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RESEARCH ARTICLE

Serotonin, but not dopamine, controls the stress response and anxiety-like behavior in the crayfish *Procambarus clarkii*

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ABSTRACT

In the animal kingdom, biogenic amines are widespread modulators of the nervous system that frequently interact to control mood. Our previous investigations in crayfish (*Procambarus clarkii*) have established that stress induces changes in brain serotonin (5-HT) concentrations that are responsible for the appearance of anxiety-like behavior (ALB). Here, we further analyze the roles of 5-HT and another biogenic amine, dopamine (DA), on the crayfish response to stress. We show that the intensity of crayfish ALB depends on the intensity of stressful stimulation and is associated with increased concentrations of 5-HT in the brain. These 5-HT levels were significantly correlated, before, as well as after stress, with those of DA, which were approximately 3- to 5-times less abundant. However, whereas the degree of ALB was clearly correlated with brain 5-HT concentrations, it was not significantly correlated with DA. Moreover, in contrast to injections of 5-HT, DA injections were not able to elicit a stress response or ALB. In addition, 5-HT and DA levels were not modified by treatment with the anxiolytic chlordiazepoxide, confirming that suppression of ALB by this GABA-A receptor ligand acts downstream and is independent of changes in crayfish bioamine levels. Our study also provides evidence that the anxiogenic effect of 5-HT injections can be prevented by a preliminary injection of 5-HT antagonists. Altogether, our results emphasize that the rises in brain concentrations of 5-HT, but not DA, play a role in controlling the induction and the intensity of crayfish ALB.

KEY WORDS: 5-HT, Octapamine, Benzodiazepines, GABA, Anxiety, Crustacea

INTRODUCTION

All living animals face danger and develop strategies to avoid potentially harmful situations. Danger elicits a stress response that may result in the escape reflex, freezing or aggression and requires the mobilization of energy stores, generally under hormonal control. When stress is prolonged, it can elicit long-lasting behavioral adaptations, resulting in a sustained apprehension of the environment, which persists even in a new context and in the absence of stressor. This state of higher alertness is called anxiety (Blanchard and Blanchard, 1988; Belzung and Philippot, 2007; Steimer, 2011). Recently, it has been shown that anxiety-like behavior (ALB) is also observable in crustaceans (Fossat et al., 2014), allowing the study of primitive forms of anxiety and of their neurobiological correlates.

Monoaminergic systems generally intervene in coordinating responses to stress. More particularly, the biogenic amines serotonin (5-HT) and dopamine (DA) have been involved in fear responses and anxiety in mammals (Bonhomme and Esposito, 1998; Nutt, 2001; Graeff and Zangrossi, 2010; Canteras and Graeff, 2014; Fossat et al., 2014; Zangrossi and Graeff, 2014). In crayfish and other decapods, 5-HT has been shown to control the stress response by raising secretion of crustacean hyperglycemic hormones (CHHs), leading to an increase in blood glucose (Lorenzon et al., 2005; Fossat et al., 2014). DA has been also claimed to have such a role, although conflicting observations have been reported (Sarojini et al., 1995; Zou et al., 2003; Lorenzon et al., 2005). Downstream from the stress response, 5-HT is able to trigger ALB in crayfish (Fossat et al., 2014), but the possible role of DA on this behavior has not yet been investigated.

The first aim of this study was to compare the roles of 5-HT and DA in the stress response and subsequent ALB in the crayfish *Procambarus clarkii*. For this purpose, we submitted animals to electric field stress, and then transferred them to a dark/light plus maze, as previously described (Fossat et al., 2014). In order to verify whether ALB is dependent on the intensity of the aversive stimulus, we applied various stress intensities and measured the brain levels of 5-HT and DA. Then, we analyzed the possible effects of exogenous DA injections on the stress response and ALB, in order to compare results with the effects already described for 5-HT (Fossat et al., 2014). In addition, we verified that injection of 5-HT antagonists prevented the anxiogenic effect of 5-HT and we also studied the consequences on 5-HT and DA levels of anxiolytic treatment with chlordiazepoxide, a ligand of the GABA-A receptor (Snyder et al., 2000), to check for any interaction between 5-HT, DA and GABA neuromodulation.

