J Comp Physiol A (2005) 191: 481–489
DOI 10.1007/s00359-005-0607-y

ORIGINAL PAPER

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Decrease in excitability of LG following habituation of the crayfish escape reaction

Received: 30 June 2004 / Revised: 11 January 2005 / Accepted: 22 January 2005 / Published online: 5 March 2005

Abstract Crayfish escapes from threatening stimuli to the abdomen by tailflipping upwards and forwards. This lateral giant (LG)-mediated escape reaction habituates readily upon repetitive sensory stimulation. Using an isolated abdominal nerve cord preparation, we have analyzed the change in LG activity by applying additional sensory stimulation after different periods following habituation to characterize the retention of LG habituation. Results show that the LG mediated response habituates more quickly, but the retention time is shorter, as repetitive sensory stimulation is applied at progressively shorter inter-stimulus time intervals. The spike response of LG recovers quickly, within several minutes after habituation, but they fail to spike when an additional stimulus is applied after specific long periods following habituation. The critical period of the delay for this decrease in excitability of LG is dependent on the inter-stimulus time interval of the initial repetitive stimulus. As the inter-stimulus interval became longer, the delay needed for decrease in excitability became shorter. Furthermore, the local injection of 10^{-6} mol l^{-1} octopamine into the neuropil just following habituation promotes the achievement of decrease in excitability. No effects were observed when 10^{-6} mol l^{-1} serotonin and tyramine were injected. These results suggested octopamine promotes decrease in excitability of LG following habituation.

Keywords Retention · Octopamine · Tailflip · Learning · Memory

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Abbreviations EPSP: Excitatory post-synaptic potential · LG: Lateral giant · ISI: Inter-stimulus interval · Oct: Octopamine · 5-HT: Serotonin

Introduction

The neural mechanisms underlying the lateral giant (LG)-mediated escape reaction of crayfish have been analyzed in detail (e.g. Edwards et al. 1999). A strong tactile stimulus applied to the abdomen of crayfish evokes a rapid flexion of the abdominal musculature that leads to an escape response directed up and forwards (Wine and Krasne 1972). The LG acts as a command neuron by receiving sensory inputs directly from extero- and proprioceptive afferents via both electrical and chemical synapses and indirectly from sensory interneurons (Wine and Krasne 1972; Newland et al. 1997; Araki and Nagayama 2003) and by directly exciting motor giant (MoG) motor neurons in anterior abdominal segments (Wine 1984). The rapid flexion of the abdomen is triggered within 10 ms following spikes in LG.

The LGs are inactivated readily upon repetitive sensory stimulation (Krasne and Woodsmall 1969) as a result of a decline in the efficacy of chemical synaptic transmission from exteroceptive afferents to both LG and the sensory interneurons (Zucker 1972; Araki and Nagayama 2003) this results in crayfish becoming unresponsive to similar sensory stimuli. Zucker (1972) showed that this phenomenon reflects the characteristics of habituation described by Thompson and Spencer (1966), and it is now regarded as an example of habituation, or reduction in reflexive response caused by neural plasticity (not by sensory adaptation or effector fatigue) induced by repetition of a constant stimulus (Christoffersen 1997). In intact animals, habituation can be retained for several hours, however in preparations in which the abdominal nerve cord is isolated this habituation declines rapidly within several minutes (Krasne

1969). Krasne and Teshiba (1995) suggested that tonic descending inhibitory inputs are necessary to maintain LG habituation.

Since habituation is a simple form of non-associative learning, characterizing the change of responsiveness of the LG itself following habituation is important for further understanding the physiological bases of the retention of memory and learning (Krasne and Edwards 2002). In this paper, using an isolated abdominal nerve cord preparation, we have analyzed, quantitatively, the response of the LGs to repetitive sensory stimulation at different inter-stimulus intervals (ISIs). Moreover, we have also characterized the change in the excitability of LGs to additional sensory stimuli after different periods following the onset of habituation. Our results showed that the spike response of the LGs recovered quickly within minutes after habituation, but the LGs became less excitable when additional sensory stimuli were applied after a long period following habituation. The delay following habituation needed for a decrease in excitability of LG depended on the ISIs preceding habituation, and octopamine promoted the decrease phase of LG excitability.

Materials and methods

Animals and preparations

Adult male and female crayfish, *Procambarus clarkii* Girard (6–11 cm body length from rostrum to telson) were used in all experiments. Crayfish were purchased commercially (Sankyo Labo Service, Tokyo Japan and ME Suisan, Miyagi, Japan) and maintained in laboratory tanks, fed weekly on a diet of chopped potato and liver. Prior to experiments, crayfish were isolated individually in small tanks (20×35×25 cm) for at least 1 week. There were no significant differences in results according to sex, body length, or supplier.

