response to both intracellular activation and tactile stimulation. After induction by tactile stimulation, L30 AUG can decrease SWR circuit output and SWR duration for >1 min (Fischer and Carew, 1995).

Here we show that a pharmacological blockade of synaptic inhibition alters post-turbulence but not in-turbulence regulation of the SWR, confirming that these phases of regulation depend on distinct cellular processes. We then demonstrate that L30 AUG is the process that mediates post-turbulence regulation. Interactions with other cellular processes, however, cause the expression of L30 AUG to vary over time, so that it regulates the

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SWR after brief but not long turbulence. Our results demonstrate a specific behavioral function for AUG and show how interactions between cellular processes can confer temporal sensitivity in the network regulation of behavior.

Some of these results have been presented previously in abstract form (Calin-Jageman and Fischer, 2002).

Materials and Methods

Animals

Wild-caught adult *Aplysia californica* (150–300 gm) were obtained commercially (Marinus Inc., Long Beach, CA) and maintained at 15°C in a 600 l aquarium with continuously circulating artificial sea water (ASW; Instant Ocean). Animals were housed separately in floating colanders, fed dried seaweed twice a week (not on testing days), and maintained on a 12 hr light/dark cycle. Animals were excluded from these studies if an egg mass was detected in their home colander, because mating and egg laying can alter siphon behavior (Levy et al., 1994) and influence *Aplysia* neuronal physiology (Goldsmith and Byrne, 1993).

Cellular studies

Experimental preparation. Cellular experiments were conducted with reduced preparations consisting of the tail, siphon, mantle shelf, and CNS (Fischer and Carew, 1995). Animals were anesthetized by injection of isotonic $MgCl_2$ (50% of body weight) into the body cavity. The siphon and mantle, tail, and CNS, along with the siphon and tail (P9) nerves, were then removed from the animal. The preparation was transferred to a Sylgard-coated, two-chambered recording dish. The ring ganglia (pleural, pedal, and cerebral ganglia) and abdominal ganglia, which constitute most of the *Aplysia* CNS (excluding the buccal ganglion), were placed in a separate compartment from the rest of the preparation. This allowed for physiological recordings in a compartment isolated from turbulent conditions [described below and in Fischer et al. (2000)]. The mantle and tail were both secured dorsal side up by pinning to the Sylgard using 26 ga hypodermic needles. The abdominal ganglion was pinned ventral side up using minuten pins, and the left hemiganglion was surgically desheathed. Throughout the experiment, the preparation was perfused continuously with ASW (19–22°C) through three perfusion lines: one implanted in the tail, one in the siphon artery, and one to the chamber containing the central ganglia. At least 45 min of recovery time after dissection was allowed before physiological recordings, at which time tail stimulation could evoke siphon responses. In the event that we were unable to evoke siphon responses (e.g., resulting from nerve damage during dissection), the preparation was discarded.

Standard intracellular recording techniques were used. Neurons were impaled with glass microelectrodes (resistance 10–15 M Ω) containing either 3 M KCl, 3 M KAc, or 0.6 M K_2SO_4 • 20 mM KCl. Electrical potentials were amplified on Axoclamp 2-A (Axon Instruments, Burlington, CA) or IX2-700 (Dagan) amplifiers and then digitized (Powerlab 8SP; AD Instruments) for computer analysis.

Cell identification. LFS motor neurons (MNs) were identified by visually monitoring siphon movements that are characteristically produced by intracellular activation of these MNs (Hickie and Walters, 1995). L29 interneurons were identified on the basis of size and position, their ability to recruit recurrent IPSPs when intracellularly activated, and their characteristic cellular response to the siphon tap stimulus (Hawkins et al., 1981; Fischer and Carew, 1993). L30 interneurons were identified on the basis of their recurrent synaptic relationship with L29: L30 interneurons are excited by L29 activation and in turn produce IPSPs back onto L29 (Hawkins et al., 1981; Fischer and Carew, 1993).

Experimental procedures. These experiments were designed to identify the cellular processes that contribute to environmental regulation of the SWR. To produce environmental regulation, the siphon, mantle, and tail were exposed to water turbulence, generated by blowing compressed air underwater through two tubes constructed from 2 ml serological pipettes (Fischer et al., 2000). *Aplysia* commonly encounter turbulent environments in their natural habitat (Kupfermann and Carew, 1974). In intact animals and reduced preparations, turbulence does not elicit SWRs but does regulate the SWR response to siphon stimulation (Fischer et al., 2000). In our experimental preparations, the siphon was stimulated by

brief, 60 msec taps applied by a glass probe attached to a stimulator-driven relay. The interstimulus interval (ISI) was 10–11 min, an interval that does not decrement the complex EPSP (Fischer et al., 1997). The response of the SWR circuit was monitored as polysynaptic (complex) EPSPs in identified LFS siphon motor neurons (hyperpolarized to -80 mV to prevent action potentials). Circuit responsiveness was quantified as the area (mv•ms) underneath the initial 500 msec of the evoked complex EPSP, the approximate duration of the evoked response under baseline conditions. This measure is sensitive to changes in both the duration and amplitude of the evoked complex EPSP. For measures of L30 AUG, L30-evoked IPSCs were measured in L29s using two-electrode voltage clamp. L29s were clamped at a holding potential of -80 mV, at which the evoked IPSCs from L30 interneurons appear inward (see Figs. 4, 6).

