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RESEARCH ARTICLE

Predation risk modifies behaviour by shaping the response of identified brain neurons

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ABSTRACT

Interpopulation comparisons in species that show behavioural variations associated with particular ecological disparities offer good opportunities for assessing how environmental factors may foster specific functional adaptations in the brain. Yet, studies on the neural substrate that can account for interpopulation behavioural adaptations are scarce. Predation is one of the strongest driving forces for behavioural evolvability and, consequently, for shaping structural and functional brain adaptations. We analysed the escape response of crabs *Neohelice granulata* from two isolated populations exposed to different risks of avian predation. Individuals from the high-risk area proved to be more reactive to visual danger stimuli (VDS) than those from an area where predators are rare. Control experiments indicate that the response difference was specific for impending visual threats. Subsequently, we analysed the response to VDS of a group of giant brain neurons that are thought to play a main role in the visually guided escape response of the crab. Neurons from animals of the population with the stronger escape response were more responsive to VDS than neurons from animals of the less reactive population. Our results suggest a robust linkage between the pressure imposed by the predation risk, the response of identified neurons and the behavioural outcome.

KEY WORDS: Visual stimuli, Escape, Crab, Population, Ecology

INTRODUCTION

The power of predation pressure in producing within-species behavioural variability has frequently been demonstrated (e.g. Hemmi et al., 2006; Herczeg et al., 2009). Therefore, it is expected that selective pressure stemming from predation will induce adaptive changes in the brain. However, the effects of such ecological force in sculpting brain functions have remained largely unexplored.

Behaviour is determined by the dynamic interactions of many neurons, which imposes a serious challenge to understanding the neural basis of behaviour at the cellular level. The difficulties are lessened in some invertebrates, in which neural circuits composed of individually identifiable neurons allow the activity of particular neurons to be related to the behaviour of the animal (Kristan, 2008). This is especially valid in the case of escape behaviours, where the velocity required for the successful avoidance of a predatory attack implies rather straightforward circuits containing giant neurons (Fotowat et al., 2011; Herberholz and Marquart, 2012).

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The crab *Neohelice* (previously *Chasmagnathus*) *granulata* (Dana 1851), is an established invertebrate model for studying the neurobiology of visually guided behaviours, learning and memory (for reviews, see Hemmi and Tomsic, 2012; Tomsic and Maldonado, 2014; Tomsic and Romano, 2013). In nature, these crabs are preyed upon by gulls and other seabirds, for which reason they are highly prone to escape from visual danger stimuli (VDS) presented either in the field (Fathala and Maldonado, 2011; Hemmi and Tomsic, 2015) or in the laboratory (Oliva and Tomsic, 2012; Sztarker and Tomsic, 2008). An extensive amount of evidence indicates that the crab response to VDS involves a group of at least four distinct classes of motion-sensitive lobula giant (LG) neurons. These are central elements that arise in the lobula (third optic neuropile) and project their axon through the protocerebral tract, presumably to premotor centres in the midbrain (Berón de Astrada and Tomsic, 2002; Medan et al., 2007, 2015; Sztarker et al., 2005). The response strength of LG neurons correlates closely with the intensity of the escape response of crabs to VDS across a broad range of conditions (Oliva et al., 2007; Sztarker and Tomsic, 2008, 2011; Tomsic et al., 2003). The time course of LG responses also correlates well with the temporal dynamics of the escape response (Berón de Astrada et al., 2013; Oliva and Tomsic, 2014; Tomsic et al., 2009), suggesting that these neurons process most of the relevant visual information that drives the escape behaviour. Three classes of LG neurons respond not only to visual information but also to proprioceptive input from the legs (Berón de Astrada and Tomsic, 2002; Medan et al., 2007). This may allow them to process some of the contextual information during predator escape, such as path integration information, which has been shown to influence the escape and burrow defence behaviour of fiddler crabs in the field (Hemmi and Zeil, 2003a,b).