The nerve chain from the 2nd to 6th (terminal) abdominal ganglion with relevant nerve roots was isolated from the abdomen and pinned, dorsal-side-up, in a Sylgard-lined perfusion chamber (4 ml volume), containing cooled Van Harreveld's (1936) solution. Fresh saline was constantly perfused through the chamber at a rate of 4.5 ml min^{-1} using an Eyla micro tube pump (MP-3). The dorsal ganglionic sheath of the terminal ganglion was surgically removed with fine forceps to allow micropipette access into the neuropil.

Stimulation and recording

The spike activity of LG was recorded extracellularly from the 4–5th abdominal connective using a suction electrode. Nerve roots 2, 3 and 4 of the terminal abdominal ganglion, that contain the mechanosensory afferents innervating the uropods and telson, were electrically stimulated simultaneously using a single oil hook electrode. Square pulses (0.01–0.05 ms duration; 1–20 V intensity) were delivered through the stimulating electrode.

Experimental procedure

After 15 min of rest following dissection, the sensory evoked spike threshold of the LG was determined by gradually increasing the intensity of stimulation to the sensory nerves. After the LG spike threshold was determined, the intensity of stimulation was set so that the stimulus was just suprathreshold. The preparation was rested for a further 5 min before repetitive sensory stimulation was applied with an ISI ranging from 1 s to 300 s until the LG failed to give rise to spikes following five continuous trials. The spike rate of the LGs was calculated by averaging each trial of stimulation and is shown as a habituation curve. Following habituation, a single stimulus pulse of the same intensity as the initial one (= test stimulus) was applied following delays varying from 2 s to 60 min to determine retention time of habituation (see Fig. 2a).

To examine the effect of serotonin, octopamine or tyramine on the decrease of excitability of LG following habituation, the drugs were applied locally into the neuropil immediately after the LG response habituation. For drug application, the tips of micropipettes were broken manually under a microscope to a tip diameter of about 10 μm. Each drug was applied under pressure into the neuropil of the terminal ganglion, near the dendritic branches of LG, at depths of about 250 μm from the dorsal surface with N_2 gas controlled by a pneumatic picopump (PV820, WPI) at 18 psi for 50 ms. All drugs were purchased from Sigma Chemical and their effect analyzed statistically using the χ^2 test for independence. Results are based on recordings from more than 500 preparations. The stimulus conditions used in each series of experiments is represented by insets in some figures.

Results

Habituation curve

Sensory stimulation applied to nerve roots 2–4 of the terminal abdominal ganglion gave rise to a spike in the LG interneuron. Upon repeated stimulation, the response of LG gradually declined, or habituated, and failed to give rise to a spike. When the stimulus was repeated with a 1 s ISI, the LG response immediately showed a rapid habituation, decreasing by 80% within four trials of stimulation, and by 95% after the 20th trial (filled circles in Fig. 1). As the ISI was increased, the decrease in the LG response became slow. For example, at a 5 s ISI, the response of LG declined by 80% after 20 trials (open circles in Fig. 1) and at ISIs of 20 s or 60 s, the response of LG decreased by 50% after 20 stimuli

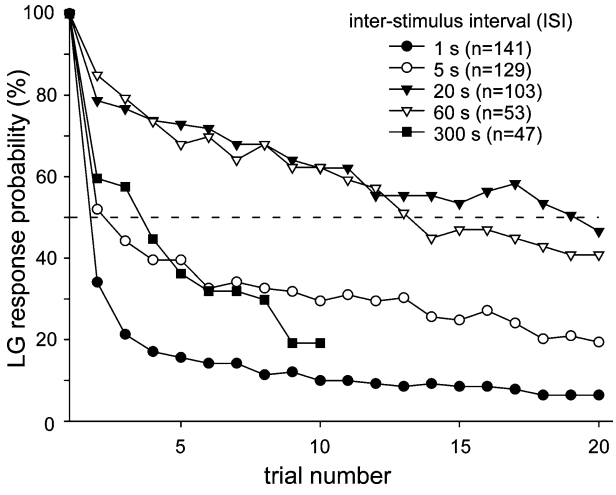

Fig. 1 Habituation curves of the response of LG to repeated sensory stimulation. The occurrence of LG spikes was plotted against 20 repeated stimulations at different ISI (ten trials in the stimulation at a 300 s ISI). The LG response probability (%) was calculated as the number of animals in which LG responded with spike/total number of animals. The animals in which LG failed to give rise to a spike following five continuous trials were judged as being habituated. The number of animals examined at each ISI is indicated by number *n*

(Fig. 1: filled triangles for 20 s intervals and open triangles for 60 s intervals). When a stimulus was repeated with a 300 s ISI, however, habituation occurred more readily (filled squares in Fig. 1). The response of LG declined by 80% after only ten trials, and the habituation curve was similar to that at a 5 s ISI.

The number of stimuli necessary to cause habituation increased when the ISI was increased, with the exception of a 300 s ISI. For example, the number of stimuli needed to cause habituation at a 5 s ISI was significantly greater than at a 1 s ISI ($P < 0.001$; log-rank test). With 20 s and 60 s ISIs, furthermore, the number of stimuli significantly increased compared to that with a 5 s interval ($P < 0.005$ and $P < 0.001$, respectively; log-rank test).