Each experiment contrasted the effects of turbulence under two conditions: (1) a Control condition and (2) an experimental condition designed to block a process or set of processes in the SWR circuit. In the control condition, baseline responding was characterized with two pretest measures. The siphon, tail, and mantle were then exposed to turbulence. In-turbulence regulation was measured by timing exposure so that the next siphon tap occurred during turbulence (see Fig. 2A). Post-turbulence regulation was measured by timing exposure so that the next siphon tap always occurred 1 min after turbulence. The same protocol was used in the experimental condition except that a manipulation was introduced before or during turbulence to block a set of processes in the SWR circuit.

Experimental manipulations. In one set of experiments, we blocked inhibitory transmission in the SWR circuit by incubating the CNS with 100 μ M D-tubocurarine (D-TC) (Sigma-Aldrich, St. Louis, MO) dissolved in ASW. Incubation always began at least 15 min before data collection and continued throughout the experimental protocol. In every preparation tested, D-TC markedly increased the area of tap-evoked complex EPSPs in LFS motor neurons (see Fig. 2B), an effect indicative of the efficacy of D-TC at blocking inhibitory transmission (Trudeau and Castellucci, 1993). The effects of D-TC were stable during data collection because we observed no significant differences between consecutive baseline measures taken in D-TC ($t < 1$ for each experiment).

In another set of experiments, we blocked the induction of L30 AUG during turbulence by simultaneously inactivating two to three of the three known L30s in the SWR circuit (Hawkins et al., 1981). L30s were inactivated during turbulence by current-clamp hyperpolarization to -80 mV, which prevented activity (compare Figs. 5A, 8B) and thus the induction of AUG.

We also blocked the induction of L30 AUG during turbulence by applying a mild electric shock (1 sec, 60 mA AC) to the tail 10 min before turbulence. Previous research has shown that this protocol induces metaplasticity in L30 interneurons that drastically attenuates the ability of L30 activity to induce AUG (Fischer et al., 1997). This effect is selective and does not produce overt behavioral effects such as sensitization (Fischer et al., 1997). Consistent with these observations, there were no significant differences between baseline measures taken before and after tail shock ($t < 1$).

Behavioral analysis

Surgery. To facilitate viewing of the siphon during behavioral experiments, the parapodia (skin flaps covering the mantle) were removed surgically (Fischer et al., 2000).

Experimental procedures. Behavioral experiments were conducted to replicate our findings at the cellular level and had a design similar to our cellular experiments. Individual *Aplysia* were placed in a plastic testing container (25 × 45 cm long × 20 cm deep) that was suspended in a larger tank to maintain temperature and water exchange through openings cut in the bottom. Turbulence was generated by bubbling air from four nozzles located at the bottom of the testing chamber. The SWR was evoked at an 11 min ISI by brief stimulation (flick) with a pliable nylon bristle (clamped in surgical hemostats) in the inner lumen of the siphon, which bent freely after contact with the siphon. SWR duration was scored from the time of stimulus onset to the time at which the siphon just began to relax (Marcus et al., 1988). To prevent measurement bias, the experimenter stimulating the siphon would signal SWR onset and offset but

would not observe the actual time score for each evoked response. SWR onset and offset were signaled to a PC-type computer via a remote switch and an analog-to-digital input/output board. SWR duration was recorded by Inquisit Data Collection Software (Millisecond Software, Seattle, WA).

For experiments involving tail shock, each animal was placed briefly in a holding tank 15 min before data collection. In the holding tank, animals were given either a brief tail shock (1 sec, 60 mA AC) or a sham shock (1 sec touch with the shock wand, no current passed). After 5 min to recover, animals were returned to their testing chamber. The experimenter collecting data did not observe the tail-shock procedures and remained blind to shock condition.

Statistics

Pretest measures were averaged to characterize baseline responding. Data are expressed as percentage change from average baseline, so that a measure of 0% indicates no change in SWR response. The effects of experimental manipulations were assessed using *t* tests for independent groups. Within groups, test measures were compared with baseline measures using *t* tests for repeated measures. A 0.05 α level was used to assess significance. All probability values are two tailed. Summary data are presented as means $\pm$ SEM.

