Brief Communication

Desensitization of Postsynaptic Glutamate Receptors Contributes to High-Frequency Homosynaptic Depression of *Aplysia* Sensorimotor Connections

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Withdrawal reflexes of *Aplysia* are mediated in part by a monosynaptic circuit of sensory (SN) and motor (MN) neurons. A brief high-frequency burst of spikes in the SN produces excitatory postsynaptic potentials (EPSPs) that rapidly decrease in amplitude during the burst of activity. It is generally believed that this and other (i.e., low-frequency) forms of homosynaptic depression are entirely caused by presynaptic mechanisms (e.g., depletion of releasable transmitter). The present study examines the contribution that desensitization of postsynaptic glutamate receptors makes to homosynaptic depression. Bath application of cyclothiazide, an agent that reduces desensitization of non-NMDA glutamate receptors, reduced high-, but not low-frequency synaptic depression. Thus, a postsynaptic mechanism, desensitization of glutamate receptors, can also contribute to homosynaptic depression of sensorimotor synapses.

Withdrawal reflexes of *Aplysia* are mediated in part by a monosynaptic circuit of sensory neurons (SNs) and motor neurons (MNs; for reviews, see Byrne et al. 1991; Cleary et al. 1995). Stimuli sufficient to elicit withdrawal reflexes evoke a high-frequency burst of spikes in the SNs (Byrne et al. 1978a,b; Walters et al. 1983; Stopfer and Carew 1996; Frost et al. 1997; Antonov et al. 1999; Phares et al. 2003). Such bursts of SN activity induce homosynaptic depression of the sensorimotor synapses, a mechanism that limits the response of MNs to peripheral stimuli (Byrne et al. 1978b; Walters et al. 1983; Stopfer and Carew 1996; Antonov et al. 1999; Phares et al. 2003).

For the past thirty years, homosynaptic depression of *Aplysia* sensorimotor synapses has been attributed exclusively to presynaptic mechanisms (Castellucci and Kandel 1974; Byrne 1982; Gingrich and Byrne 1985; Bailey and Chen 1988; Eliot et al. 1994; Armitage and Siegelbaum 1998; Royer et al. 2000; Gover et al. 2002; Zhao and Klein 2002). Moreover, it has been repeatedly suggested that homosynaptic depression of the sensorimotor synapse does not have a postsynaptic contribution (Castellucci and Kandel 1974; Armitage and Siegelbaum 1998; Royer et al. 2000).

The transmitter at sensorimotor synapses is most likely glutamate (Dale and Kandel 1993; Trudeau and Castellucci 1993; Schacher et al. 1997; Armitage and Siegelbaum 1998; Conrad et al. 1999; Levenson et al. 2000b; Chin et al. 2002; Antonov et al. 2003). Because glutamate receptors exhibit pronounced desensitization during high-frequency bursts (Jones and Westbrook 1996), desensitization may also contribute to high-frequency homosynaptic depression of the *Aplysia* sensorimotor synapse. The present study examines this issue by taking advantage of cyclothiazide, an agent that reduces desensitization of non-NMDA glutamate receptors (Patneau et al. 1993).

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Aplysia californica (150–300 g) were obtained from Alacritty Marine Biological and Marinus Inc. Animals were housed in aquaria at 15°C on a 12-h/12-h light/dark cycle and were fed dried seaweed three times a week. Animals were anaesthetized by injection of isotonic MgCl₂ (0.5 mL/g of body weight). To reduce synaptic transmission, removal and desheathing of pleural-pedal ganglia were performed while the ganglia were bathed in high-Mg²⁺, low-Ca²⁺ artificial seawater (pH 7.65) containing the following: 400 mM NaCl, 10 mM KCl, 80 mM MgCl₂, 20 mM MgSO₄, 1 mM CaCl₂, 2.5 mM NaHCO₃, and 10 mM HEPES. During experiments, the ganglia were bathed in high-divalent artificial seawater (pH 7.65), which reduces activity in polysynaptic pathways by increasing spike threshold (Byrne et al. 1978b). This solution contained the following: 368 mM NaCl, 8 mM KCl, 80 mM MgCl₂, 20 mM MgSO₄, 13.8 mM CaCl₂, 2.5 mM NaHCO₃, and 10 mM HEPES. The desheathing and testing procedures were conducted in a static bath, which was maintained at 15°C.