RESULTS

Stress intensity and ALB in crayfish

An electric field applied to crayfish for a 30 min period generates an aversive reaction (generally a series of tail flips) and induces ALB that can be observed thereafter in a sub aquatic dark/light plus maze (examples in Fig. 1, compare crayfish routes in A and B). In order to analyze the effects of stress intensity on ALB, we measured several behavior variables after submitting animals to various electric field intensities (see Materials and methods) and performed principal component analysis (PCA, Fig. 1C,D). This analysis revealed that electric field intensity lower than 4 mA did not elicit significant ALB (Fig. 1C). ALB started with a stressor intensity of 4 mA and then progressively increased with higher intensities, as shown by PCA barycenters shifted on the right part of the *x*-axis, which represents the main component (Comp 1, Fig. 1C). This axis

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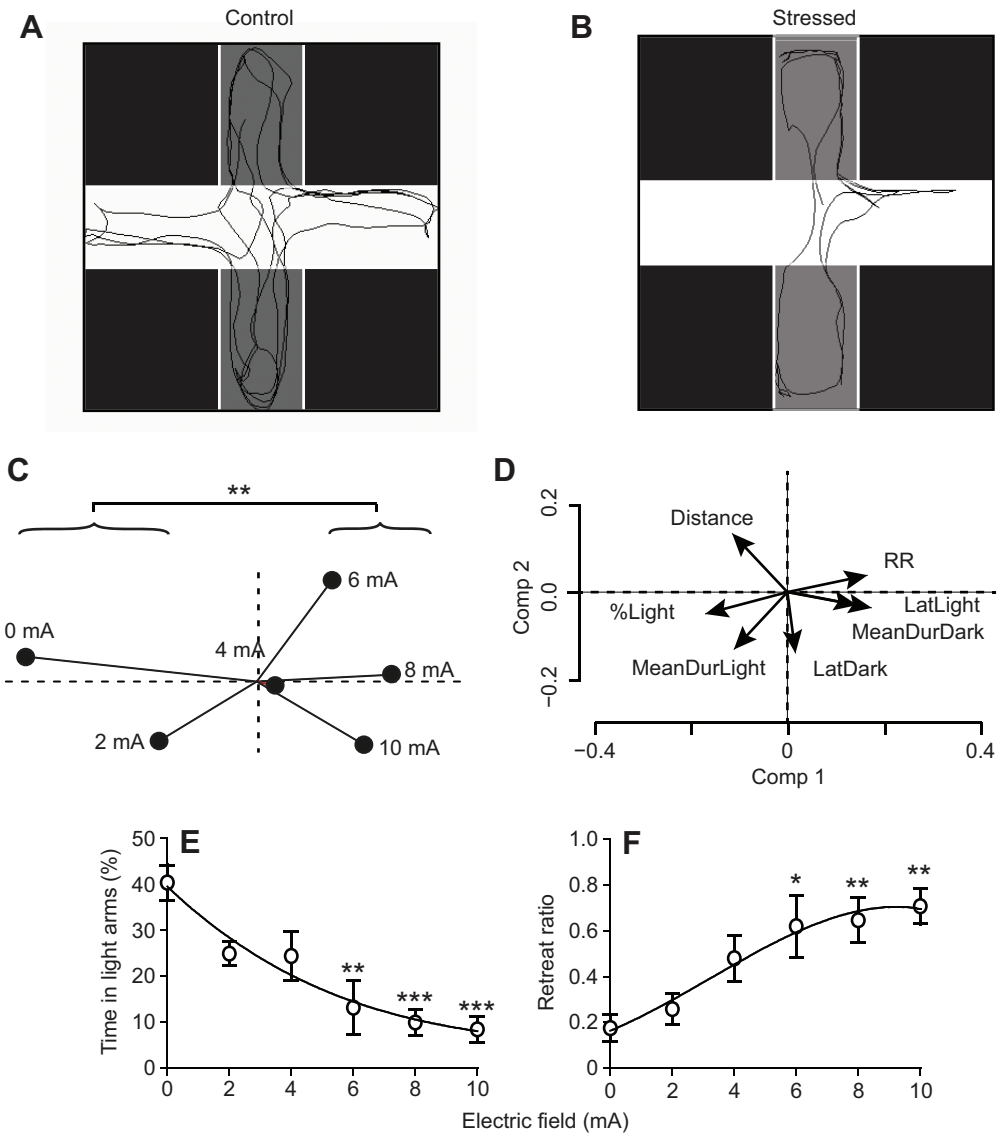