Populations of *N. granulata* are restricted to environments from hyposaline estuaries to hypersaline bays, both of which are associated with salt marshes, which are separated by hundreds of kilometres along the southwest Atlantic coast (Luppi et al., 2013). In two of these isolated populations, the density of crabs per area is similar (Luppi et al., 2013) but, as revealed by a 10 year census (Blanco and Carbonell, 2001), the number of shorebirds is much higher in the northern than in the southern location. In addition, a specialized crab-eating gull, *Larus atlanticus*, as well as other terrestrial birds whose diets include crabs, are present in large number in the northern location, but are almost absent in the southern one (Berón et al., 2011; Copello and Favero, 2001). This provides an excellent opportunity to investigate whether a meaningful ecological difference, such as the predation risk, may determine interpopulation differences of behaviour that can be tracked down to the neuronal level. Our results indicate that, indeed, the risk of predation affects the crab's behavioural performance by shaping the functioning of the LG neurons, and we discuss whether this might be due to evolutionary change or phenotypic plasticity.

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MATERIALS AND METHODS

Animals and populations

Neohelice granulata is a grapsoid crab reaching a size of up to 36 mm across the carapace. The crabs inhabit the intertidal zone, both mud flats and salt marshes (areas densely vegetated with cord grasses). Individual animals dig burrows for protection because they are preyed upon by several species of seabirds (Bachmann and Martinez, 1999; Blanco and Carbonell, 2001; Berón et al., 2011; Copello and Favero, 2001; Spivak and Sánchez, 1992), for minimization of adverse environmental conditions (Luppi et al., 2013), and for reproductive activities (Sal Moyano et al., 2012). The current study was performed on the populations inhabiting the coastal inlets of Mar Chiquita (37°40′S) and San Antonio Oeste (40°48′S), Argentina (Fig. 1A, B). The incidence of avian predators and predator attacks in each of the two populations was evaluated by 12 focal observations per day, one every 40 min, on an area of 25 m^2, carried out during the low tides over 5 days (a total of 60 observation episodes). We defined an avian event as the presence of one or more birds within the sample area in each observation episode. Events were differentiated between those corresponding to walking predators (WP), flying predators (FP) and flying non-predators (FNP). Care was taken not to double-count individuals.

Behaviour assessments in the laboratory

Male adult crabs (2.7–2.9 cm carapace width) collected from the two locations were transported to the laboratory and kept as previously described (e.g. Oliva and Tomsic, 2012). The experiments were performed during the first week following the animals' arrival at the laboratory.

Our methods for behavioural and electrophysiological recordings have been described in detail elsewhere (Tomsic et al., 2003). Briefly, the VDS consisted of the displacement of a black rectangle (subtended angle 30 deg×10 deg) positioned 25 cm above the crab, which simulated the motion of an impending natural threat (see