In each preparation, a single tail MN was identified in the pedal ganglion, based on previously described criteria (Zhang et al. 1994), and impaled with two microelectrodes (10–15 MΩ, filled with 3 M KAc). One electrode was used for recording the membrane potential and the other for current injection. During testing, the MN was hyperpolarized to –80 mV. Input resistance of the MN was measured by intracellular injection of a hyperpolarizing current pulse (–1 nA, 1 sec) while the MN was at its resting potential.

Glutamate was ejected by applying pressure (20 psi, 100 msec) to a blunt glass pipette (5 μm, filled with 100 mM L-glutamate) with a Picospritzer (General Valve Co.). The pressure applications were comparable to those used in previous studies (Dale and Kandel 1993; Trudeau and Castellucci 1993; Zhu et al. 1997; Storozhuk and Castellucci 1999). Ejections of glutamate were paired, at interpulse intervals (IPI) of 100 or 200 msec (measured from offset of the first pulse to onset of the second). The paired-pulse ratio (PPR) was defined as the amplitude of the second response divided by the amplitude of the first response and

multiplied by 100%. While the MN was hyperpolarized to -80 mV, glutamate was ejected onto the neuropil of the pedal ganglion, in the vicinity of the MN cell body, eliciting fast transient depolarizing responses in the MN. We believe that the responses were evoked by direct action of glutamate on the MN, and not by transmitter released from occult presynaptic neurons that were activated by the glutamate puffs, for two reasons. First, these puffs were applied in medium containing high Mg^{2+} , high Ca^{2+} , which prevents passive depolarizations from reaching threshold and triggering spikes (Byrne et al. 1978b; Trudeau and Castellucci 1995). Second, the kinetics of the MN responses were slower than typical EPSPs, reflecting the relatively long application of glutamate (i.e., 100 msec; cf. Fig. 1A to Fig. 2A).

In experiments that examined synaptic depression, a SN in the ventrocaudal cluster of the pleural ganglion was impaled with a single microelectrode (10–15 M Ω , 3 M KAc). Once a synaptically connected pair of cells was identified, the preparation was allowed to rest for 10 min before testing began. Initially,

Figure 1 Desensitization of receptors limits their repetitive responses to exogenous glutamate. (A1) Two consecutive puffs of glutamate at a short interpulse interval (IPI) evoked a pair of depolarizing responses, the second of which was smaller than the first. The two traces shown are the responses of the same MN to two pairs of glutamate puffs at the 100- and 200-msec intervals. (A2) Average paired-pulse ratio (PPR) ($\pm$ SEM) at the 100- and 200-msec IPI. At the IPI of 100 msec, the second response to glutamate was 64.9% $\pm$ 9.3% of the first response, whereas at the 200-msec IPI, the second response was 83.0% $\pm$ 9.0% of the first. The difference between the two PPRs was statistically significant. This indicates that 200 msec after the first glutamate puff, there was a smaller degree of desensitization of receptors than at 100 msec after the puff. (B1) Responses of an MN to two puffs of glutamate (IPI = 200 msec), before and after application of CTZ. After CTZ, the first response was unaffected, but the second response was increased. (B2) Average PPR ($\pm$ SEM) at the 200-msec interval, before and after CTZ. The PPR at 200 msec IPI increased from 67.4% $\pm$ 13.9% (Control) to 107.2% $\pm$ 18.1% following application of CTZ (+CTZ). The difference between the two PPRs was statistically significant.