Fig. 1. Relationship between stress intensity and anxiety-like behavior in the crayfish. (A) Example of unstressed crayfish route in the dark/light plus maze (control). Note that crayfish spontaneously visited all the arms of the maze. (B) Example of stressed crayfish route showing that crayfish almost never visited light arms limiting exploration to dark part of the maze. (C,D) PCA of ALB under increased stressor intensity. (C) Location of barycenters from each animal group in the plane defined by the first and second components of the PCA. This analysis showed that stimulations at 0 or 2 mA were significantly separated from those between 6 to 10 mA on the first component. (D) Contribution of each variable to the variance in the first and second components of the PCA. This method revealed that two variables (% time in light, %Light: 3365 and retreat ratio, RR: 2715) were mostly responsible for animal variance in the first component, i.e. horizontal axis that can be interpreted as the 'anxiety' axis. Similarly, two variables, walked distance (Distance: 2536) and latency to first entry in dark arms (LatDark: 5765) were mostly responsible for animal variance in the second component, i.e. vertical axis that can be interpreted as the 'locomotion' axis. Other variables with minor contributions: mean duration of visits in dark arms (MeanDurDark) and light arms (MeanDurLight), latency to first entry in light arms (LatLight). (E,F) Levels of ALB (means±s.e.m.), assessed by the percentage of time spent in light arms (E) and the retreat ratio value (F), are correlated with stress intensity (Kruskall–Wallis followed by *post hoc* Dunn tests: **P*<0.05; ***P*<0.01; ****P*<0.001).

corresponded to a higher level of ALB, as illustrated by the variable plot (Fig. 1D), and can be interpreted as the 'anxiety' axis. Moreover, this analysis highlighted the two major variables describing ALB, the percentage of time spent in light arms and the retreat ratio (defined as the number of aborted attempts to enter into a light compartment on the total number of entries, see Fossat et al., 2014). The contribution of these two variables to the variance of component 1 was larger than the mean contribution of all variables to that component. Therefore, we mainly focused on these two variables to evaluate ALB in our study: we plotted the intensity of electric field with the percentage of time spent in the light arms and the retreat ratio (Fig. 1E,F). These curves showed the progressive increase of ALB with stressor intensity. ALB was dependent on stress intensity, with a maximum effect for the higher stressor intensity. After exposure to 10 mA stressor intensity, crayfish spent significantly less time in the light arms of the dark/light plus maze (Fig. 1E; 40±4% in controls versus 8.4±2.7% in stressed crayfish; N=14, P<0.0001, Mann–Whitney). Simultaneously, the retreat ratio was significantly increased (Fig. 1F; 0.017±0.06 in control versus 0.71±0.08 after stress; N=14, P<0.001, Mann–Whitney).

Bioamine levels and ALB in stressed crayfish

The levels of 5-HT and DA in the brains of isolated crayfish were analyzed using HPLC. Our results in unstressed controls showed that 5-HT levels (277±24 pg mg^{-1} brain fresh weight, i.e. 1.57±0.14 pmol mg^{-1}) were significantly higher than DA levels (82±6.7 pg mg^{-1}, i.e. 0.53±0.04 pmol mg^{-1}; P<0.0001, Mann–Whitney, N=14). After stress stimulation (10 mA, 30 min), the amount of brain 5-HT increased significantly (Fig. 2A, 451±41 pg mg^{-1} in stressed crayfish; N=11, i.e. a 69% increase from unstressed controls described above, P<0.001, Mann–Whitney). The amount of DA did not significantly increase (Fig. 2A, 103±12 pg mg^{-1} in stressed crayfish; N=11, i.e. a 26% increase from the unstressed controls, P=0.08, Mann–Whitney). However, a clear correlation was found between individual levels of 5-HT and DA both in the control (R^2=0.36, P<0.05) and after stress (R^2=0.79, P<0.001) (Fig. 2B). We also analyzed individual 5-HT/DA ratios (calculated from pmol results), because such ratios were insensitive to any possible problem during extraction or HPLC procedure and independent of brain mass measurements (they can be used on individuals for which the brain fresh mass was not estimated). Interestingly, the mean of such individual 5-HT/DA ratios

