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RESEARCH ARTICLE

Social status-dependent modulation of LG-flip habituation in the crayfish

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SUMMARY

Strong stimuli applied to the tailfan of the crayfish *Procambarus clarkii* evoked lateral giant interneurone (LG)-mediated tailflips. When the sensory stimulus was applied repeatedly, the response of the LG habituated until it failed to give rise to a spike. We found that this LG-flip habituation was dependent on social status. With a short interstimulus interval of 5 s, the rate of habituation of the LG in both socially dominant and subordinate crayfish was lower than that in socially isolated animals. By contrast, with a long interstimulus interval of 60 s, the rate of habituation of subordinate animals was lower than that of both socially isolated and dominant animals. The excitability of the LGs following habituation was also dependent on social status. Following habituation, the spike response of LGs recovered within several minutes; however, they showed significant depression with a decrease in excitability. With a 5 or 60 s interstimulus interval, subordinate animals showed longer delays of depression compared with dominant animals. A decrease in the rate of habituation and a delay of depression in subordinate crayfish would be advantageous for maintaining an active escape response to evade repeated attacks of dominant animals and a reduced learning ability to adapt to social status.

Key words: crayfish, tailflip, social hierarchy, habituation, retention.

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INTRODUCTION

Habituation is a well-known form of non-associative learning (Thompson and Spencer, 1966) in which reflexive behavioural responses gradually reduce upon repeated stimulation. Habituation is subject to change, as has been shown in the siphon withdrawal reflex in *Aplysia* (Kandel, 2009) and the lateral giant interneurone (LG)-mediated tailflip of crayfish (Krasne and Woodsmall, 1969; Zucker, 1972; Araki and Nagayama, 2003; Nagayama and Newland, 2011). In crayfish, exteroceptive and proprioceptive information from the tailfan is transmitted to the LGs as excitatory post-synaptic potentials (EPSPs) directly *via* electrical and chemical synapses from sensory afferents and indirectly through sensory interneurones (Newland et al., 2000). The LGs are inactivated rapidly upon repeated sensory stimulation. This results in crayfish becoming unresponsive to similar sensory stimuli and reduces the effectiveness of the escape behaviour. Habituation is controlled by descending inhibition from the brain in intact animals (Shirinyan et al., 2006), and using isolated abdominal nerve cord preparations, habituation has also been found to be caused by a decline of chemical transmitter from exteroceptive afferents. Synaptic efficacy recovers readily after several minutes (Krasne, 1969), restoring escape behaviour to control levels. The excitability of LGs, however, decreases after a 10 min, or more, delay after habituation and this depression of the LGs is maintained for more than 60 min (Araki and Nagayama, 2005).

In both vertebrates and invertebrates, the establishment of social hierarchy through conflict over living resources has so far been reported to change agonistic and non-agonistic behaviours of individuals according to the acquired social status (Yeh et al., 1997; Dyakonova et al., 1999; Hofmann et al., 1999; von Holst et al., 1999; Herberholz et al., 2003; Song et al., 2006). The avoidance reactions of crayfish are one of the interesting examples of social status-dependent behavioural plasticity. When a crayfish is in a stationary resting posture, gentle mechanical stimulation to the tailfan evokes an avoidance reaction (Nagayama et al., 1986). Small crayfish show an escape-like dart response while larger animals show a defensive-like turn response. When two small crayfish encounter each other, a winner–loser relationship is established following several combats, with the winner changing reaction to show a turn response (Fujimoto et al., 2011). A similar response change is observed when mechanical stimulation is applied to the crayfish abdomen (Song et al., 2006; Issa et al., 2012).

The LG-mediated tailflip is triggered by the sudden attack of predators to the rear of the animal (Herberholz et al., 2004) and is also observed during agonistic encounters between crayfish (Sato and Nagayama, 2012). Thus, habituation of the LG appears to be affected by the establishment of social status of crayfish as dominant animals perform more aggressively and subordinate animals show mainly submissive acts like retreat and tailflip in response to the attacks of dominant animals (Sato and Nagayama, 2012; Ueno and Nagayama, 2012). There is only a small body of evidence that indicates state-dependent modulation of habituation in both vertebrates and invertebrates. One example is the proboscis extension response in honeybees. Hungry bees need more trials for habituation than fed bees (Braun and Bicker, 1992) and this lower degree of habituation is due to a higher responsiveness to sucrose than in satiated bees (Scheiner, 2004). In this study, we analysed whether habituation of LG-mediated tailflip changes depending on social status.