while still bathed with control saline (i.e., high-divalent artificial seawater), sensorimotor synapses were tested twice at frequencies of 10 and 1 Hz (i.e., four tests). Each test consisted of three EPSPs evoked at either 10 or 1 Hz, and the amplitudes of the second and third EPSPs were normalized to the amplitude of the first EPSP in each test. The tests at 10 and 1 Hz were alternated at 10-min intervals. The normalized amplitudes of the EPSPs were averaged for each frequency to provide the baseline amount of synaptic depression at 10 and 1 Hz, before pharmacological treatment. Basal synaptic transmission was measured as the average peak amplitude of first EPSPs from both 10- and 1-Hz tests. Only a single sensorimotor synapse was tested in each preparation. All reported values of Student's *t* statistic refer to results of two-tailed *t*-tests for paired observations, and *F* ratios refer to results of two-way repeated measures ANOVA. In cases in which MN responses summated (i.e., paired puffs of glutamate or three EPSPs elicited at 10 Hz), their peak amplitude was measured from the extrapolated decay of the previous depolarization of the membrane.

Initially, we examined whether repeated, brief application of glutamate could desensitize receptors on the MN. Two puffs of glutamate were applied at an IPI of either 100 or 200 msec. Each puff of glutamate evoked fast transient depolarizations of the MN. The response to the first glutamate pulse averaged 6.3 $\pm$ 0.9 mV (mean $\pm$ SEM). At both the 100- and 200-msec IPI, the response to the second pulse was smaller than the response to the first (Fig. 1A; $n = 7$, $F_{(1,6)} = 6.8$, $p < 0.05$). In addition, the PPR at 200 msec was significantly greater than the PPR at the 100-msec interval (Fig. 1A; $n = 7$, $t(6) = 2.7$, $p < 0.05$).

Several lines of evidence indicated that the depression of the second response was not caused by nonlinear summation as might occur if the membrane potential approached the reversal potential of the response (i.e., +10 mV; Dale and Kandel 1993). First, MNs were initially held at the hyperpolarized potential of -80 mV, and the summated response was not greater than 15 mV. Second, the second response could reach a higher level of depolarization at the long IPI than the short one, in the same neuron, although in both cases the membrane potential at the onset of the response was very similar (e.g., Fig. 1A1).

Depression of the MN response to the second glutamate puff is consistent with the hypothesis that the first puff of glutamate desensitizes the receptors, which slowly recover from desensitization during the IPI. To test this hypothesis, we treated the ganglia with cyclothiazide (CTZ), an agent that reduces desensitization of non-NMDA receptors in vertebrates (Patneau et al. 1993; Trussell et al. 1993; Arai and Lynch 1998a,b; Rozov et al. 2001). CTZ (Sigma Chemicals) was applied to the bath (dissolved in 0.2% DMSO) at a final concentration of 0.3 mM, a concentration that is frequently used in studies of vertebrate synapses (Partin et al. 1994; Fedeles and Raiteri 1996; Rammes et al. 1996; Cowen and Beart 1998).

As described above, MNs were stimulated with paired pulses of exogenous glutamate at the 200-msec IPI, before and 5 min after the application of CTZ (Fig. 1B). The 200-msec IPI was selected to reduce temporal overlap between the two responses. The PPR was increased significantly following application of CTZ (Fig. 1B; control: 67.4% $\pm$ 13.9%; +CTZ: 107.2% $\pm$ 18.1%; $n = 5$, $t(4) = 3.7$, $p < 0.05$), whereas CTZ did not affect the amplitude of the first response to glutamate (e.g., Fig. 1B; control: 10.2 $\pm$ 3.9 mV; +CTZ: 8.7 $\pm$ 3.5 mV; $n = 5$, $t(4) = 2.4$, $p = 0.08$). These results indicate that a desensitization mechanism could influence the response to high-frequency bursts of activity at the sensorimotor synapse. In addition, the results imply that CTZ is an appropriate tool for examining that possibility.