Recovery and decrease in excitability of LG following habituation

The habituation of LG is thought to be the result of synaptic depression from mechanosensory afferents to both the LGs and sensory interneurons (Zucker 1972; Araki and Nagayama 2003), and descending tonic inputs are thought necessary to maintain habituation for long periods (Krasne and Teshiba 1995).

The time course of change in LG activity following habituation was analyzed by applying additional sensory stimuli at fixed periods following LG habituation (insets in Figs. 2, 4). In these experiments habituation was defined as a failure of LG to fire in response to five successive stimuli. For each preparation, a test stimulus was applied only once following habituation, so the data

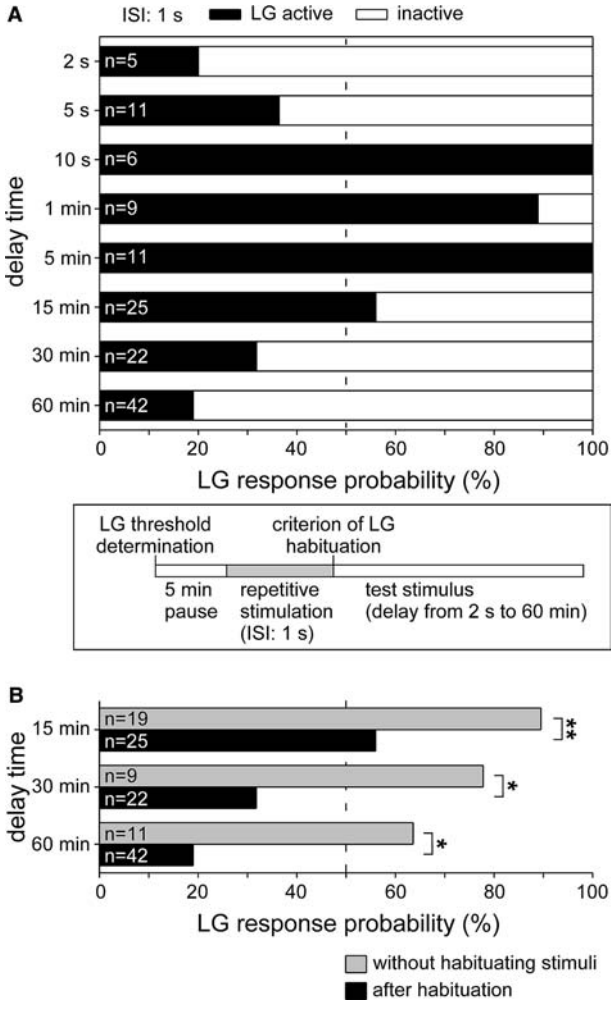

Fig. 2 **a**, **b** Decrease in excitability of LG following habituation. **a** Occurrence probability of LG spikes to the test stimulus after various delay periods following habituation caused by a repeated sensory stimulation at 1 s inter-stimulus interval (ISI). The spike response of LGs recovered within 10 s of habituation but further decrease in excitability of LG was observed 15 min after habituation. The number of animals examined in each condition is indicated by number *n*. *Inset* indicates the experimental protocol showing the timings of stimulation. **b** Decrease in LG responsiveness over time. The occurrence probabilities of LG spikes to the test stimulus after delay periods following single sensory stimulation were measured (*gray bar*). The responsiveness of LGs decreased slowly over time, but it was still significantly higher than that of previously habituated preparations (*filled black bar*) for each delay period (*$P < 0.05$, **$P < 0.01$ with χ^2 test for independence)

were derived from different preparations. The LGs recovered quickly from habituation following repeated stimulation with a short ISI. A test stimulus applied 2 s following habituation with an ISI of 1 s failed to elicit LG spikes in 80% of preparations examined (2 s in Fig. 2a) and 60% of preparations failed to produce LG spikes with a delay of 5 s (5 s in Fig. 2a). When the test stimulus was applied 10 s following habituation, by contrast, LG gave rise to a spike in all preparations examined (10 s in Fig. 2a). After a delay of 1 min or

5 min following habituation, most LGs also gave rise to a spike in response to the test stimulus (1 min and 5 min in Fig. 2a). Thus, in most preparations the LG response recovered within 10 s following habituation.

By contrast, in animals rested for 15 min following habituation with no additional stimulation, the test stimulus failed to elicit LG spikes in more than 40% of preparations (15 min in Fig. 2a). After 30 min of the rest, the test stimulus failed to elicit LG spikes in more than 65% of preparations (30 min in Fig. 2a). In more than 80% of preparations (34 out of 42 preparations), LG failed to give rise to a spike in response when the test stimulus was applied 60 min after habituation (60 min in Fig. 2a). These observations indicate while synaptic efficacy of sensory afferents recovered quickly following habituation (since the response of LG recovered in a 10 s period) that a decrease in the excitability of LG occurred following relatively long delays.