MATERIALS AND METHODS

Animals

Adult male crayfish, *Procambarus clarkii* (Girard 1852) (6–9 cm body length from rostrum to telson) were used in all experiments. Crayfish were purchased commercially from a local supplier; they were maintained in laboratory freshwater tanks and fed weekly on a diet of chopped potato and liver. Prior to experiments, crayfish were isolated individually in small opaque containers (19×33×15 cm, width×length×height) filled with water to a depth of 10 cm, for at least 30 days on a 12 h:12 h photoperiod. Crayfish that moulted within a week of the experiments were not used in this study.

Establishment of social hierarchy

Experimental trials were carried out in a dimly lit laboratory at a room temperature of ~23°C. Two crayfish of similar size (length difference less than 10%) were selected and paired in a new opaque container of 26×38×24 cm (width×length×height) filled with water of about half-depth. Prior to each trial, an opaque plastic divider was placed in the centre of the tank, separating it into two areas; a single crayfish was placed on each side of this barrier and allowed to acclimate for at least 10 min before the divider was removed.

After the pairing, the crayfish started agonistic behaviour, e.g. approach and fighting, and a winner–loser relationship (dominance hierarchy) was established within 30 min (Sato and Nagayama, 2012; Ueno and Nagayama, 2012). Before dominance order was established, the crayfish that initiated the approach was frequently beaten in the following bouts by their opponent. After the establishment of dominance order, however, subordinate crayfish almost always showed a retreat or escape tailflip following the dominant animal's approach without a fight. We determined the dominance order of paired crayfish when the subordinate crayfish showed a retreat or tailflip following the dominant's approach at least three times in succession. After 1 h of pairing, dominant and subordinate crayfish were re-isolated for physiological experiments.

Habituation curve

Dominance order is maintained for more than a week (Hemsworth et al., 2007). Within 18 h (from 2 to 18 h) of formation of the dominant–subordinate relationship, dominant and subordinate animals were dissected to analyse physiologically the habituation of the LG-mediated tailflip. For control experiments, isolated crayfish without the experience of agonistic encounters were used. Crayfish were quickly decapitated and the abdomen pinned in a dissection chamber containing cooled van Harreveld's solution (van Harreveld, 1936). The nerve chain from the 2nd to terminal (6th) abdominal ganglion with relevant nerve roots was isolated from the abdomen and transferred and pinned, dorsal-side up, in a Sylgard-lined perfusion chamber, containing van Harreveld's solution at ~18°C. The spike activity of LG was monitored extracellularly from the 4th to the 5th abdominal connective using a suction electrode. Nerve roots 2, 3 and 4 of the terminal abdominal ganglion, which contain mechanosensory afferents innervating the uropods and telson, were electrically stimulated simultaneously using a single oil hook electrode. Square stimulus pulses (0.01–0.05 ms duration, 1–20 V intensity) were delivered through the stimulating electrode. The stimulus intensity required to elicit the LG spike was somewhat variable from preparation to preparation because of the inconsistency of electrode attachment to the three nerve roots.

After 15 min rest following dissection, the spike threshold of the LG to sensory stimulation was determined by gradually increasing the intensity of stimulation of the sensory nerves with a 20 s inter-stimulus interval. After the LG spike threshold was determined, the stimulation intensity was set so that the stimulus was just suprathreshold. The preparation was rested for a further 5 min before repeated sensory stimulation was applied with an inter-stimulus interval of either 5 or 60 s, until the LG failed to give rise to spikes following five continuous stimuli. Preparations that did not show habituation after 40 trials of stimulation were counted as 'non-habituated' preparations. The spike rate of the LGs was calculated by averaging each trial of stimulation and was plotted on a habituation curve.

In some preparations, following habituation, a single stimulus pulse of the same intensity as the initial one (the test stimulus) was applied following a delay of 30 min, in the case of inter-stimulus intervals of 5 s, and 10 min with inter-stimulus intervals of 60 s, to determine whether depression of LGs occurred. The delay time of each inter-stimulus interval was selected as the critical period for LG depression (see Araki and Nagayama, 2005).