To examine the possible role of desensitization at the sensorimotor synapse, SNs of the pleural ganglion were stimulated to fire three action potentials at either 10 or 1 Hz, before and 15

Figure 2 Desensitization of postsynaptic receptors limits the responses (EPSPs) of MNs to high-frequency stimulation of a presynaptic SN. (A1) EPSPs evoked at 10 Hz, before and 15 min after the application of CTZ. CTZ increased the amplitudes of the second and third EPSPs, without affecting the first EPSP. (A2) Average relative amplitude of EPSPs 2 and 3 at 10 Hz was significantly different between control and CTZ. The same synapses were stimulated with 2 trains at 10 Hz and 2 trains at 1 Hz (see B), alternating at 10-min intervals, before application of CTZ. The same stimulation regime was repeated after application of CTZ. (B1) EPSPs evoked at 1 Hz, before and after the application of CTZ (data are from the same pair of monosynaptically connected sensory and motor neurons as in A1). The relative depression during 1 Hz activity was not affected by CTZ. (B2) Average relative amplitude of EPSPs 2 and 3 at 1 Hz, from 2 trains repeated at 10-min intervals, was not significantly different between control and CTZ.

min after application of CTZ (0.3 mM), while the monosynaptic EPSPs were recorded from the MN soma. A frequency of 10 Hz is in the intermediate range of the physiological activity of sensory neurons (Byrne et al. 1974, 1978a,b; Walters et al. 1983; Clatworthy and Walters 1993; Stopfer and Carew 1996; Frost et al. 1997; Antonov et al. 1999, 2003; Phares et al. 2003), and 1 Hz is the highest frequency that has been previously used to investigate synaptic depression in sensorimotor synapses (Castellucci and Kandel 1974; Byrne 1982; Eliot et al. 1994; Armitage and Siegelbaum 1998; Royer et al. 2000; Gover et al. 2002). Because the puff-evoked responses of MNs indicated that the receptors remain desensitized for at least 200 msec after their activation (see Fig. 1A2), synaptic stimulation at the 100-msec intervals (10 Hz) should engage desensitization of postsynaptic receptors.

The SN action potentials evoked three progressively decreasing EPSPs in the postsynaptic MN (e.g., Figs. 2A1, 2B1). CTZ did not affect the peak amplitude of first EPSPs (control: 3.7 ± 0.9 mV; +CTZ: 3.1 ± 0.8 mV; $n = 5$, $t(4) = 1.1$, $p = 0.34$), which indicates that basal transmission is not affected by postsynaptic desensitization, in agreement with a previous report (Storozhuk and Castellucci 1999). In addition, MN input resistance was stable throughout testing and was not affected by application of CTZ (control: 30.7 ± 8.6 M Ω ; +CTZ: 30.4 ± 9.3 M Ω ; $n = 5$). Although MN resistance was tested at the cell body, it is assumed that if CTZ had affected the membrane resistance at postsynaptic

sites, it would have also affected the resistance at the cell body, because CTZ was applied to the entire bath.

Contrary to its lack of effect on the first EPSPs, CTZ increased the peak amplitude of the second and third EPSPs during the 10-Hz test (e.g., Fig. 2A1), thus reducing their depression (Fig. 2A2, $n = 5$, $F_{(1,4)} = 11.5$, $p < 0.05$). The inhibition of desensitization may have unmasked paired-pulse facilitation (Jiang and Abrams 1998), contributing to the observed increase of EPSP2 after application of CTZ. The effect of CTZ in decreasing synaptic depression appeared to be limited to activity at 10 Hz, as the depression evoked by 1 Hz in the same sensorimotor synapses was not significantly affected by CTZ (Fig. 2B; $n = 5$, $F_{(1,4)} = 1.1$, $p = 0.34$). Although the contribution of desensitization to 1 Hz synaptic depression in *Aplysia* sensorimotor synapses cannot be excluded, these results indicate that synaptic transmission at least at 10 Hz, is partially limited by desensitization of postsynaptic receptors.