Results

Synaptic inhibition is necessary for post-turbulence regulation

Exposure to turbulence changes SWR responsiveness to siphon stimulation, decreasing the duration of elicited SWRs during turbulence and for >1 min after brief turbulence (Fischer et al., 2000). In siphon–tail preparations, turbulence produces analogous effects in the way LFS motor neurons respond to siphon stimulation, reducing the area of tap-evoked complex EPSPs during turbulence and for >1 min after brief turbulence (Calin-Jageman and Fischer, 2003). Here we use this cellular correlate of turbulence-induced regulation to identify the processes operating in the SWR circuit that contribute to this form of regulation.

The decrease in circuit responsiveness produced by turbulence could be the result of (1) a decrease in excitatory transmission (such as homosynaptic depression or sensory adaptation) or (2) an increase in network-level inhibition (such as L30 AUG) (Fig. 1). To assess the relative contributions of excitation and inhibition during environmental regulation of the SWR circuit, we used CNS exposure to 100 μ M D-TC. Acetylcholine mediates the known forms of inhibitory transmission in the SWR circuit (Storozhuk and Castellucci, 1999), and 100 μ M D-TC effectively blocks cholinergic inhibitory transmission in the circuit (Trudeau and Castellucci, 1993).

In-turbulence regulation

We began by examining the effects of D-TC on in-turbulence regulation of the SWR circuit. The experimental design is diagrammed in Figure 2A. Reflex input to siphon motor neurons was measured as tap-evoked complex EPSPs in identified LFS motor neurons. In the control condition ($n = 9$), baseline responding was characterized as the average of two pretest measures (ISI = 11 min). Turbulence was then applied to the tail and siphon for 12 min. Test measures were taken in turbulence 1 and 12 min after turbulence onset. In the D-TC condition ($n = 9$), the same procedure was followed after incubating the CNS with 100 μ M D-TC for 15 min. In most cases (75%), preparations were exposed to both the control and D-TC treatments, in that order, with a 15 min rest interval between experiments.

We found that D-TC did not alter motor neuron regulation during turbulence. Measures taken 1 min into turbulence were reduced by 42% in the D-TC condition and 42% in the control condition, a nonsignificant difference ($t < 1$). Similarly, mea-

Figure 1. Simplified schematic of the SWR reflex network emphasizing the elements examined in the present work. Siphon-evoked input to LFS siphon motor neurons is mediated through two groups of identified excitatory neurons (L29s and L34s) and sensory neurons. Both the L29s and L34s are subject to L30-mediated inhibition via activity-dependent short-term plasticity (asterisks). L30s receive excitatory input via a recurrent circuit with the L29s and also receive sensory input from both siphon and tail pathways. Additional inhibitory neurons (signified by ?) also provide synaptic input to the L29s (e.g., L16, L35), although their functional significance under the conditions explored here are unknown. Siphon sensory input from innocuous tactile stimulation is mediated by an unidentified population of low-threshold sensory neurons (Frost et al., 1997); tail sensory input is mediated by a poly-synaptic pathway (Cleary et al., 1995). This diagram has been modified from Frost and Kandel (1995).

asures taken 12 min into turbulence were decreased by 45% in the D-TC condition and 43% in the control condition, a nonsignificant difference ($t < 1$). There were no between-group differences in measures taken 11 min after turbulence.

Post-turbulence regulation

We next used D-TC to examine the contribution of synaptic inhibition to post-turbulence regulation. Because the time course of post-turbulence regulation depends on exposure duration, we used 10 sec, 1 min, 5 min, and 10 min exposures in both the control and D-TC conditions. The experimental design is diagrammed in Figure 3A. In the control condition ($n = 9$ per turbulence duration), baseline responding was characterized by the average of two measures taken before turbulence (11 min). Post-turbulence regulation was probed by taking measures 1 and 12 min after turbulence offset. In the D-TC condition ($n = 9$ per turbulence duration), the same procedure was used after incubation of the CNS with 100 μ M D-TC for 15 min. In most cases (60%), preparations were exposed to both the control and D-TC treatments, in that order.

Consistent with our previous observations (Calin-Jageman and Fischer, 2003), in the control condition there was a persistent inhibition of LFS responsiveness after brief (10 sec–5 min) but not long (10 min) turbulence (Fig. 3B). For example, measures taken 1 min after turbulence were reduced by 25% of baseline after 10 sec of turbulence ($t_{(8)} = 5.19$; $p < 0.01$) but were statistically identical to baseline after 10 min of turbulence ($t < 1$). Turbulence durations of 1 and 5 min produced a persistent inhibition similar to that observed with a 10 sec exposure; it is not yet clear whether intermediate duration (6–9 min) would produce regulation intermediate between the 10 sec and 10 min exposures.