We analyzed the viability of the preparations over time by analyzing the responses of LG to test stimuli applied in the absence of habituating stimulus series (Fig. 2b). In approximately 90% of tests (17 out of 19 animals), LG gave rise to a spike in response to the test stimulus when it was applied after 15 min. The LG response probability was reduced to 78% (seven out of nine preparations) and 64% (7 out of 11 preparations) when the test stimuli were applied after 30 min and 60 min, respectively. We also analyzed the response of interneuron A to sensory stimulation in which the stimulus intensity was set just suprathreshold to evoke spikes in interneuron A. In approximately 80% of tests (9 out of 11 preparations), interneuron A responded with a spike when the stimulus was applied 30 min after rest without further stimulation, while about 70% (five out of seven preparations) produced a spike in response to sensory stimulation after a 60 min delay. Thus, neural activity was slightly reduced over the time course of the experiments (over 60 min), independent of the initial habituation. However, habituation induced a decrease in the excitability of LG that was unrelated to the time-depended decrease in viability, since the LG response in the presence of habituating stimulus series was significantly lower than that of the controls after each rest period (Fig. 2b) ($P < 0.01$ in 60 min, $P < 0.05$ in 15 min and 30 min; χ^2 test for independence). Lack of a significant decline in the viability of the LGs over time was further supported by the observations that in nine out of ten animals, LG continued to respond with a spike in response to direct electrical stimulation in which the stimulus intensity was just suprathreshold and set 60 min before each test. The temporal change in excitability of LGs following habituation, therefore, implies that two parallel mechanisms of depression in excitability of LG occur by repeated sensory stimulation.

The response of LGs to a test stimulus following habituation gives us information as to whether the spike threshold of LGs changes over time, but does not show how much it changes. We therefore measured the threshold of LG over time following habituation (Fig. 3). Threshold of LGs that just showed habituation and rested for 60 min was determined by the gradual increase (1 V each) in the stimulus intensity from the initial spike threshold (7–10 V) at the intervals of 5 s. Threshold of LGs that rested for 5 min was determined by the gradual increase in the stimulus intensity from about 70% of the initial spike threshold at 5 s intervals. When the LG response showed habituation, the spike threshold of LG increased significantly, $117.3 \pm 4.8\%$ (mean ± SE; $n = 10$), relative to its initial spike threshold ($P < 0.05$; paired t test). In preparations rested for 5 min following habituation, the threshold of LG decreased to $91.0 \pm 1.9\%$ ($n = 12$) of its initial level while its threshold increased again to $143.9 \pm 10.1\%$ ($n = 14$) when preparations were rested for 60 min without stimulation following habituation. This was significantly higher ($P < 0.05$; two sample t test) than that of LG axons that had just been habituated. These results suggested that the responsiveness of LGs to sensory stimulation recovered quickly after habituation but depressed if no additional stimuli were applied for relatively long periods. Since repeated stimulation was necessary to determine the LG threshold, this, in itself, could reduce the responsiveness of LG. Thus, threshold changes in LG with and without habituating stimulus series were compared in animals rested for 60 min from the initial stimulus. In crayfish without a series of habituating stimuli, the spike threshold increased slightly ($105.8 \pm 6.3\%$; $n = 9$) relative to the initial level, that was significantly lower ($P < 0.01$; two sample t test) than that of LG rested for 60 min after habituation (Fig. 3). Since a decrease in excitability of LGs following habituation was accomplished by a significantly higher threshold of LGs, it is likely that some physiological change may also occur in the LGs during the period of delay following habituation.

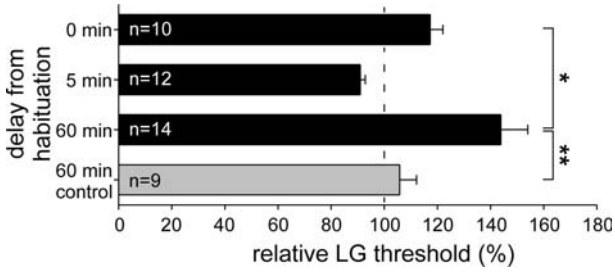

Fig. 3 Relative changes in LG threshold following habituation. The intensity of electrical stimulation needed to elicit an LG spike immediately (*top*), 5 min (2nd) and 60 min (3rd) following habituation expressed as percentages of the initial intensity of stimulation. The *bottom* indicates the intensity of electrical stimulation needed to elicit an LG spike 60 min after no series of habituating stimuli as control. *Error bars* indicate the standard error. *Asterisks* indicate that the LG threshold after a 60 min delay differed significantly from the threshold just after habituation (*$P < 0.05$ with two sample t test) and threshold after a 60 min delay without habituating stimulus series (**$P < 0.01$ with two sample t test). The number of animals examined for each condition is indicated by number n