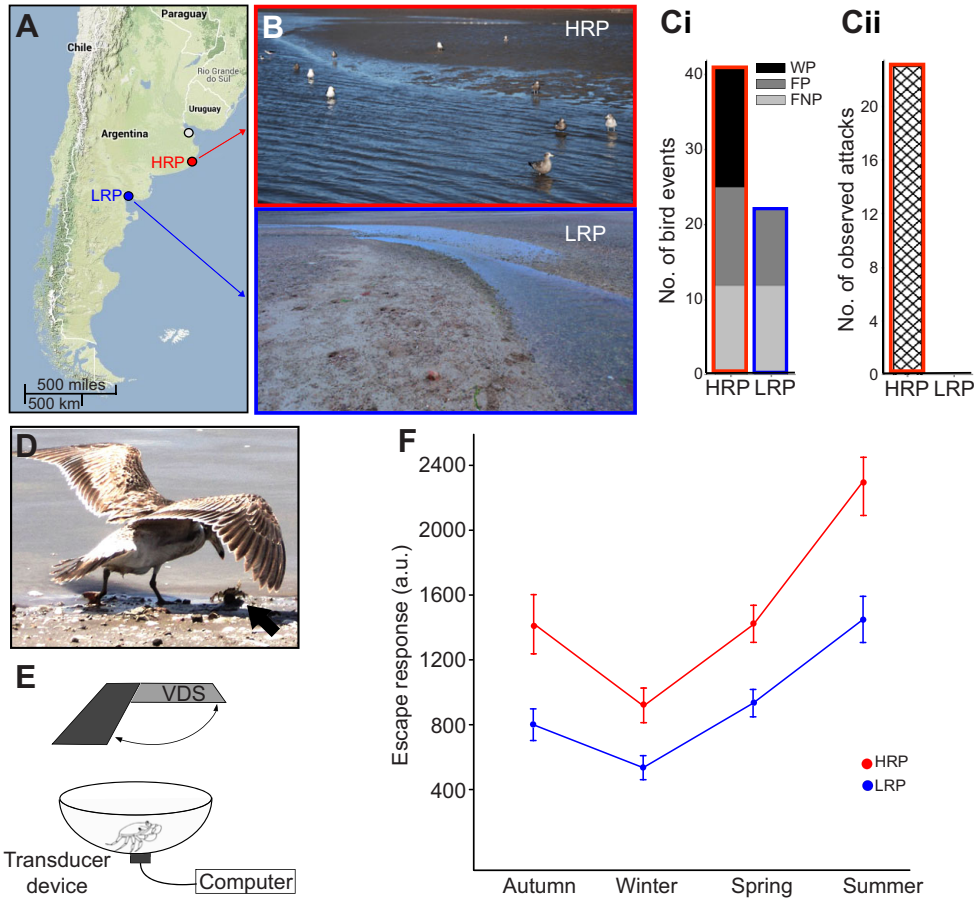

Fig. 1. Crabs from populations exposed to a different risk of avian predation show different escape intensity to visual danger stimuli (VDS). (A) Map of Argentina showing the two locations inhabited by the crab *Neohelice granulata* that were considered in the current study. This species is confined to the brackish water of estuaries and marshes, which are separated by long seashore distances. The red and blue dots mark the sites occupied by the populations with high and low risk of avian predation, respectively. The white dot indicates the location of the laboratory where the experiments were performed. (B) Pictures of the region occupied by the high-risk population (HRP), where crab-eating gulls are abundant (upper picture), and the region occupied by the low-risk population (LRP), where predatory birds are scarce (lower picture). (Ci) Number of bird events within an area of 25 m^2, during 60 observation episodes in the HRP and the LRP regions. In the HRP site, each event often included up to 5 birds, while in the LRP site, each event was represented by a single bird. Events were distinguished between those corresponding to walking and flying predators and flying non-predators (WP, FP, FNP, respectively). (Cii) Number of bird attacks upon crabs within the same area during 60 observation periods for each population. (D) A seagull chasing after a crab of the HRP (arrow). (E) The VDS used to elicit the crab's escape response in the laboratory consisted of the overhead displacement of a black rectangle. The intensity of escape was recorded by a transducer device at the bottom of the bowl containing the crab (see Materials and methods and Fig. 3B). (F) Comparison of the escape intensity (a.u., arbitrary units; see Materials and methods) of crabs from the HRP and LRP in response to the VDS presentation, across the four seasons (means±s.e.m.). Each data point was obtained by pooling and averaging responses of 150–240 animals from three separated capture efforts per season and per population, over 2 years. Two-way ANOVA disclosed a significant difference between populations (P<0.001) and across seasons (P<0.001).

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Fig. 1D,E). The motion cycle of the VDS comprised a 90 deg clockwise and counter-clockwise excursion that was completed in 2.2 s. The intensity of the escape response was evaluated in an actometer (Fig. 1E) consisting of a bowl-shaped container with a steep concave wall. The container was connected to a transducer device such that locomotion by the crab inside the container was translated into voltage changes recorded by a computer. The moving stimulus and recording method have been used extensively in our laboratory to study the escape response of the crab (reviewed in Tomsic and Romano, 2013).