We found that D-TC attenuated the persistent inhibition observed after brief turbulence. In the D-TC condition, 10 sec of turbulence produced only a 7% reduction in measures taken 1

Figure 2. In-turbulence regulation of the SWR circuit does not depend on synaptic inhibition. *A*, Experimental protocol. The siphon was stimulated at 11 min intervals (arrows). After two baseline measures, the siphon and tail were exposed to 12 min of turbulence (shaded bar). Test measures were taken 1 min (Turb-1) and 12 min (Turb-12) after turbulence onset and again 11 min (Post-11) after turbulence offset. Synaptic inhibition was blocked by incubating the CNS with 100 μ M D-TC (hatched bar) for 15 min before data collection. *B*, Representative physiology: tap-evoked complex EPSPs in a single LFS motor neuron during control (top) and D-TC (bottom) experiments. For each measure, the motor neuron was hyperpolarized to -80 mV by current clamp. In both conditions, turbulence reduced siphon-evoked responses at both Turb-1 and Turb-2. *C*, Results: summary data ($M \pm SEM$) of changes in tap-evoked complex EPSP area during turbulence in the control (circles) and D-TC (squares) conditions. Each data point represents nine experiments. $*p < 0.01$ for within-group contrast to baseline measure.

min after turbulence, a significant difference compared with the 25% reduction observed in the control condition ($t_{(16)} = 3.20$; $p < 0.01$). Similarly, 1 min of turbulence produced an 8% reduction in the D-TC condition, less than the 26% reduction observed in the control condition ($t_{(16)} = 2.70$; $p < 0.05$). Finally, 5 min of turbulence produced a 3% reduction in the D-TC condition, a significant difference from the 30% reduction observed in the control condition ($t_{(16)} = 5.68$; $p < 0.01$). Thus, D-TC blocked 69–90% of the persistent inhibition normally observed 1 min after brief turbulence. In the 10 min condition, which does not produce a persistent inhibition of circuit responsiveness, D-TC produced no effect. Measures taken 1 min after turbulence had

Figure 3. Post-turbulence regulation of the SWR circuit requires synaptic inhibition. *A*, Experimental protocol. The siphon was stimulated at 11 min intervals (arrows). After two baseline measures, the siphon and tail were exposed to turbulence for 1 sec, 1 min, 5 min, or 10 min (shaded bars). Exposure onset was timed so that the next measure (Post-1) always occurred 1 min after turbulence. Synaptic inhibition was blocked by incubating the CNS with 100 μ M D-TC (hatched bar). *B*, Results: Post-1 measures ($M \pm SEM$) in the control (solid bars) and D-TC (hatched bars) conditions by exposure duration. Each bar represents nine experiments. $*p < 0.01$ for within-group contrast to baseline measure.

returned to within 1% of baseline in the D-TC condition and within 3% of baseline in the control condition. The between-group comparison was not significant ($t < 1$). Regardless of turbulence duration, there were no between-group differences in measures taken 12 min after turbulence ($t < 1$ for each comparison; data not shown).

These results confirm that different cellular processes mediate in-turbulence and post-turbulence SWR regulation. Treatment with D-TC does attenuate the decrease in circuit responsiveness that occurs for 1 min after brief turbulence, indicating that this phase of regulation is likely caused by cholinergic inhibitory transmission. Treatment with D-TC does not alter the decrease in circuit responsiveness that occurs during turbulence, so this phase of regulation must be caused by either a decrease in network excitation or an unidentified form of D-TC-insensitive synaptic inhibition.

Turbulence produces L30 AUG

We next sought to identify the inhibitory cellular process that mediates post-turbulence regulation. A number of lines of evidence led us to consider the role of L30 AUG. First, D-TC blocks L30 transmission (Lieb and Frost, 1997), and we observed that this blockade renders AUG completely ineffective (Fig. 4). Second, previous research has shown that brief tactile stimulation, such as brushing the tail for 5 sec, can induce L30 AUG, which then inhibits SWR responsiveness for ~ 1 min (Fischer and Carew, 1995). Third, manipulations that prevent the induction of L30 AUG attenuate the inhibition normally observed after brief tactile stimulation (Fischer et al., 1997).

Turbulence-induced L30 activity

AUG is an activity-dependent form of synaptic plasticity, so we began by measuring the effects of turbulence on L30 activity. L30

Figure 4. D-TC (100 μ M) blocks L30 transmission and renders AUG ineffective. Each panel shows L30-evoked IPSCs 20 sec before (left) and 20 sec after (right) intracellular activation of the L30 (5 Hz for 5 sec). Under normal conditions (top), L30 activation produces significant AUG. During incubation with D-TC (bottom), L30 transmission is blocked and AUG is ineffective. For this recording, L30-evoked IPSCs were recorded in an L29 interneuron voltage clamped at a holding potential of -80 mV, below the reversal potential for the L30–L29 synapse. For this reason, the IPSCs appear inward.

activity was recorded in reduced preparations while the tail and siphon were exposed to turbulence ($n = 4$). L30s were not active in the absence of tactile stimulation. In every preparation tested, turbulence produced strong initial L30 activation, averaging 2.3 Hz over the first 10 sec (Fig. 5). This activation began to decay immediately, reaching a steady state of 0.0–0.4 Hz within 1 min. There was no “off” response after turbulence offset. These results indicate that turbulence produces time-dependent L30 activation, a strong initial activation lasting 10–50 sec, and a low-level steady-state activation that persists throughout a long exposure to turbulence.