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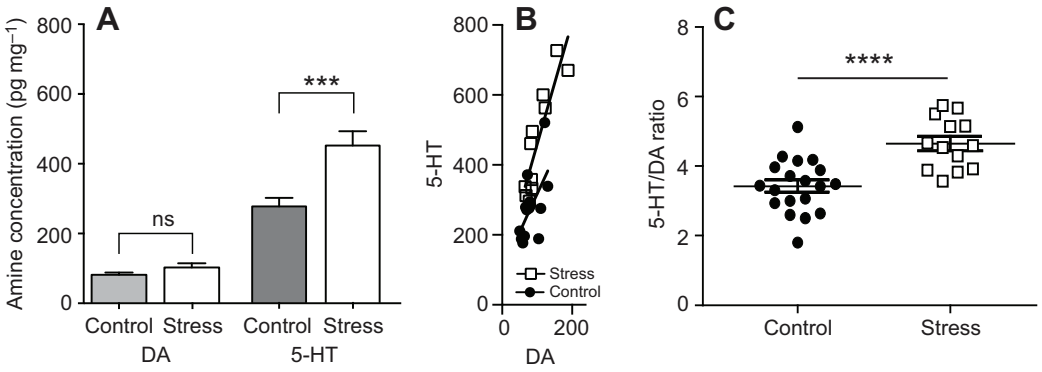

Fig. 2. Levels of 5-HT and DA in brains of unstressed and stressed crayfish. (A) Bioamine concentrations in brains expressed as pg mg^{-1}: levels of 5-HT were significantly increased after stress in crayfish brains (***P=0.0003, N=14 and 11 for control and stress, respectively), whereas levels of DA only showed a trend to a slight increase, that was not significant (P=0.08). (B) A high correlation was found between 5-HT and DA concentrations, before (controls: black dots) as well as after stress (white squares, P=0.0002). (C) Brain 5-HT/DA ratios clearly increased after stress (****P<0.0001, N=19 and 13 for control and stress, respectively). Horizontal bars represent means±s.e.m.

significantly increased from 3.4±0.2 in controls to 4.7±0.2 (P<0.0001, Mann–Whitney) after stressful stimulation (Fig. 2C).

We next analyzed whether the amounts of brain 5-HT and DA were correlated to ALB intensity, by focusing on the two main variables emphasized by PCA, namely the percentage of time in light arms and retreat ratios (Fig. 3), which were more relevant than correlation with electric field intensities. We found that the amount of brain 5-HT was significantly correlated to both the percentage of time in light arms (Fig. 3A, R^2=0.34, P<0.01) and retreat ratios (Fig. 3B, R^2=0.33, P<0.01). By contrast, in spite of its correlation with 5-HT, DA was not significantly correlated to ALB, either with percentage of time in light (Fig. 3C, R^2=0.034, P=0.45) or with retreat ratios (Fig. 3D, R^2=0.038, P=0.38). As a consequence, 5-HT/DA ratios were not as well correlated as 5-HT levels to ALB variables (not shown, R^2=0.28 and 0.34, respectively, for percentage time in light and retreat ratios, P<0.05). Altogether, our results show that 5-HT concentrations increase significantly in the brains of stressed crayfish and are particularly correlated to the level of ALB. By contrast, DA concentrations, although correlated to 5-HT levels before and after stress, only show a weak and insignificant increase after stress, without clear correlation to ALB.

5-HT and DA in the stress response

To evaluate the possible role of DA on the stress response and ALB, and to compare with the role of 5-HT (which has already been investigated, see Fossat et al., 2014), we injected exogenous bioamines at a concentration sufficient to penetrate the brain. We verified that, after abdominal injection of either 5-HT or DA at 5 µg g^{-1}, a clear increase in the brain concentrations of these bioamines was detected using RP-HPLC 15 min after injection (Fig. 4A, 1350±176 pg mg^{-1}, N=8, P<0.0001; Mann–Whitney, for 5-HT and 1750±450 pg mg^{-1}, N=3, P<0.05; Mann–Whitney, for DA). We first assessed activation of the stress axis by measuring glucose titers in the hemolymph (Fig. 4B). We observed that 5-HT, but not DA, elicited a significant increase in blood glucose, suggesting that 5-HT alone is involved in the control of the physiological stress response.