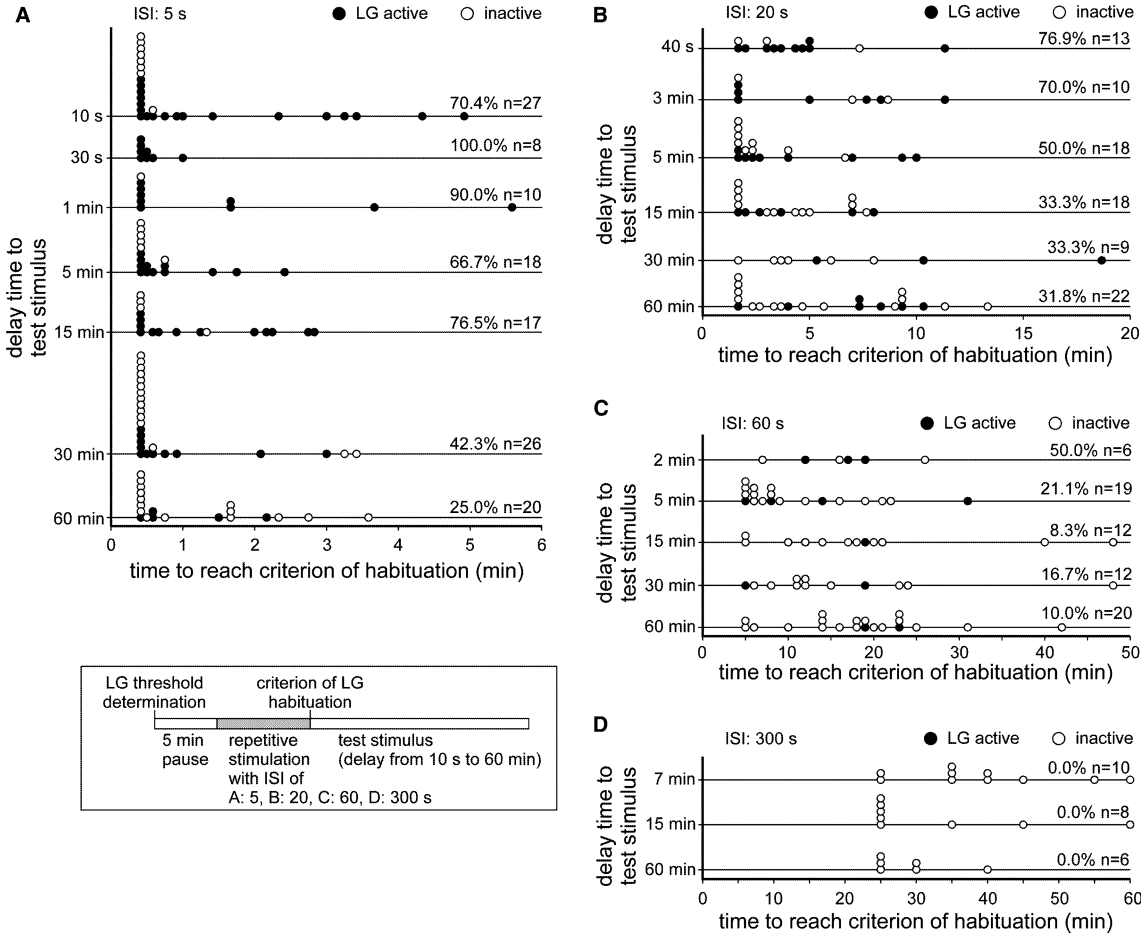

Fig. 4 a–d Effect of ISI on the decrease in excitability of LG following habituation. The LG response to the test stimulus was plotted against the time at which LG habituated. Filled circles represent responses in which LG produced a spike and open circles represent responses in which LG failed to spike in response to test stimuli after various delays following habituation. The occurrence probability of LG spikes to the test stimulus in each rest period following habituation is indicated on the right. The LG response habituated upon repetitive stimulation at ISIs of 5 s (**a**), 20 s (**b**), 60 s (**c**) and 300 s (**d**). The number of animals examined in each condition is indicated by number *n*. The LG consistently failed to respond with a spike to the test stimuli following habituation when repetitive stimulation was applied at a 300 s ISI (**d**). Inset indicates the experimental protocol showing the timings of stimulation

To examine whether the decrease in the excitability of LG following habituation also occurred at longer ISIs, the LG response probabilities were examined after various delays following habituation (Fig. 4). In Fig4 the LG response to test stimuli in each preparation is shown plotted against the time required to reach habituation, and the response probabilities of LGs for each delay time are shown in insets on the right. With ISI of 5 s, the response of LG fully recovered within 1 min, and the excitability of LG decreased after a delay of 15 min (Fig. 4a). For longer ISIs of 20 s and 60 s a short recovery and decrease in excitability of LG also occurred, but the delay time needed to depress LG activity decreased (Fig. 4b, c). For example, with ISIs of 20 s and 60 s, the maximum recovery of the LG responses were about 77% and 50%, respectively, and the decrease in LG response started from a delay time of 5 min. At an ISI of 300 s, no preparation responded with a LG spike after delay time of 7–60 min (Fig. 4d).