Statistical analyses

The difference in the number of stimuli required for LG to habituate was analysed statistically using a log-rank test and a Fisher's exact test (Sigma Plot ver.11).

RESULTS

Sensory stimulation applied to nerve roots 2–4 of the 6th abdominal ganglion gave rise to a spike in the LG interneurone. There was no significant difference in the spike threshold of the LGs between socially isolated (i.e. control), dominant or subordinate crayfish.

Habituation with a 5 s inter-stimulus interval

When the sensory stimulus was applied repeatedly with a 5 s inter-stimulus interval, the response of the LG gradually declined until it failed to give rise to a spike (Fig. 1A). Socially isolated crayfish showed a rapid habituation of the LG, with a decrease in firing probability by 50% within four trials of stimulation, and by 75% after 25 trials. By contrast, socially subordinate crayfish showed a slower rate of habituation. After the 20th trial of stimulation, only 50% of subordinate animals showed habituation, while 35% still responded with a spike after 40 trials. Dominant crayfish were also found to show a slow decline in the rate of habituation. Approximately 20 trials were needed to decrease LG firing probability by 50%, while more than 40% of LGs still produced a spike after 40 trials. To show the different distributions of the number of trials needed to produce habituation, preparations were plotted according to the stimulus number after the LG failed to show a spike response. In control animals (Fig. 1B), 37.5% of preparations (N=33 out of 88 preparations) showed habituation from the 2nd sensory stimulus, and this percentage decreased quickly with the increase in the number of stimuli. Approximately 65% of preparations showed habituation after the 10th stimulus, while only 16% (N=14) showed no habituation within 40 trials (filled bar in Fig. 1B). In dominant (Fig. 1C) and subordinate crayfish (Fig. 1D), less than 40% of preparations showed habituation within 10 trials, while 20 out of 45 dominant animals (44.4%) and 16 out of 44 subordinate animals (36.4%) did not habituate (filled bars in Fig. 1C,D). The number of stimuli required to habituate LG in both the dominant and subordinate animals increased significantly in comparison with that for socially isolated crayfish (P<0.01, log-rank test). For animals that showed habituation, the number of trials (mean ± s.e.m.) required for habituation was 8.2±1.1 in control (N=74), 8.2±1.8 in dominant (N=25) and 11.9±2.2 in subordinate animals (N=28). There was no significant difference between crayfish that showed habituation in each group using a log-rank test.