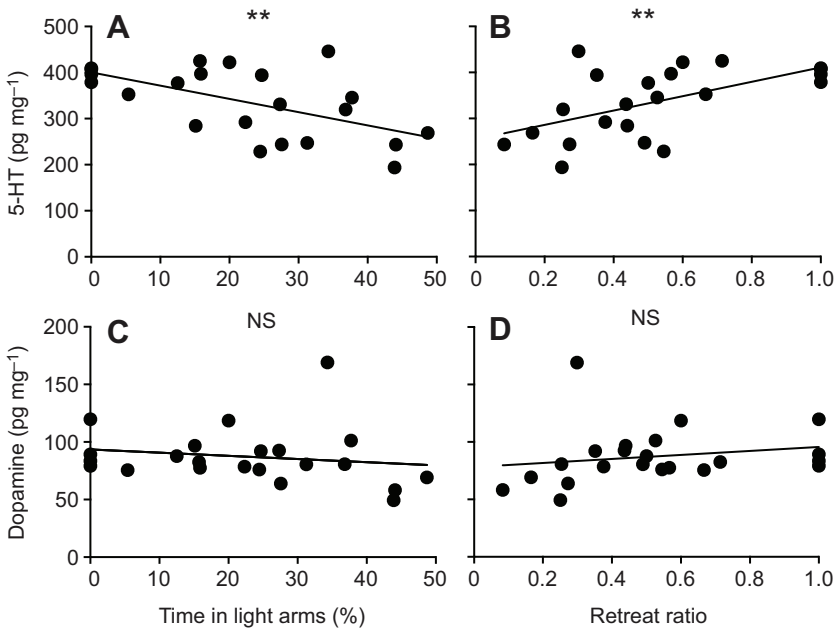

Fig. 3. Correlation between ALB and the concentration of bioamines. (A) Correlation between 5-HT levels in stressed crayfish and the percentage of time spent in light arms. This correlation was highly significant (**P=0.004). (B) Correlation between 5-HT levels and retreat ratios (**P=0.005). (C,D) By contrast, DA levels and ALB variables were not significantly correlated with the percentage of time spent in light arms (C) or with retreat ratios (D).

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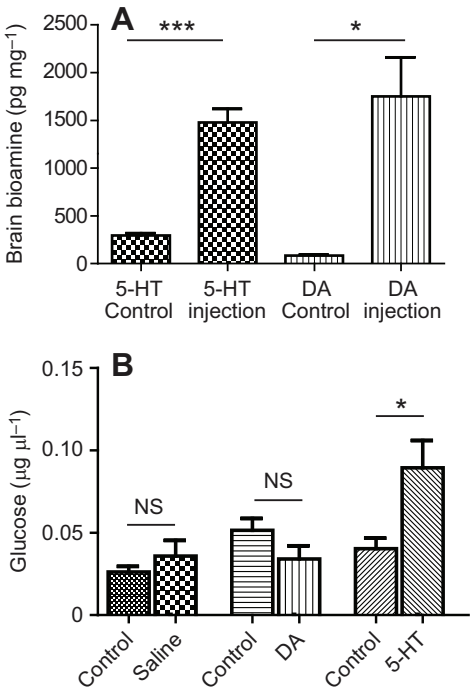

Fig. 4. Effects of 5-HT and DA injections on glucose titer. (A) 5-HT or DA injections in crayfish induce a huge and significant increase of their respective levels in brains, as revealed by HPLC measurements. (B) Hemolymph glucose titers (in µg µl^{-1}) were measured on the same animals before (control) and after injection of saline, DA or 5-HT. Saline and DA injections did not change significantly the concentrations of glucose, while 5-HT promoted a significant increase. Histograms indicate means+s.e.m. ***P<0.001, *P<0.05. NS, not significant.

5-HT and DA in ALB

We next compared the consequences of injections of DA and 5-HT on ALB (Fig. 5A,B). As previously described (Fossat et al., 2014), we found that injection of 5-HT was able to elicit ALB: the percentage of time spent in light arms was significantly decreased compared with controls (Fig. 5B and D, 37±6% in saline injected, n=9 versus 7±4% in 5-HT injected; N=8, P<0.01, Dunn's test). Retreat ratios were significantly increased (Fig. 5E, 0.19±0.06, N=9, in saline injected versus 0.81±0.13, N=8, in 5-HT injected; P<0.05, Dunn's test). The effect of 5-HT was abolished when 5-HT was injected after a mixture of large spectrum 5-HT antagonists (see Materials and methods and Fig. 5C,E). By contrast, injection of DA had no significant effect on the percentage time in light arms or retreat ratios (Fig. 5D,E, 36±8%, N=8; P>0.05 and 0.25±0.09, respectively; P>0.05 compared with saline injected, N=8, Dunn's test).