Turbulence-induced AUG

We next examined whether turbulence-induced L30 activation is sufficient to produce AUG. L30 to L29 IPSCs were measured by evoking single L30 spikes. After characterizing baseline responding with three pretest measures, the tail and siphon were exposed to turbulence for 10 sec or 10 min. To test for AUG, IPSC amplitude was measured every 20 sec after turbulence offset for 2 min.

We observed post-turbulence AUG after brief but not long turbulence (Fig. 6). After 10 sec of turbulence, AUG persisted for >60 sec. IPSC amplitude was enhanced by 80% of baseline 20 sec after turbulence ($t_{(5)} = 8.21$; $p < 0.01$), by 38% of baseline 40 sec after turbulence ($t_{(5)} = 2.99$; $p < 0.05$), and by 28% of baseline 60 sec after turbulence ($t_{(5)} = 3.20$; $p < 0.05$). After 80 sec, IPSC amplitude was within 2% of baseline, a nonsignificant difference ($t < 1$). Subsequent measures remained within 4% of baseline.

After 10 min of turbulence, there was no post-turbulence AUG. In fact, IPSC amplitude was significantly reduced by 19% of baseline 20 sec after turbulence ($t_{(4)} = 3.10$; $p < 0.05$). IPSC

Figure 5. Turbulence produces time-dependent L30 activation. *A*, Representative physiology: representative example of L30 activity during a 10 min exposure to turbulence. The neuron was not active in the absence of turbulence (pre- and post-turbulence). *B*, Results: summary data (M $\pm$ SEM) of L30 activity before (-1 min), during (0–10 min), and just after ($+1$ min) a 10 min exposure to turbulence. Activity is expressed in spikes per second in 10 sec bins. Each data point represents four experiments. L30s are not spontaneously active, and there was no activity before or after turbulence.

amplitude was also reduced by 25% of baseline 40 sec after turbulence and by 18% of baseline 60 sec after turbulence, but these reductions were not statistically significant ($t_{(3)} = 1.71$, $p = 0.18$; $t_{(4)} < 1$, respectively), possibly because of the small sample size. From 80 sec on, IPSC amplitude remained within 10% of baseline.

In the 10 min condition, IPSC amplitude was measured every 30 sec throughout turbulence, although these measures were usually contaminated by turbulence-induced inputs to L29s. Sufficient data for analysis ($n = 4$) were obtained for the 30 sec, 8 min, and 10 min time points (data not shown). These measures confirmed that turbulence produces an initial enhancement of L30 IPSCs that declines over the course of a 10 min exposure to turbulence. IPSC amplitude was enhanced by 228% of baseline after 30 sec in turbulence ($t_{(3)} = 4.70$; $p < 0.05$), depressed by 16% of baseline after 8 min in turbulence ($t < 1$), and depressed by 27% of baseline after 10 min in turbulence ($t_{(3)} = 5.20$; $p < 0.05$).

L30 AUG is necessary for post- but not in-turbulence regulation

We next examined whether turbulence-induced L30 AUG contributes to the effects of turbulence on the SWR circuit. To do this, we simultaneously inactivated all or most of the three L30s in the SWR circuit via direct hyperpolarization, preventing the induction and expression of AUG.

In-turbulence regulation

We began by measuring the effects of L30 inactivation on LFS regulation during turbulence. The experimental design was similar to that described in Figure 2*A*, except the ISI was 10 min and only one measure was taken during turbulence (1 min in turbu-

Figure 6. Turbulence produces L30 AUG that persists after brief (10 sec) but not long (10 min) turbulence. *A*, Representative physiology: IPSCs evoked by L30 spikes after 10 sec (top) or 10 min (bottom) of turbulence measured in L29 neurons under TEVC (hold = -80 mV). In the 10 sec condition, IPSC amplitude was enhanced for up to 60 sec after turbulence. In the 10 min condition, IPSC amplitude was depressed for ~20 sec after turbulence. *B*, Results: summary data ($M \pm SEM$) of changes in IPSC amplitude after 10 sec (circles; $n = 6$) and 10 min (squares; $n = 5$) of turbulence.

lence). For each experimental preparation, we measured baseline regulation in a control condition ($n = 5$) and then repeated the experiment with all three of the known L30s inactivated during the 1 min turbulence measures (Turb-1). L30s were inactivated by current clamping them to -80 mV. L30 activity was not recorded during the control phase of the experiment.

We found that L30 inactivation did not alter LFS regulation during turbulence (Fig. 7). Measures taken 1 min after turbulence were reduced by 54% in the control condition and by 48% in the L30-inactivation condition. The between-group comparison was not significant ($t < 1$). Thus, similar to the effect of blocking synaptic inhibition with D-TC, L30 inactivation does not alter in-turbulence regulation of the SWR circuit.