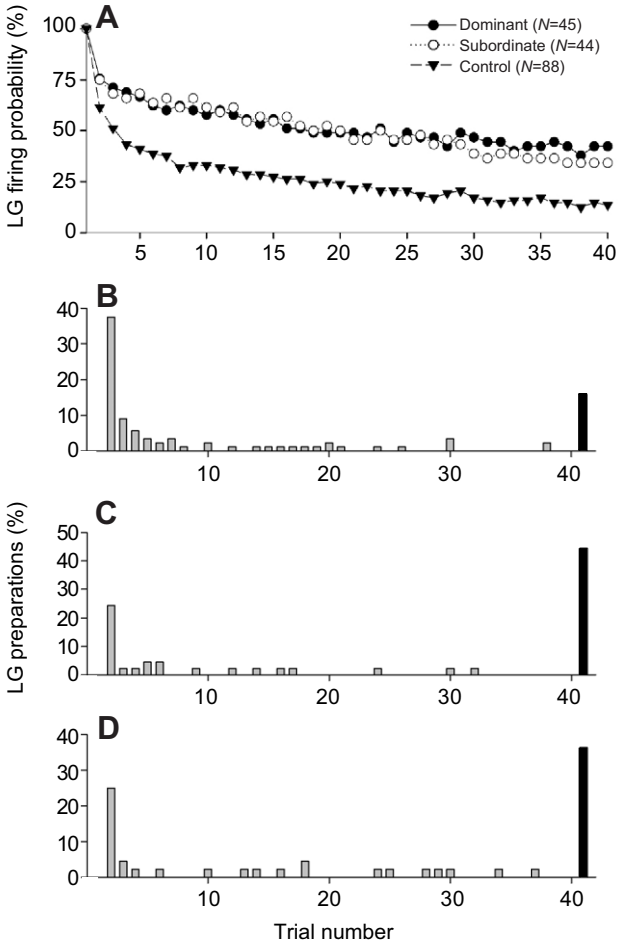

Fig. 1. Effect of social status on the habituation of the lateral giant interneurone (LG) spike response to repeated stimulation with a 5 s inter-stimulus interval. (A) Habituation curves of the response of the LG to repeated sensory stimulation. The LG firing probabilities of control, dominant and subordinate animals are plotted as the percentage of animals in which the LG fired on a given trial. *N*, number of animals used. (B–D) Distribution of stimulus numbers required to reach the onset of habituation for (B) control (*N*=88), (C) dominant (*N*=45) and (D) subordinate (*N*=44) animals. The black bars indicate animals that did not show habituation within 40 trials.

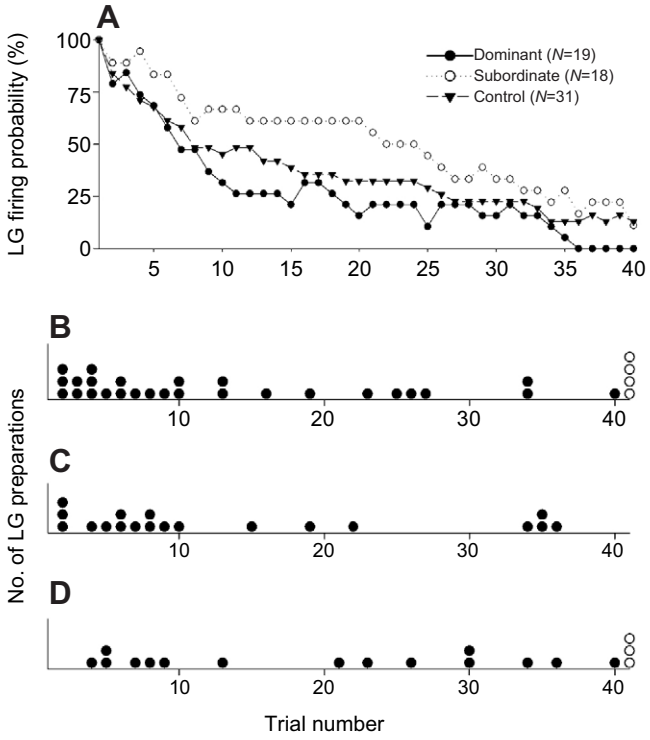

Fig. 2. Effect of social status on habituation of the LG spike response to repeated stimulation with a 60 s inter-stimulus interval. (A) Habituation curves of the response of the LG to repeated sensory stimulation. The LG firing probabilities of control, dominant and subordinate animals are plotted as the percentage of animals in which the LG fired on a given trial. *N*, number of animals used. (B–D) Distribution of stimulus numbers required to reach the onset of habituation for (B) control, (C) dominant and (D) subordinate animals. Open circles indicate animals that did not show habituation within 40 trials.

Habituation with a 60 s inter-stimulus interval

When the sensory stimulus was applied repeatedly with a 60 s inter-stimulus interval, the decrease in the LG response was less rapid (Fig. 2A). In the control and dominant animals, the response of LG declined to 70% after five trials and to 20–30% after 20 trials. Approximately 10% of control animals still responded with a spike after 40 trials, while all dominant animals showed habituation within 36 trials. In subordinate animals, the response of the LG declined to 80% after five trials, to 60% after 20 trials and to 10% after 40 trials. To determine the different distributions of the number of trials required to for habituation, preparations were plotted according to the stimulus number after the LG failed to show a spike response. In control (Fig. 2B) and dominant crayfish (Fig. 2C), more than 50% of preparations (*N*=16 out of 31 control and *N*=12 out of 19 dominant animals) showed habituation within 10 trials, while only 6 out of 18 subordinate crayfish (33.3%) showed habituation (Fig. 2D). A further 40% of subordinate animals (*N*=8) habituated after 20 trials while the remaining 16.6% (*N*=3) showed no habituation within 40 trials (open circles in Fig. 2D). In comparison, 12.9% of control animals (*N*=4; open circles in Fig. 2B) and none of the dominant animals failed to show habituation. Thus, the number of stimuli necessary to cause habituation in subordinate animals increased compared with that of control and dominant animals (significantly different from dominants, $P<0.05$, log-rank test). For animals that showed habituation, the number of trials (mean ± s.e.m.) for habituation was 14.1±2.7 in control (*N*=27), 13.9±2.8 in dominant animals (*N*=19) and 19.4±3.3 in subordinate animals (*N*=15). Again, in subordinate animals more trials were required although this was not statistically significant ($P=0.141$ *versus* control and $P=0.276$ *versus* dominants, log-rank test).