Anxiolytics and bioamines in crayfish

Beyond the clear role of 5-HT in activation of the stress response and of ALB, we analyzed the consequences of treatment with a classical anxiolytic on ALB and bioamine levels. Previous investigations (Fossat et al., 2014) have shown that chlordiazepoxide (CDZ) is able to abolish ALB. Because 5-HT is involved in the metabolic response to stress as well as in ALB, we assessed the consequence of CDZ treatment on glucose and 5-HT levels after stress. As in our previous study, CDZ at 15 µg g^{-1} abolished ALB in crayfish by restoring the percentage of time spent in the light arms and decreasing retreat ratios (Fig. 6A,B; percentage of time in light arms: 8.4±2.7% in stressed animals versus 24±4% in CDZ injected, N=9, P<0.05, Mann–Whitney; retreat ratios: 0.69± 0.09 and 0.35±0.07, N=9, P<0.05, Mann–Whitney). Glucose levels were not significantly different in animals that received saline or CDZ before stress (Fig. 6C). Similarly, the amounts of 5-HT were not significantly changed in the brain of stressed crayfish that received CDZ compared with those that did not receive CDZ (Fig. 6D; 384± 62 pg mg^{-1} in stressed crayfish injected with CDZ versus 466± 47 pg mg^{-1} in stressed crayfish; N=6 and 13, respectively, P=0.29, Mann–Whitney). Similar observations were made with DA (not shown) and, as a consequence, 5-HT/DA ratios after CDZ treatment remained identical to those of stressed animals (Fig. 6E; 4.59±0.22 in stressed crayfish and 4.7±0.15 in stressed injected with CDZ; N=13 and 6, respectively, P=0.58, Mann–Whitney). Finally, after CDZ injection, the correlation between 5-HT levels and ALB parameters (percentage of time in light arms

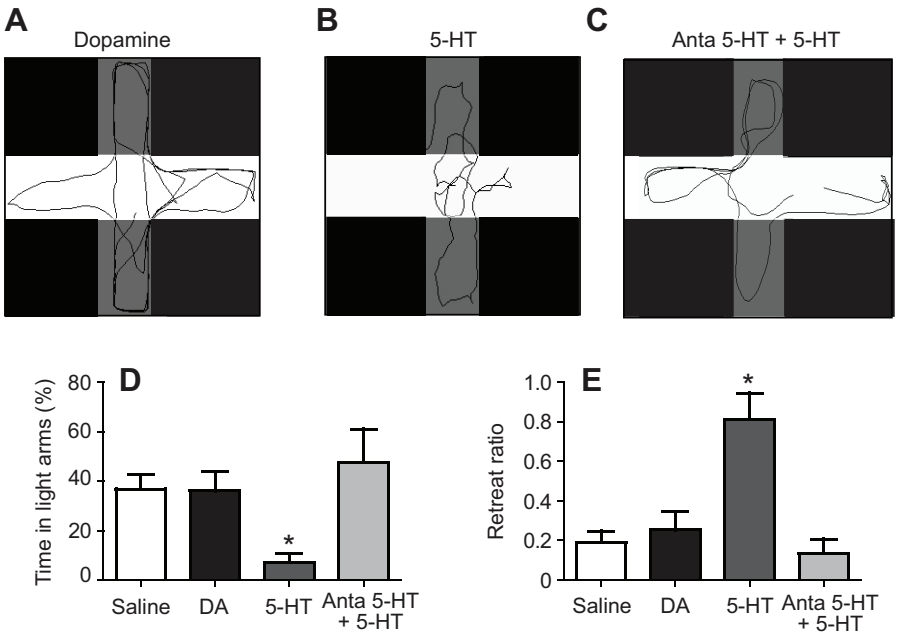

Fig. 5. Injection of 5-HT but not DA elicits ALB. (A–C) Examples of crayfish routes after injection of DA (A), 5HT (B) or a mixture of 5-HT antagonists before 5-HT (C). Note that after 5-HT injection, crayfish exploratory behavior was restrained to dark arms, while after DA injection, as well after a 5-HT injection following treatment with antagonists, crayfish moved inside the whole maze. (D,E). After 5-HT injection, but not after injection of either DA or 5-HT antagonist mixture plus 5-HT (Anta 5-HT+5-HT), the percentage of time in light arms and retreat ratios were significantly different from those of saline injected crayfish. Histograms indicate means+s.e.m. *P<0.05.