To control whether the result obtained with the VDS was specific for visual stimuli signalling potential threats, we evaluated the animal's responses to a visual harmless stimulus, the optomotor response to the rotation of the panorama, and to a non-visual nociceptive stimulus, the avoidance response to an electrical shock. The optomotor response was elicited by the rotation of a drum with a pattern of black and white vertical stripes. Upon rotation of the panorama (2 cycles min^{-1}), the animal, within a transparent bowl located inside the drum, rotates in attempt to stabilize the optic flow. The circular base of the bowl was divided by 8 radial lines and the response was quantified as the number of lines crossed by the longitudinal axis of the animal in a period of 2 min. To evaluate the response to a non-visual stimulus, we used a mild electrical shock delivered by wire electrodes attached to one side of the animal's carapace. The avoidance response was assessed in the tethered crab walking on a freely rotating Styrofoam ball. The walking distance was measured by the rotation of the ball recorded with two optical mice (for further details, see Oliva and Tomsic, 2012).

Electrophysiology

Intracellular recordings were performed in the optic lobes of intact living animals according to methods previously described (e.g. Tomsic et al., 2003). Briefly, the crab was firmly held in an adjustable clamp and the eyestalks were cemented to the carapace in their normal seeing position. To access the optic ganglia, we removed a small section of cuticle (about 500 µm in diameter) from the tip of the eyestalk without causing damage to the ommatidia area and inserted a glass microelectrode through the opening in the cuticle. Microelectrodes (borosilicate glass, 1.2 mm outer diameter, 0.68 mm inner diameter) were pulled on a Flaming-Brown micropipette puller (P-97, Sutter Instruments), yielding tip resistances of 40–60 MΩ when filled with 3 mol l^{-1} KCl. A bridge balance amplifier was used for intracellular recordings (Axoclamp 2B, Molecular Devices). Signals were digitized at 10 kHz (Digidata 1320, Molecular Devices) and recorded with Clampex (Molecular Devices) for off-line analysis using pClamp 9.

Wide-field tangential neurons of the lobula can be identified based on their stronger response to motion stimuli compared with stationary changes of illumination (Berón de Astrada and Tomsic, 2002; Medan et al., 2007). Once the identity of a LG neuron was established, a black curtain was lowered to prevent uncontrolled visual stimulation and the animal was left undisturbed for 10 min before the experiment began. All intracellular recordings were performed at membrane resting potential. Only one neuron per animal was evaluated. Cells were recorded by experimenters blinded to the population to which the animal belonged.

Data analysis

The escape response was transduced and recorded in the actometer as a train of voltage changes, the magnitude of which reflects the intensity of the animal's attempts to get away from the VDS. The response intensity was estimated by the area of voltage changes recorded during the periods of visual motion stimulation. However, because the crab is constrained within the bowl and cannot really escape, the values are reported in arbitrary units (Tomsic et al., 2003). The neuronal response was estimated by the number of spikes recorded during the periods of stimulation. The stimulus-evoked excitatory postsynaptic potential (EPSP) was measured as the area of the electrical response after action potentials had been digitally removed from the records (for further details, see Tomsic et al., 2003). Two-way ANOVA and two-sample *t*-tests were used to compare the behavioural and the neural responses between crabs of the two populations.