Depression of LG activity following habituation

Our previous study (Araki and Nagayama, 2005) showed that the spike response of the LGs recovered within several minutes of habituation; however, the LGs failed to spike when an additional stimulus was applied after specific periods following habituation. This critical period for LG depression, a decrease in the excitability of LG following habituation, was dependent on the inter-stimulus interval of the initial repetitive stimulus. We examined whether social status also caused a change in the LG excitability following habituation. Twenty control, 11 dominant and 17 subordinate crayfish were tested following habituation with a 5 s inter-stimulus interval (Fig. 3A). A test stimulus was applied only once, 30 min after habituation. In control crayfish, 8 out of 20 LGs gave rise to

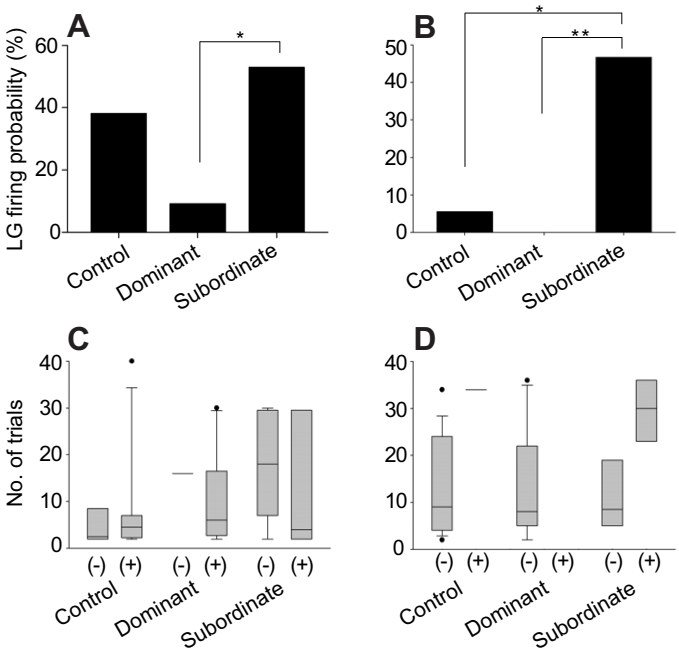

Fig. 3. Decrease in the excitability of the LG following habituation. (A) Occurrence probability of a LG spike in response to a test stimulus 30 min after habituation caused by repeated sensory stimulation with a 5 s inter-stimulus interval. (B) Occurrence probability of a LG spike in response to a test stimulus 10 min after habituation caused by repeated sensory stimulation with a 60 s inter-stimulus interval. For A and B, asterisks indicate significant differences (*P<0.05, **P<0.01). (C) Number of stimuli required to reach the onset of habituation to the repeated stimulus with a 5 s inter-stimulus interval. Preparations that did not respond with a spike to a test stimulus 30 min after habituation (–) and preparations that responded with a spike to a test stimulus (+) are shown. (D) Number of stimuli required to reach the onset of habituation to the repeated stimulus with a 60 s inter-stimulus interval. Preparations that did not respond with a spike to a test stimulus 10 min after habituation (–) and preparations that responded with a spike to a test stimulus (+) are shown. For C and D, lines within the boxes indicate the median values, the boxes represent the 25% and the 75% quartiles, the error bars indicate the 90th and 10th percentiles, and filled circles indicate outlying points.