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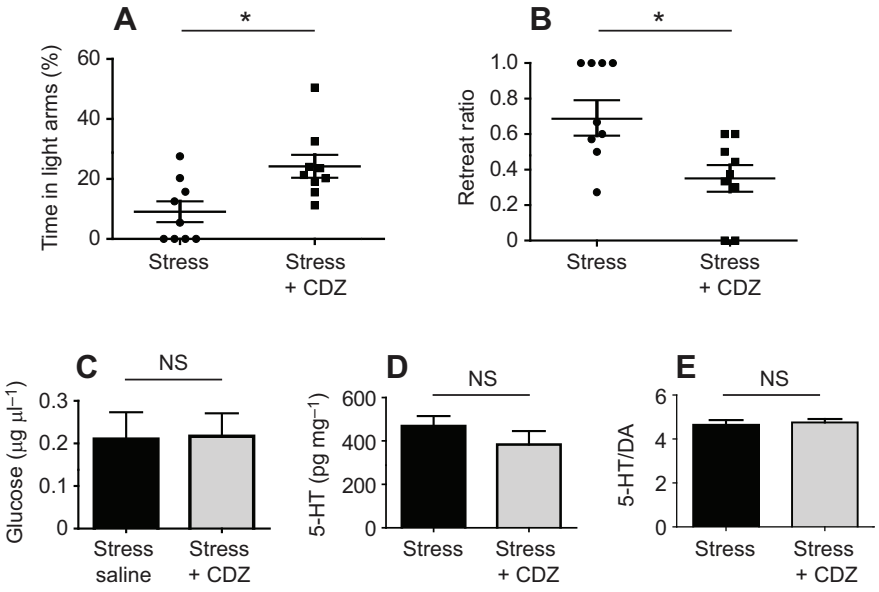

Fig. 6. CDZ abolished ALB but did not modify bioamine levels. (A,B) When stressed crayfish were injected with CDZ, the percentage of time spent in the light arms and retreat ratios were significantly different from stressed animals (*P<0.05, Mann–Whitney) and reached values similar to those of unstressed crayfish. (C) Glucose titers increase induced by a stress protocol was similar in saline or CDZ injected crayfish. (D,E) 5-HT and 5-HT/DA ratios were not affected by CDZ injection and were similar to stressed crayfish. Horizontal bars indicate means±s.e.m. NS, not significant.

and retreat ratio) was completely suppressed (Fig. 7): for 5-HT, R^2=0.09 and 0.08 for percentage time in light and retreat ratios, respectively (Fig. 7A,B); for DA, R^2=0.14 and 0.05 for percentage time in light and retreat ratios, respectively (Fig. 7C,D).

DISCUSSION

Correlation between stress and ALB intensity

The intensity of ALB in crayfish was dependent on the intensity of previous stressful stimulation: minimal electric field intensity was required to cause a significant change in ALB, then ALB increased with electric field intensity, until it reached a plateau. A similar effect on ALB intensity was also previously observed in crayfish with the modification of stimulation time (Fossat et al., 2014). All these results indicate that both stress intensity and the duration of stress exposure generate various degrees of ALB, and a combination of the two probably will do also. Such a quantitative relationship between stress and anxiety is also a very common feature of human and vertebrate anxiety, for which the excess of stress can lead to pathological disorders (Angst and Vollrath, 1991). Our previous and present observations in crayfish were only focused on a single and relatively short period of stress, but experiments are now in progress to evaluate what could happen with a chronic stress or with the combination of various stress sources.

Intensity of ALB and 5-HT

Our present data clearly show that animals displaying increased avoidance of illuminated maze areas, as well as higher retreat ratios, also had higher brain concentrations of 5-HT. The fact that 5-HT concentrations could increase in crayfish brain in correlation with the intensity of ALB, which is by itself a consequence of stress intensity, suggests that the amount of 5-HT in the brain could be a direct consequence of stress intensity. Such a rise in 5-HT, which could accumulate proportionally with intensity and duration of stress, is reminiscent of observations made in mammals on fatigue and overtraining, which can also be considered as stress responses (see review by Meeusen et al., 2007).