RESULTS

Population differences and escape response

We analysed behavioural and neural responses from two isolated populations of the crab *N. granulata*, located hundreds of kilometres apart (Fig. 1A). Fig. 1B illustrates common views of these sites that herein we identify as the high- and the low-risk populations (HRP and LRP). As expected from the literature (Blanco and Carbonell, 2001; Berón et al., 2011; Copello and Favero, 2001), we detected the occurrence of more bird events in the HRP than in the LRP (Fig. 1Ci). In addition, in the LRP, each avian event was always represented by the presence of a single bird, while in the HRP, each event often included up to 5 birds. Fig. 1Ci shows that the number of events corresponding to non-predatory birds was the same in the two populations, but the number of predator events was different, in particular because there were not walking predators in the LRP. During an equivalent number of observations, we detected 23 predator attacks upon crabs in the HRP (65% were successful and crabs were eaten), but none in the LRP (Fig. 1Cii). *Neohelice granulata* is known to be preyed upon by various avian species, which approach the crab using several different strategies including walking, surface seizing and surface plunging (Blanco and Carbonell, 2001; Spivak and Sánchez, 1992). In our observations, however, all the attacks were performed by the predator landing nearby and ultimately walking towards the crab or just approaching by walking from far away (Fig. 1D). Therefore, in our study, the difference in predator attacks found between the HRP and the LRP was related to the different incidence of walking predators observed between the two populations.

Field observations indicated that crabs from the HRP flee from a person walking along the mudflat more readily than crabs from the LRP, suggesting that they are more responsive to visual threats. This impression was confirmed by measuring the escape response, which in nature is evoked by a chasing gull (Fig. 1D), when it was evoked by a simulated VDS in the laboratory (see Fig. 1E and Materials and methods). Our study included 12 capture efforts of crabs carried out simultaneously in the two locations, at different times of the year, over 2 years. For every single capture, behavioural experiments were performed with 50–80 individuals from each population. In Fig. 1F, each data point represents the mean response of 150–240 animals pooled from three separate capture efforts within the same season over 2 years. A two-way ANOVA of these data shows that the individuals of the HRP performed stronger escape responses to a VDS than those from the LRP ($P<0.001$), and that the difference was maintained despite the seasonal effect (Sztarker and Tomsic, 2008) on the level of escape in the two populations ($P<0.001$).

It could be argued that the difference found in the strength of the escape response between animals from the HRP and LRP could be caused by factors unrelated to the predation risk. For instance, if food was less available for crabs in the LRP area, this might have an effect

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on the sensory or the motor capability of the animals. In such cases, the difference of responsiveness should also be reflected in other behaviours. To investigate whether the difference observed between crabs of the HRP and the LRP was specific for the VDS-elicited escape response or was a more general difference in responsiveness, we evaluated the animals' performance in two distinct behavioural assays. First, we assessed the optomotor response to the rotation of the visual panorama, i.e. a visually guided behaviour that does not entail danger. Second, we evaluated the avoidance response to an electrical stimulus, i.e. a motor response to a non-visual nociceptive stimulus. The results showed no differences in the optomotor response (P=0.98) or in the shock avoidance response (P=0.4) between animals of the two populations (Fig. 2). These results are consistent with the notion that the difference found in the escape performance is specific for visual stimuli representing impending threats.

Neural response differences reflect the impact of predation

The response strength of LG neurons to VDS has been found to correlate closely with the escape response of the crabs across a wide range of conditions (Berón de Astrada et al., 2013; Oliva et al., 2007; Sztarker and Tomsic, 2008, 2011; Tomsic et al., 2003, 2009). Hence, we investigated whether the difference in the escape response between the HRP and the LRP could be partially accounted for by a difference in the response of the LGs to the VDS.

Comparisons between the LGs from animals of the two populations revealed no significant differences in the mean spontaneous activity or the mean resting membrane potential (Fig. 3A,B). Between 30% and 40% of the recorded neurons from each population showed a compound evoked EPSP (Fig. 3C, left),