a spike in response to a test stimulus such that their firing probability was consistent with our previous work (Araki and Nagayama, 2005). Only one out of 11 dominant crayfish gave rise to a LG spike, while in subordinates more than 50% (n=9 out of 17) gave rise to a spike. This difference between dominants and subordinates was statistically significant (P<0.05, Fisher's exact test). Eighteen control, 19 dominant and 16 subordinate crayfish were also tested following habituation to repeated stimulation with a 60 s inter-stimulus interval (Fig. 3B). After 10 min of rest following habituation, the test stimulus failed to elicit LG spikes in more than 95% of control (N=17 out of 18) and 100% of dominant animals (N=19). In contrast, 7 out of 15 subordinate animals gave rise to a LG spike. The rate of LG firing of subordinate animals was significantly higher than in control (P<0.05, Fisher's exact test) and dominant animals (P<0.01, Fisher's exact test). Thus, the process of LG depression following habituation was prolonged or suppressed in subordinate animals.

Next, we compared the process of habituation in both LG firing and non-firing animals in response to the test stimulus. In the 5 s inter-stimulus interval group (Fig. 3C), the number of trials (mean ± s.e.m.) for habituation was 8.4±3.2 in non-firing control animals (N=12), 7.9±4.4 in LG firing control animals (N=8), 10.4±3.1 in non-firing dominant animals (N=10), 16 in LG firing dominant animals (N=1), 12.6±5.3 in non-firing subordinate animals (N=8) and 17.2±3.6 in LG firing subordinate animals (N=9). There were no significant differences between LG firing and non-firing animals in each group or different groups (log-rank test). By contrast, as shown in Fig. 3D, in the 60 s inter-stimulus interval group, the number of trials with LG firing subordinate animals increased significantly compared with non-firing subordinate animals (P<0.05, log-rank test), and compared with control (P<0.01, log-rank test) and dominant animals (P<0.05, log-rank test). The number of trials (mean ± s.e.m.) for habituation was 12.9±2.5 in non-firing control animals (N=17), 34 in LG firing control animals (N=1), 13.9±2.8 in non-firing dominants (N=19), 11.9±3.3 in non-firing subordinates (N=8) and 28.0±4.1 in LG firing subordinates (N=7). Crayfish that habituated slowly did not show depression following habituation.

DISCUSSION

This study using isolated abdominal nerve cords shows that the rate of habituation of LG-mediated tailflips and the excitability of LGs following habituation change depending on the social status of the crayfish.

Habituation of subordinate and dominant animals

With a 5 or a 60 s inter-stimulus interval, the rate of habituation of LGs in subordinate animals was lower than in control animals. There was a slow decline in spike activity of the LGs to repeated sensory stimulation. As the LG-mediated tailflip is a highly stereotyped behaviour to allow animals to escape from threatening stimuli such as an attack from predators or a conspecific (Wine and Krasne, 1972; Sato and Nagayama, 2012), a decrease in the rate of habituation is crucial to evade repeated attacks of dominant animals. In dominant crayfish, the rate of habituation in response to repeated stimulation with a 5 s inter-stimulus interval was also lower than in control animals. With a 60 s inter-stimulus interval, by contrast, a dominant status had no effect on the rate at which habituation occurred. A decrease in the rate of habituation in dominant animals might appear contradictory as dominant individuals appear not to need to evade encounters from subordinates. The advantage of the prevention of habituation for dominants remains to be clarified in the future.

Neuromodulators, such as serotonin and octopamine, play a key role in dominance hierarchy formation (Huber et al., 1997; Huber and Delago, 1998). Direct injection of serotonin or octopamine into the systemic circulation of crayfish and lobsters induces dominant-like or subordinate-like posture, respectively (Livingstone et al., 1980; Tierney and Mangiamele, 2001). In shore crabs, serotonin levels in the haemolymph increase during a fight while octopamine levels decrease in dominant crabs but increase in subordinate animals (Sneddon et al., 2000). Furthermore, application of serotonin increases the LG response to sensory stimulation in dominant crayfish, while it decreases the LG excitability in subordinate animals (Yeh et al., 1997). These results strongly suggest that serotonin and octopamine are linked with social hierarchy. Serotonin and octopamine increase the number of stimuli required to habituate the LG response to sensory stimulation with a 5 s inter-stimulus interval (Araki et al., 2005). Both amines enhance the synaptic responses of the LG to sensory stimulation but do so through two different signaling cascades: serotonin-induced synaptic enhancement of the LGs is mediated by an increase in cAMP levels following activation of adenylate cyclase (Araki et al., 2005) while octopamine-induced enhancement is mediated by an increase in inositol trisphosphate (IP_3) levels following activation of phospholipase C (Araki and Nagayama, 2012). These findings