For longer ISIs longer training periods were needed to reach habituation. It was therefore possible that the fewer recoveries and shorter delays to depress the LG response could be attributed to degradation of preparations over time. If this was true, rapidly habituated preparations would respond with LG spikes to test stimulus more than slowly habituated ones. There was however, no such correlation indicating that there was run-down in the preparations. For example, with ISI of 60 s, the time to reach habituation ranged from 5 min to 58 min, and the probability of LG spike response in preparations that habituated less than 15 min and over 15 min were about 14% (out of 35 preparations) and 21% (out of 34 preparations), respectively.

The effect of inter-stimulus interval on the time-course of excitability

There was a large difference in the time elapsed during five successive failures of the LG response needed to produce habituation between different ISIs. We there-

fore investigated the effect of ISI on the time course of the decrease in LG excitability by comparing the LG response probabilities to test stimuli following a delay from the onset of habituation (Fig. 5). The change in response probabilities of LGs revealed that the decrease in excitability of LG occurred with shorter delay periods when habituated with long ISIs (20–60 s). After a delay period of 0–10 min more than 60% of preparations responded with spike to test stimuli with 1–20 s intervals, while a significantly lower proportion of preparations (about 30%) responded with 60 s interval ($P < 0.001$ 60 s vs. 1 s and 5 s, $P < 0.01$ 60 s vs. 20 s; χ^2 test for independence). During the next delay period of 10–20 min, the LG response probability with 20 s and 60 s intervals decreased to less than 60% of the 0–10 min interval, but only small decreases or consistent responses were observed with 1 s and 5 s intervals. The response probability with 1–5 s interval was still significantly higher than at 60 s ($P < 0.01$; χ^2 test for independence), while the response probability with 20 s intervals decreased significantly compared to intervals of 5 s ($P < 0.05$; χ^2 test for independence). During delay periods of 20–40 min, the response probability with 1 s and 5 s intervals decreased to less than 60% of the 0–10 min interval and there was no significant difference between intervals as in the later delay periods of 60 min and over.

Effects of serotonin and octopamine on decrease in excitability of LG activity

As a first step to understanding the neural mechanism underlying the decrease in excitability of LG following habituation, the effects of serotonin and octopamine on the response of the LGs to test stimuli were analyzed. Following habituation of LG with repeated stimulation at 1 s ISI, 10^{-6} mol l^{-1} serotonin or 10^{-6} mol l^{-1} octopamine was injected locally into the neuropil near the dendritic branches of the LGs (inset in Fig. 6a). When the test stimulus was applied 2 s or 5 s following habituation, the occurrence probability of the spike

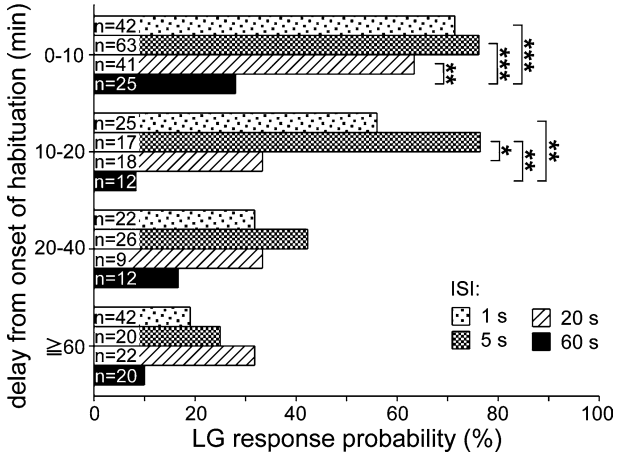

Fig. 5 Relationship between ISI and decrease in excitability of LGs following onset of habituation. The LG response probability to test stimuli after various delay periods following the onset of habituation were recalculated from the data obtained in Figs. 2 and 4, and compared between ISIs in each delay period. Asterisks indicate statistical significance; $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ with χ^2 test for independence. The number of preparations used in each condition is indicated by number *n* on the left side of each bar