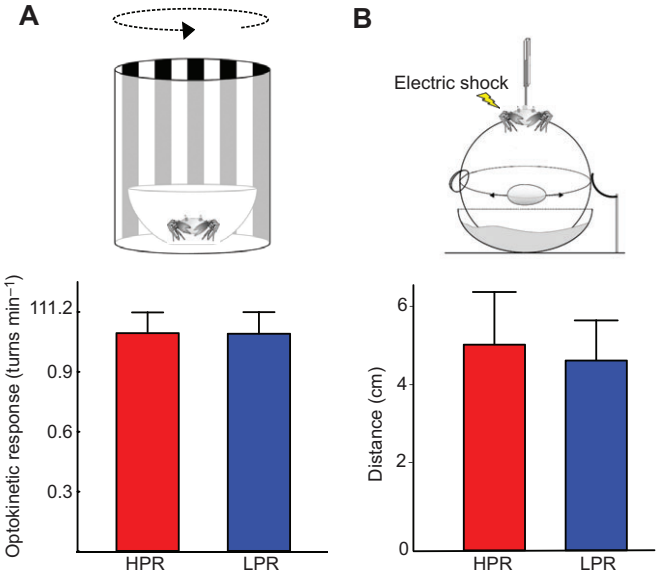

Fig. 2. Responses to a visual harmless stimulus and to a non-visual nociceptive stimulus are similar for crabs of the HRP and LRP. (A) The optomotor response was elicited by the rotation of a cylinder with a pattern of black and white vertical stripes. The rotation of the panorama (2 cycles min^{-1}) induces the animals to rotate in an attempt to stabilize the optic flow. The response was quantified as the number of complete turns made by the animal in a period of 2 min. There was no significant difference between the responses of the two populations (N=20 per group, t-test, P=0.98). (B) Avoidance response to a mild electrical shock. The shock was delivered to one side of the animal and the locomotor response was measured by the rotation of the floating ball recorded with two optical mice. There was no significant difference between the two populations (N=11 per group, t-test, P=0.4). Graphs show means and s.e.m.

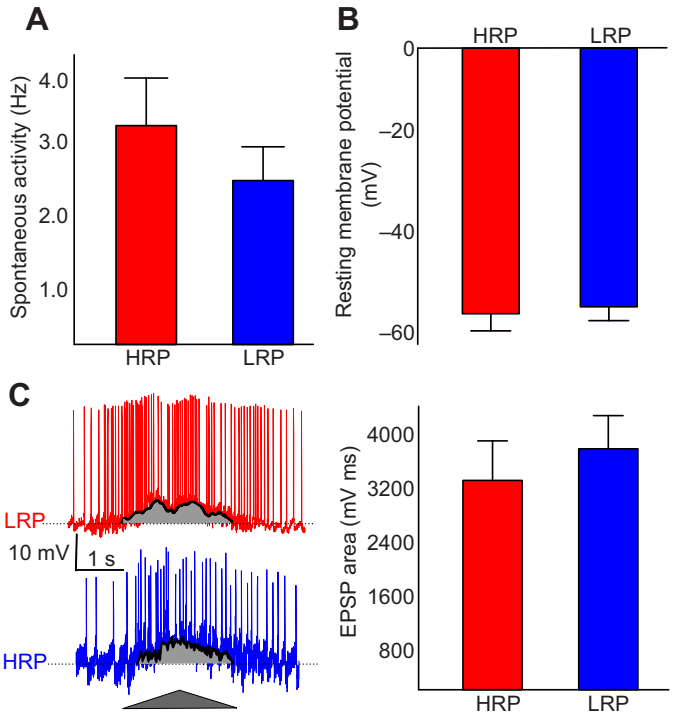

Fig. 3. Properties of lobula giant (LG) neurons from crabs of the HRP and LRP measured by *in vivo* intracellular recordings. (A) The mean spontaneous firing activity and (B) the mean resting membrane potential were not significantly different between crabs from the HRP (N=25) and LRP (N=28; t-test, P=0.2 and P=0.75, respectively). (C) Left, the upper and lower traces show an LG response to the VDS of crabs from the HRP and LRP, respectively. After removing the action potentials, the area of the compound excitatory postsynaptic potential (EPSP) elicited by the VDS (grey area) was calculated for each cell in which the input signal could be reliably measured. The two top sides of the triangle below the traces represent the two directions of motion (see Fig. 1E). Right, the mean area of compound EPSPs obtained from crabs of the HRP (N=10) and LRP (N=9). There was no significant difference between them (t-test, P=0.67). Graphs in A–C show means±s.e.m.

which corresponds to impalements performed close to the dendritic region (Medan et al., 2007). This allowed us to compare the magnitude of the input signal (the EPSP area) elicited by the VDS between neurons of the two populations. There were no significant differences (Fig. 3C, right).