Post-turbulence regulation

We next examined the effects of L30 inactivation on post-turbulence regulation. The experimental design was similar to

Figure 7. In-turbulence regulation of the SWR circuit does not depend on L30 AUG. *A*, Representative physiology: in-turbulence regulation of a single LFS motor neuron during control (top) and L30-inactivation (bottom) conditions. Three L30s were inactivated during the test measure taken in turbulence. *B*, Results: summary data ($M \pm SEM$) of changes in tap-evoked complex EPSP area during turbulence in the control (circles; $n = 5$) and L30-inactivation (squares; $n = 5$) conditions. In all experiments, three L30s were hyperpolarized. * $p < 0.01$ for within-group contrast to baseline measure.

the one diagrammed in Figure 3A, except that only two turbulence durations were used: 10 sec and 10 min, chosen because they produce different time courses of regulation. We measured baseline regulation in a control condition ($n = 5$ per group) and then repeated the experiment with two (of three) L30s inactivated throughout turbulence ($n = 5$ per group).

We found that L30 inactivation attenuated the persistent inhibition normally observed after brief turbulence (Fig. 8). After 10 sec of turbulence, measures taken 1 min after turbulence were reduced by 33% in the control condition but reduced only 11% in the L30-inactivation condition, a significant between-group difference ($t_{(3)} = 3.67$; $p < 0.05$). Thus, inactivation blocked ~60%

Figure 8. Post-turbulence regulation of the SWR circuit depends on L30 AUG. *A*, Representative physiology: tap-evoked complex EPSPs in the same LFS motor neuron before (left), 1 min after (middle), and 12 min after (right) exposure to 10 sec of turbulence. Responses were measured under control conditions (top) and with two L30s inactivated during turbulence (inset at bottom). *B*, Results: summary data ($M \pm SEM$) of changes in tap-evoked complex EPSP area during turbulence in the control condition (circles; $n = 5$) and L30-inactivation (squares; $n = 5$) conditions. * $p < 0.01$ for within-group contrast to baseline measure.

of the persistent inhibition observed 1 min after turbulence in the control condition. L30 inactivation did not alter the rapid recovery normally observed after long turbulence. After 10 min of turbulence, measures taken 1 min after turbulence were reduced by 7% in the control condition and elevated by 17% in the L30-inactivation condition. The comparison across conditions was not significant ($t < 1$). Taken together, these results show that L30 AUG makes an important contribution to post-turbulence regulation of the SWR circuit, mediating the persistent inhibition that occurs after brief but not long turbulence.

L30 AUG mediates post-turbulence regulation of the SWR circuit and behavior

To document the behavioral relevance of L30 AUG during post-turbulence regulation, we took advantage of the fact that mild tail shock attenuates the induction of L30 AUG (Fischer et al., 1997) in the absence of other behavioral effects (e.g., sensitization). The utility of this manipulation is that it can be applied to both intact animals as well as reduced preparations. We conducted the same post-turbulence experiment on both reduced preparations and intact animals. Shock (1 sec, 60 mA AC) was administered to the tail immediately after the first baseline measure, 11 min before the second baseline. In both experiments the first and second baselines were statistically identical in the tail-shock condition, confirming that this intensity of tail shock does not produce sensitization. This is consistent with previous experiments using the same behavioral protocol (Fischer et al., 2000).

Physiological experiment

In reduced preparations, tail shock produced effects similar to D-TC and L30 inactivation, attenuating the persistent inhibition observed after brief (10 sec) turbulence (Fig. 9A). Measures taken 1 min after turbulence were reduced by 41% in the control condition but elevated by 2% in the tail-shock condition, a significant between-group difference ($t_{(12)} = 2.90$; $p < 0.05$). Tail shock did not alter the rapid recovery normally observed after long (10 min) turbulence. Measures taken 1 min after turbulence were within 3% of baseline in the control condition and within 12% of baseline in the tail-shock condition. The between-group comparison was not significant ($t_{(13)} = 1.266$; $p < 0.22$). Regardless of turbulence duration, there were no effects of tail shock on measures taken 12 min after turbulence (data not shown).

Behavioral experiment

Tail shock produced the same pattern of results on SWR duration in intact animals (Fig. 9B). In the control condition, 10 sec of turbulence produced a 36% reduction in measures taken 1 min after turbulence. In the tail-shock condition, there was only a 2% reduction, a significant between-group difference ($t_{(23)} = 2.48$; $p < 0.05$). Tail shock did not alter the rapid recovery normally observed after long (10 min) turbulence. Measures taken 1 min after turbulence were elevated by 27% of baseline in the control condition and elevated by 11% of baseline in the tail-shock condition. The between-group comparison was not significant ($t < 1$). Regardless of turbulence duration, there were no between-group differences in measures taken 12 min after turbulence (data not shown).