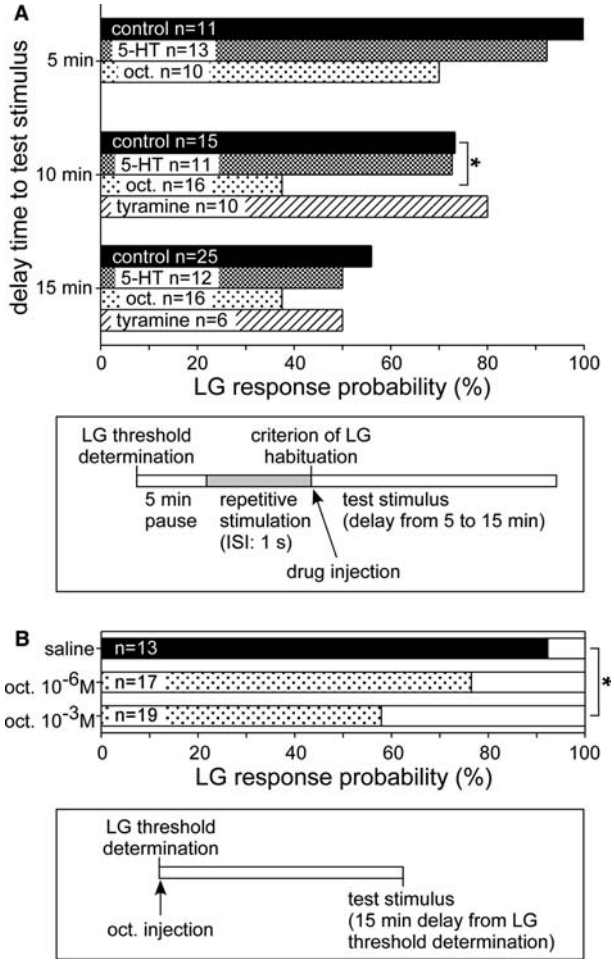

Fig. 6 **a** Effect of 10^{-6} mol l^{-1} serotonin, octopamine and tyramine upon the decrease in excitability of LG following habituation. Each drug was injected locally into the neuropil just after LG showed habituation to repetitive stimulation at a 1 s ISI. The occurrence probability of LG spikes to the test stimulus after 5 min, 10 min, and 15 min following habituation. *Asterisks* indicate the occurrence probability of LG spikes differed significantly between control and 10^{-6} mol l^{-1} octopamine-injected crayfish ($P < 0.05$ with χ^2 test for independence). The number of animals examined in each condition is indicated by number *n*. *Inset* indicates the experimental protocol showing the timings of stimulation. **b** Effect of octopamine on LG activity without repeated sensory stimulation. After the LG threshold was determined, animals were tested 15 min later with and without local injection of octopamine or saline (= control) into the neuropil. The occurrence probability of LG spikes to the same intensity of sensory stimulation was plotted. *Asterisks* indicate that the occurrence probability of LG spikes differed significantly between control and 10^{-3} mol l^{-1} octopamine-injected crayfish ($P < 0.05$ with χ^2 test for independence). Inset indicates the experimental protocol showing the timings of stimulation

response of LG increased slightly during the injection of both serotonin and octopamine (not shown). The effect of serotonin was inconsistent when the test stimulus was applied after a longer delay (i.e. 5 min, 10 min, and 15 min) following habituation (Fig. 6a). For example, after a delay of 10 min, LG in about 75% of preparations responded with a spike in response to the test stimulus in both the control and test preparations in which serotonin was applied (Fig. 6a). By contrast, the local injection of octopamine promoted the achievement of decrease in excitability of LG. The probability of the occurrence of the LG spike response to the test stimulus after 5 min, 10 min, or 15 min following habituation was less in octopamine injected preparations compared to control animals. In particular, after 10 min of rest, the LG response probability of preparations treated with octopamine declined significantly compared to controls ($P < 0.05$; χ^2 test for independence). The test stimulus elicited LG spikes in less than 40% of the octopamine-treated preparations, but the same stimulus elicited spikes in about 70% of the control preparations (Fig. 6a). Since Saudou et al. (1990) reported that the tyramine receptor has an affinity for octopamine, the possibility that octopamine could act via a tyramine receptor was tested by applying tyramine into the neuropil following habituation. Pressure injection of 10^{-6} mol l^{-1} tyramine had no effect on the LG response probability to the test stimulus after 10 min and 15 min following habituation (Fig. 6a).

To examine the possibility that octopamine could trigger neural processes underlying the decrease in excitability of LG, the effect of octopamine was analyzed using preparations that were rested for 15 min without further stimulation after determination of the spike threshold (Fig. 6b). In control animals in which just saline was injected into the neuropil, 12 out of 13 preparations gave rise to a spike when the test stimulus was applied after a delay of 15 min. In preparations that were injected with 10^{-6} mol l^{-1} octopamine, the response probability of the LGs to the test stimulus decreased to about 75% (13 out of 17 crayfish). When 10^{-3} mol l^{-1} octopamine was applied, the response probability of the LG significantly decreased to less than 60% (11 out of 19 crayfish) ($P < 0.05$; χ^2 test for independence). Thus, octopamine increased the spike threshold level of LG during a delay of 15 min.

Discussion

Recovery from LG habituation

We have shown, using isolated abdominal nerve cord preparations, that the responsiveness of the LG recovers rapidly following habituation to repetitive sensory stimulation with short ISIs of less than 1 min. For example, 10 s after the LG response had fully habituated with stimuli of 1 s ISI, additional sensory stimulation again elicited spikes in LG. Furthermore, a 1 min delay following habituation was sufficient for LG to recover when stimulated with a 5 s ISI. The results of this study, therefore, support the early work of Krasne (1969) that a reduction of EPSP amplitude in LG caused by repetitive sensory stimulation at a 5 s ISI recovered within 5 min. This habituation was caused by a decline of transmitter release from mechanosensory afferents (Zucker 1972), but synaptic efficacy could recover readily after a short delay.