However, when we compared the number of action potentials elicited by the VDS, we found a clear difference between neurons of the two populations, which parallels the difference observed in the strength of the escape response. Fig. 4A,B shows representative LG and behavioural responses from crabs of the HRP and LRP, respectively. The neuron from the crab of the HRP fired more spikes in response to the VDS than the neuron from the crab of the LRP (see also Fig. 3C, left), thus reflecting the difference in the strength of escape. This was confirmed by statistical analyses of results obtained from animals from the different capture efforts performed over the 2 years of study. In fact, the mean neuronal response and the mean behavioural response elicited by the VDS were both significantly higher in crabs of the HRP than in crabs from the LRP (Fig. 3C,D).

DISCUSSION

It has been found that fiddler crabs from different populations show differences in their social signalling display that correlate with the

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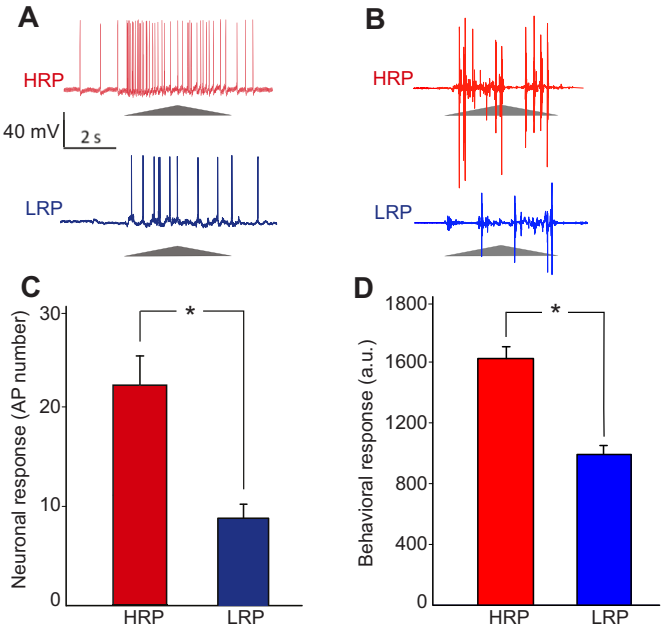

Fig. 4. Comparison of the neuronal and behavioural responses to the VDS of crabs from the HRP and LRP. (A) *In vivo* intracellular recorded responses of two LG neurons to the VDS, one neuron from a crab of the HRP and the other from a crab of the LRP. (B) Recordings of two individual behavioural responses to the VDS, one from a crab of the HRP and the other from a crab of the LRP. The escape response was transduced and recorded as a train of voltage changes, with peaks corresponding to running steps of the escape reaction. The two top sides of the triangles below the traces represent the two directions of motion (see Fig. 1E). (C,D) Averaged neuronal (AP, action potential) and behavioural responses from experiments such as those illustrated in A and B, respectively. The neuronal response was assessed as the number of action potentials elicited by the VDS. The behavioural response was estimated by the area of voltage deflections during the periods of visual motion stimulation. Neurons: HRP N=25, LRP N=28; behaviour: HRP N=753 crabs, LRP N=746 crabs. The graphs show means±s.e.m.; t-test, *P<0.001.

level of predatory birds present in each population (Hemmi et al., 2006). In the present study, we show that crabs from a region where the risk of avian predation is high display more intense escape responses to an impending visual threat than crabs from a region where the predation risk is low. In contrast, the optomotor response to panoramic motion and the startle response to an actual nociceptive stimulus were very similar between animals of the two populations. The results from these control experiments rule out the possibility that the difference in escape is due to peripheral or non-specific effects on the visual or motor performance. Thus, the results suggest that a difference between the two populations resides in the central nervous system and that it is specific to the visually guided escape behaviour.