Discussion

Two processes mediate environmental regulation of behavior

Exposure to an environmental stimulus, such as water turbulence, produces two phases of SWR regulation: (1) an in-turbulence decrease in responsiveness and (2) a post-turbulence period of recovery. We have shown previously that exposure duration effects post-turbulence but not in-turbulence regulation (Calin-Jageman and Fischer, 2003). Here we show that these phases of SWR regulation also show different sensitivities to D-TC: treatment with D-TC has no effect on in-turbulence regulation but blocks up to 90% of post-turbulence regulation. This differential effect of D-TC indicates that different cellular processes mediate in-turbulence and post-turbulence regulation.

Cellular basis of post-turbulence regulation

We have identified L30 AUG as the process that mediates regulation after brief turbulence, demonstrating that (1) turbulence

A. CELLULAR EXPERIMENT

B. BEHAVIORAL EXPERIMENT

Figure 9. Tail shock disrupts post-turbulence regulation after brief (10 sec) but not long (10 min) turbulence. *A*, Cellular experiment: post-1 measures ($M \pm SEM$) indicating changes in tap-evoked complex EPSP area after 10 sec (left) and 10 min (right) turbulence in both the control (solid bars) and tail-shock conditions (hatched bars). For each condition, there were seven experiments per exposure duration. *B*, Behavioral experiment: post-1 measures ($M \pm SEM$) indicating changes in SWR duration after 10 sec (left) and 10 min (right) turbulence in both the control (solid bars) and tail-shock conditions (hatched bars). The control condition includes 12 animals per exposure duration; the tail-shock condition includes 8 animals per exposure duration. * $p < 0.01$ for within-group contrast to baseline measure.

induces L30 activity, (2) this activity produces L30 AUG that lasts ~1 min after brief turbulence, and (3) manipulations that attenuate L30 AUG also attenuate regulation after brief turbulence but not regulation during turbulence. An interesting characteristic of

L30 AUG is its sensitivity to turbulence duration. We observed that L30 AUG is expressed after brief (10 sec–5 min) but not long (10 min) turbulence. This temporal sensitivity seems to occur because L30 activity decreases during turbulence, decaying from 2.3 to 0.4 Hz after ~1 min of exposure. This low level of activity is probably insufficient to maintain AUG, leading to a gradual decline over the course of a long exposure to turbulence. This is consistent with our measurements of the L30 to L29 synapse during turbulence, which showed an early (30 sec) enhancement that completely decayed by 8 min of exposure. The decline in L30 activity during turbulence may be a circuit-wide phenomenon, because turbulence-induced input to siphon motor neurons also declines substantially over the course of a long exposure (Fischer et al., 2000).

Cellular basis of in-turbulence regulation

During turbulence there is a decrease in SWR responsiveness that is invariant over exposure duration (Fischer et al., 2000) and insensitive to both D-TC and L30 hyperpolarization (this paper). These characteristics do not identify a small set of candidate cellular processes. First, although D-TC blocks inhibitory transmission in two of the inhibitory neuron types that are known to regulate SWR output (Lieb and Frost, 1997; Storozhuk and Castellucci, 1999), there may be additional, D-TC-insensitive forms of synaptic inhibition operating on the SWR circuit (Fig. 1). Second, the SWR circuit is abundant with processes that limit activity but do not rely on synaptic inhibition. These include sensory adaptation (Billy and Walters, 1989), spike-frequency adaptation in interneurons (Lieb and Frost, 1997), and homosynaptic depression (Byrne, 1982).

We are currently working to determine which, if any, of these candidate processes contribute to in-turbulence regulation. Our initial focus is on sensory adaptation, defined as a decrease in sensory neuron activity in response to a sustained stimulus (Laughlin, 1989). Like in-turbulence regulation, sensory adaptation reaches a steady state that is stable regardless of stimulus duration (Shapley and Enroth-Cugell, 1984). In addition, sensory adaptation operates on circuit input and thus is poised to exert a profound influence on circuit output. Determining the role of sensory adaptation during in-turbulence regulation is complicated by the fact that known siphon sensory neurons do not respond to turbulence (our unpublished observations) or mild water disturbance (Frost et al., 1997). This suggests that a yet-to-be identified population of siphon sensory neurons mediates the transduction of turbulence. Characterizing the response properties of this population will be critical to understanding the nature of sensory input to the SWR circuit in response to innocuous tactile stimulation.