Decrease in excitability of LG following habituation

Following habituation, subsequent stimulation after long delays failed to give rise to spikes in LG. This decrease in excitability of LG was maintained for more than 60 min. The critical delay period needed for decrease in excitability of LG was dependent on the ISI of the repetitive stimulation used to generate habituation. As the ISI was increased, the delay needed for decrease in excitability became shorter. This suggests that some physiological change occurs in the LGs or local circuits within the terminal ganglion following the spike response of LGs to sensory stimulation. The neural processes required to elicit decrease in excitability occur slowly since decrease in excitability occurred only after more than 10 min delay following habituation. Preparations habituated very rapidly when a repeated stimulus was applied at a short ISI. For example 80% of preparations showed habituation after the 4th trial of repeated stimulation at 1 s ISI, whereas about 50% of the preparations still responded with spikes even after the 20th trial of stimulation at a 1 min interval (Fig. 1). Therefore, as the ISI became shorter, a longer delay was necessary to generate decrease in excitability (cf. Figs. 2a, 4, 5). The observation that no recovery from habituation was observed in the LG response to repetitive stimulation at a 300 s ISI (Fig. 4d) would support the need for a delay in achievement of decrease in excitability. The LG response would shift directly from habituation to decrease in excitability in the course of a longer period of stimulation.

Since the LG response recovered quickly from habituation but soon depressed after certain periods of delay, the decrease in excitability of LG could be regarded as a form of non-associative learning. At the moment, the neural mechanisms underlying this decrease in excitability remain unclear, but the observation that the threshold of LG following decrease in excitability became significantly higher than the threshold just after habituation (Fig. 3) suggests that some physiological change could occur in the LGs during the period of the delay following habituation. Furthermore, the observations that the local injection of octopamine into the neuropil promoted decrease in excitability of LG (Fig. 6a) and the excitability of the LGs was significantly reduced by the local injection of octopamine (Fig. 6b) also support this idea. Octopamine could trigger neural processes underlying the decrease in

excitability of LG. The immediate effect of octopamine is, however, known to enhance the excitability of the LG since application of octopamine increases the synaptic response of the LG and some ascending interneurons to sensory stimulation (Glanzman and Krasne 1983; Bustamante and Krasne 1991). In this study, the occurrence of the spike response of the LGs after a short delay following habituation was increased by local injection of octopamine as well as serotonin. Furthermore, enhancement of decrease in excitability by octopamine is apparent after at least 5 min octopamine application. Thus, the effect of octopamine is to shorten the period for causing decrease in excitability via indirect effects since it is known to stimulate intracellular messenger cascades like cAMP or IP_3 systems in invertebrates (Roeder 1999) and to induce associative learning in honeybees (Hammer and Menzel 1998). Furthermore, the possibility that the probable site of change is the presynaptic terminals of the sensory afferents is still open to question. Further electrophysiological and pharmacological studies are, therefore, necessary to clarify the neural basis of the decrease in excitability of LG.

Inter-stimulus interval and time course of the habituation

The decline in LG response probability was slow when ISI increased from 1 s to 60 s, but, interestingly, at a 300 s ISI, it declined faster than a 60 s ISI (Fig. 1a). If they were plotted against the elapsed time for repetitive sensory stimulation, however, time course of the decline in LG response probability with 300 s ISI was very similar to 60 s. The 2nd, 3rd, and 4th points on the 300 s plot have similar values (60%, 57%, and 45%, respectively) and occur at the same time as the 6th, 11th, and 16th points on the 60 s plot (70%, 59%, and 47%, respectively). At this moment, we do not know what does this coincidence indicate. It may be that the time course of habituation reaches some minimum rate of change for ISI equal to 60 s or greater. To make this point clear, further studies on relationship between ISI and time course of the habituation are indispensable.

Relationship between tonic descending inputs and decrease in excitability of LG

Habituation of the LG escape reaction of the crayfish is known to be retained for several hours in intact animals (Krasne and Woodsmall 1969; Wine et al. 1975). Krasne and Teshiba (1995) suggested that descending GABAergic tonic inputs from higher centers are essential in maintaining habituation. Removing the influence of descending inputs by cutting the nerve cord between the thorax and the abdomen reduces the tendency of the LG threshold to rise during habituation. In addition, our study demonstrates that some physiological changes that reduce LG excitability may occur in the LG itself during the period following habituation. Both systems might be closely related since some descending octopaminergic interneurons in the lobster send axons into the terminal ganglion to extend branches extensively near the dendrites of the LGs (Schneider et al. 1993). As Krasne and Teshiba (1995) indicate, a long-lasting reduction of the synaptic efficacy from sensory afferents would be disadvantageous for the crayfish since sensory inputs from the tailfan elicit various behaviors other than escape tailflipping (e.g., Nagayama et al. 1986). Tonic descending control and decrease in excitability of LG could be well suited to inhibit the LG circuit selectively. Further studies are necessary to clarify the interactions between descending inhibitory pathways and the decrease in excitability of LG.

Acknowledgements We are grateful to Dr. P.L. Newland for his critical reading of this manuscript. T. Nagayama was supported by grants from the Ministry of Education, Science, Sport, Culture and Technology to carry out this work (15570060).

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