In some synapses, CTZ has been reported to affect basal, presynaptic release of neurotransmitter (Diamond and Jahr 1995; Isaacson and Walmsley 1996; Bellingham and Walmsley 1999; Ishikawa and Takahashi 2001). This does not appear to be the case in *Aplysia*. As mentioned above, the first EPSP was not changed following CTZ application, and CTZ did not affect EPSP amplitude at low-frequency stimulation. These results indicate that CTZ reduced the depression at 10 Hz, not through a presynaptic effect, but by inhibiting desensitization

of receptors. The efficacy of CTZ to reduce desensitization, in turn, is supported by its effects on puff-evoked responses (Fig. 1B). It should be noted that this use of CTZ does not permit identification of the specific kinetic step that is affected. Therefore, it is not clear if CTZ reduced desensitization of the receptors or accelerated their recovery from desensitization. Although the receptors activated by puffs of exogenous glutamate may not be the same receptors that are activated by synaptically released glutamate, we found that CTZ affected both in a similar way. This is consistent with previous use of glutamate puffs to probe the properties of postsynaptic receptors (Dale and Kandel 1993; Trudeau and Castellucci 1993, 1995; Zhu et al. 1997; Storozhuk and Castellucci 1999; Chitwood et al. 2001). Collectively, these data support the novel finding that high-frequency homosynaptic depression of *Aplysia* sensorimotor synapses is caused, in part, by desensitization of postsynaptic receptors.

The present results lead to a re-evaluation of the long-held view that homosynaptic depression in *Aplysia* sensorimotor synapses is exclusively presynaptic (Castellucci and Kandel 1974; Byrne 1982; Gingrich and Byrne 1985; Bailey and Chen 1988; Eliot et al. 1994; Armitage and Siegelbaum 1998; Royer et al. 2000; Gover et al. 2002; Zhao and Klein 2002). However, they do not challenge previous data. The apparent contradiction is resolved by considering the frequencies of stimulation that were used in the past. The sensorimotor synapses have traditionally

been stimulated with single spikes at frequencies ≤ 1 Hz (e.g., Byrne 1982). Using low frequencies of stimulation has led to an oversimplified, exclusively presynaptic, view of synaptic depression. The results presented here are still consistent with these previous reports, in that desensitization did not contribute to depression at 1 Hz as much as it did at 10 Hz. Therefore, these results extend the previous view of depression to incorporate a postsynaptic contribution that emerges when SNs are activated at a high frequency.

What is the function of high-frequency synaptic depression? In several animals, synaptic depression has been related to behavioral habituation (for review, see Christoffersen 1997). However, habituation is mimicked primarily by low-frequency synaptic depression (e.g., Byrne 1982). In contrast, we do not believe that the function of high-frequency synaptic depression in *Aplysia* sensorimotor synapses is related to habituation. Instead, this form of synaptic depression probably contributes to the transfer of information about a single stimulus from the sensory to the motor neurons (Phares et al. 2003). Because SNs respond to individual stimuli of the peripheral tissue with short-duration, high-frequency bursts of spikes, encoding of the stimulus in the response of the MN would be limited by the burst-induced depression of sensorimotor synapses (Byrne et al. 1978b; Walters et al. 1983; Stopfer and Carew 1996; Antonov et al. 1999; Phares et al. 2003).

Finally, the finding that desensitization contributes to high-frequency synaptic depression raises the possibility that this mechanism may be regulated during learning. Both short- and long-term sensitization of withdrawal reflexes correlate with enhancement of transmitter release from SNs (Bailey and Chen 1983; Dale et al. 1988; Byrne and Kandel 1996). However, a greater amount of transmitter would be expected to activate and, subsequently, desensitize a greater fraction of the postsynaptic receptors, resulting in augmented depression of the MN response. To compensate for this enhanced depression, these forms of behavioral learning would be predicted to also involve mechanisms to overcome postsynaptic desensitization. One such mechanism could be up-regulation of the postsynaptic receptors, which has been associated with short- and long-term facilitation of sensorimotor synapses (Trudeau and Castellucci 1995; Zhu et al. 1997; Chitwood et al. 2001). Another protective mechanism could be enhancement of glutamate uptake from the synapse following long-term sensitization (Levenson et al. 2000a). Further experimentation will shed light on the relative contribution of postsynaptic desensitization to sensorimotor burst transmission before versus after synaptic facilitation and learning.

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