In crayfish, social status was found to affect the escape from mechanical stimulation by affecting the response of a premotor neuron, the lateral giant neuron (Issa et al., 2012; Yeh et al., 1996). A similar result has been observed in a cichlid fish where, in comparison to subordinate males, dominant males were found to display higher startle responsiveness and increased excitability of the Mauthner cell circuit that governs this behaviour. In the latter case, the difference in escape was interpreted as a trade-off between the better reproductive opportunities of being a conspicuous dominant individual and the greater predation risk that this condition represented (Neumeister et al., 2010; Whitaker et al., 2011). In both the crayfish and fish studies, however, the behavioural and neuronal differences were observed by manipulating the social status. Therefore, the question of whether the predation risk can by itself sculpt the functioning of individual neurons had not previously been addressed in any animal.

Here, we found that the LG neurons from crabs of the HRP are much more reactive to a VDS than the neurons from animals of the LRP, and that this difference reflects the difference observed in the escape behaviour. The results indicate that the risk of predation can affect the behavioural performance by shaping the functioning of the LG neurons. The fact that the input signals (i.e. the elicited EPSPs) to the LGs evoked by the VDS were similar between the two populations (Fig. 3C) makes it unlikely that the changes observed in the output signals (i.e. number of elicited spikes) were caused by changes occurring in the LG presynaptic pathway. Accordingly, the difference in the number of elicited spikes should arise from intrinsic differences in the input–output transfer function between the LGs of the two populations. Certain learning tasks, for example, produce enduring changes in the intrinsic excitability of neurons by changing the function of voltage-gated ion channels, a change that can produce broader, even neuron-wide changes in synaptic throughput (for a review, see Zhang and Linden, 2003).

The evolution of behaviour and of neural circuits underlying behaviour is intertwined. Studying the neural elements implicated in the evolvability of behaviour among different species has the classical difficulty of distinguishing between homology and homoplasy (but see Newcomb et al., 2012), a problem that is not present when studying interpopulation differences within a single species. It has been argued that by using interpopulation comparisons, microevolutionary processes can be explicitly investigated, because more populations are likely to be found in the environments that actually shaped their brains (Gonda et al., 2011). Moreover, compared with interspecific comparisons, interpopulation comparisons more easily allow the separation of genetic variations from phenotypic plasticity. Populations of *N. granulata* are restricted to salt marshes separated by hundreds of kilometres and their larvae are unlikely to travel long distances through the ocean (Bas et al., 2010; Ituarte et al., 2012). Thus, the differences found in the intensity of responses to VDS could be genetically determined. However, the brain is one of the most plastic organs and the LG neurons are known to reflect the differences in intensity of the escape response observed among a wide range of conditions. One way to reveal whether the differences we found are due to genetic or phenotypic variations would be to rear crabs of the two populations in the laboratory. If the differences are derived from the distinct predation risk experienced during ontogeny, they should vanish in lab-reared crabs.

The aim of the present study was to evaluate whether the impact of a highly relevant ecological variable, the risk of predation, can be detected at the level of individual neurons. By comparing crabs from two isolated populations, we found a correspondence between the pressure imposed by the predation risk, the response of identified neurons to VDS and the strength of the behavioural response to such stimuli. Now we can start to investigate whether the difference between the two populations has a genetic origin or a plastic phenotypic origin, and further address the neurophysiological mechanisms underlying the response difference.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

D.T. and T.L. designed the research. T.L. and J.N. carried out the field observations and analyses. F.M. and D.T. performed the laboratory experiments and data analyses. D.T. wrote the paper.

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