suggest the increment in the number of stimuli required to habituate the LG response to sensory stimulation with a 5 s inter-stimulus interval is possibly linked to serotonin and octopamine levels of dominant and subordinate crayfish. At present, it is not clear how serotonin and octopamine affect the degree of LG habituation to sensory stimulation with a 60 s inter-stimulus interval, pointing to the need for further studies.

Descending inhibition

As we used isolated abdominal nerve cord preparations throughout these experiments, the escape circuits were disconnected from the effect of descending signals from higher centres. Thus, habituation would be mainly caused by a decline in transmitter release from afferents onto the LG itself and the interneurones presynaptic to the LG (Araki and Nagayama, 2003; Zucker, 1972). Using more intact preparations, Shirinyan and colleagues have shown that habituation largely results from inhibition that descends from the brain (Shirinyan et al., 2006). Subordinate crayfish also show inhibition of LG-mediated tailflips during agonistic encounters (Krasne et al., 1997). In this study, the spike threshold of LGs in response to sensory stimulation was not statistically different between control and subordinate animals. This would be due to the lack of descending inhibitory inputs from the brain. Thus, further experiments using whole nerve cord preparations are necessary to confirm status-dependent modulation of habituation in intact animals. Reconfiguration of the neural circuit according to social status does not necessarily depend on a continuous descending signal from a higher centre in crayfish (Fujimoto et al., 2011; Issa et al., 2012). For example, the reversal of abdominal posture and uropod motor patterns from dart to turn response in dominant animals is observed in isolated abdominal nerve cord preparations (Fujimoto et al., 2011). Our results suggest that local centres of the LG-mediated escape circuit are modified according to social status. Descending mediation and modulation of local centres must be revealed by further behavioural and neurophysiological studies.

Decrease in excitability of the LG following habituation

As the inter-stimulus interval of repeated stimulation is shortened, the rate of habituation becomes faster. A decline of transmitter release from sensory afferents to the LG is thought to be essential to generate habituation (Zucker, 1972) and the synaptic efficacy of these chemical synapses could recover readily after a short delay. With further delays of 5–15 min depending on the inter-stimulus interval of repeated stimulation, the excitability of LGs decreases again (Araki and Nagayama, 2005). With short inter-stimulus intervals of 5 s, a test stimulus applied with delays of 10–60 s following habituation evokes a LG spike. With longer delays between habituation and a further test stimulus, the probability of evoking a LG spike gradually declines to about 40% with a delay of 30 min, and to 25% with a delay of 60 min (Araki and Nagayama, 2005). With longer inter-stimulus intervals of 60 s, the probability of evoking a LG spike is 50% with a delay of 2 min, about 20% with a delay of 5 min and about 10% with a delay of 15 min (Araki and Nagayama, 2005). The neural mechanisms underlying this LG depression following habituation are still unclear, but some changes in the intrinsic properties of the LG must occur. The extent of the decrease in the excitability of LGs following habituation was quite different between dominant and subordinate crayfish. About half of the subordinate crayfish with a 5 or 60 s inter-stimulus interval did not show depression of the LG 30 or 10 min after habituation, respectively, while almost all LGs tested showed depression in dominant crayfish. For dominant animals, an enhancement of depression would be reasonable as subordinates rarely attack dominants after the establishment of social order. For subordinate crayfish, a delay of depression would be useful to maintain an escape capability to evade dominants.

There was a strong relationship between the process of habituation and the probability of evoking a spike response in the LG to a test stimulus following habituation in subordinate animals when repeated stimulation was applied with a 60 s inter-stimulus interval. The rate of habituation of subordinate crayfish that did not show depression was statistically lower than that of subordinate crayfish that showed depression. Habituation is a simple learning process, so subordinate crayfish might show a reduced learning ability to adapt to social status. This could be investigated by physiological analysis of the neural mechanism based upon reduction of the habituation process.

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