Two-process model of environmental regulation

One of the interesting aspects of our results is that L30 AUG does not contribute to regulation during turbulence, although it is expressed during early stages of exposure. One possibility is that the effects of L30 AUG are masked during turbulence by the D-TC-insensitive process that mediates in-turbulence regulation. The in-turbulence process reduces tap-evoked input to siphon motor neurons. Because the L30s are activated by the same circuit elements as the motor neurons, in-turbulence regulation may also decrease tap-evoked L30 activity, effectively “silencing” their strengthened synapses. In addition, the in-turbulence reduction in SWR responsiveness may represent a “floor” effect, so that there is no further decrement from the enhanced inhibition caused by L30 AUG. Consistent with both of these scenarios,

Figure 10. Model of the contributions of L30 AUG (dashed lines) and a D-TC-insensitive process (solid lines) to environmental regulation of behavior. In the top panel, a brief exposure to turbulence (solid bar) activates both L30 AUG and the D-TC-insensitive process. After turbulence, D-TC-insensitive process dissipates but L30 AUG persists for at least 1 min. Thus, eliciting an SWR (arrow) 1 min after turbulence offset reveals a persistent inhibition of SWR responsiveness. Over the course of a long exposure to turbulence (bottom panel), the D-TC-insensitive process causes L30 activity and L30 AUG to decay. At turbulence offset, AUG has completely decayed. Eliciting an SWR (arrow) 1 min after turbulence offset reveals that SWR duration has recovered completely.

turbulence produces an 84% reduction in tap-evoked activity of L29 interneurons (Fischer et al., 2000). The L29s are an important source of excitatory input for the L30s as well as a primary target for L30 inhibition (Fig. 1) (Fischer and Carew, 1995), so a reduction in tap-evoked L29 activity could lead to both a decrease in evoked L30 activity and a decrease in the impact of L30 AUG.

Figure 10 integrates these results into a schematic model of environmental regulation of behavior. According to this model, turbulence induces two processes: (1) L30 AUG and (2) a D-TC-insensitive process that mediates in-turbulence regulation. Although L30 AUG is induced during turbulence, its effects are masked by the D-TC-insensitive process. This process dissipates after turbulence, unmasking the effects of any remaining L30 AUG. If the exposure duration was brief (10 sec–5 min), sufficient AUG remained to produce a persistent inhibition of SWR responsiveness. If the exposure duration was long (10 min), AUG had decayed completely and SWR responsiveness rebounded rapidly. We are currently constructing a computational model of the SWR circuit to formalize and test the hypothesis that L30 AUG is regulated by the same process that alters circuit output during turbulence.

Functions of short-term plasticity

There has been increasing interest in understanding the behavioral relevance of short-term plasticity, including AUG (Nadim and Manor, 2000; Fortune and Rose, 2001). Observation of plasticity at the physiological level, however, often precludes the use of naturalistic stimuli and the observation of behavioral outputs. This makes it difficult to verify whether (1) the plasticity observed would have been induced *in vivo* and (2) the plasticity observed would have had behavioral consequences. Using reduced preparations from *Aplysia* has enabled us to overcome both of these obstacles. We have demonstrated that AUG is induced by water turbulence, an environmental stimulus that *Aplysia* often encounter in their natural habitats. Furthermore, we have shown that AUG induced by turbulence has measurable effects on the output of the SWR circuit and the duration of the behavior.

Experiments in the rat prefrontal cortex have shown that AUG

onto layer V pyramidal cells could help maintain the signal produced by a transient stimulus (Hempel et al., 2000). Interestingly, our experiments demonstrate a similar function for AUG in the *Aplysia* SWR circuit. L30 AUG maintains SWR regulation for up to 1 min after brief turbulence, functioning as a transient memory of the tactile stimulus. One potential benefit of this function of AUG is that it would allow stable behavioral regulation across multiple occurrences of a transient stimulus. For example, *Aplysia* inhabit tidal pools in which they often experience periodic tactile stimulation from waves. L30 AUG might serve as a “memory bridge” between wave peaks, maintaining stable responsiveness despite the periodic nature of stimulation. Consistent with this line of reasoning, we have found that when intact *Aplysia* were exposed to periodic turbulence (10 sec on/off cycles), SWR responsiveness remained stable across repeated changes in the environment (Fischer et al., 2002). Moreover, blocking L30 AUG with a mild tail shock eliminated this behavioral stability and caused the SWR to fluctuate with each wave cycle.

Although AUG maintains regulation after a brief stimulus, it is downregulated over the course of a long (10 min) stimulus so that SWR regulation dissipates rapidly at stimulus offset. This time-dependent regulation of AUG enables *Aplysia* to recover rapidly from noncyclic (or extremely low frequency) changes in their environment. Thus, network processes that regulate L30 AUG enable the SWR to act as a low-pass filter, producing rapid response modulation to low-frequency change and stable responding to high-frequency change.

Finally, our work highlights the difficulty of assessing the behavioral functions of synaptic plasticity. We found that L30 AUG is expressed both early in turbulence and after brief turbulence, but that it is effective at regulating the SWR circuit only after brief turbulence. The ineffectiveness of AUG during turbulence seems to be attributable to the fact that the SWR circuit is concurrently expressing additional cellular processes (possibly sensory adaptation). The upshot of these results is that the function of a cellular process is determined not only by its profile of expression but also by the state of the network in which it is embedded. This adds an additional degree of difficulty to understanding the behavioral functions of synaptic plasticity. The key to resolving this problem is having experimental control over input to a network, as well as accurate measures of network output (e.g., behavior).

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