It is presently unknown in which brain zone 5-HT accumulates in crayfish, but as it has marked effects on behavior, it appears likely that

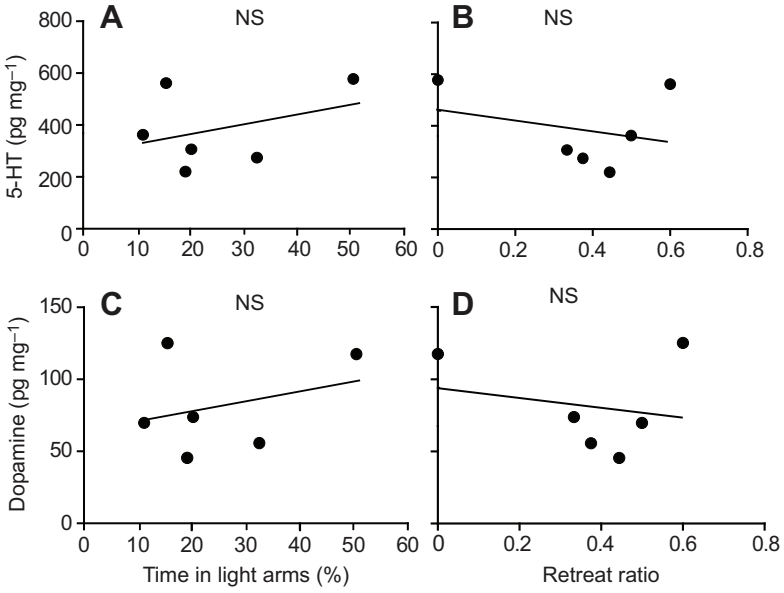

Fig. 7. After CDZ injection, ALB no longer correlates with 5-HT levels. (A,B) Correlation between ALB and 5-HT levels was completely abolished in stressed crayfish injected with CDZ as shown for time in light arms (A) (N=6, R^2=0.09, P=0.47) and RR (B) (N=6, R^2=0.08, P=0.56). (C,D) Absence of correlation between DA levels in stressed crayfish injected with CDZ and percentage of time in light arms (C) (N=6, R^2=0.14, P=0.59) and RR (D) (N=6, R^2=0.05, P=0.68). NS, not significant.

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5-HT is actively released from 5-HT neurons and invades many target neuronal networks. In line with our previous study (Fossat et al., 2014), the injection of 5-HT (5 µg g^{-1}) induces a marked ALB, close to the maximal values. Indeed, this injection dramatically enhanced the brain concentrations of 5-HT over the values reported after the highest intensity of the electrical stimulations.

Intensity of ALB and DA

Our results have shown that DA levels remain relatively stable after stress, in spite of an insignificant trend to increase. This marginal trend is possibly related to a correlation between 5-HT and DA levels, observed before stress (Fig. 2B), which could be due to the fact that these two bioamines share several enzymes involved in their biosynthesis as well as in their catabolism (Berry et al., 1996; Shih et al., 1999). However, the metabolic pathways of 5-HT and DA are clearly controlled in different ways after stress, as shown by the net increase in 5-HT/DA ratios. Moreover, in contrast to 5HT, endogenous levels of DA after stress were not significantly correlated to ALB and the injection of exogenous DA triggered neither ALB nor a stress response that was observable through glucose titers. This last observation is in accordance with previous studies showing that DA injections in crayfish have no or opposite effects on CHHs or glucose levels (Luschen et al., 1991; Zou et al., 2003; Lorenzon et al., 2004, 2005). In vertebrates too, the primary role of DA is not to mediate stress and anxiety. Nevertheless the DA system may act in several regions to accompany or enable stress or anxiety responses, by alterations of motor behavior or of learning and memory (Le Moal and Simon, 1991; Arnsten, 2000; Delaville et al., 2012). Thus, it remains possible that DA could play analogous but yet unknown roles in crayfish as a consequence of stress, but clearly not in ALB in our experimental conditions.

Another catecholamine, octopamine, the invertebrate equivalent of noradrenaline, is also frequently involved in the control of crayfish behavior and the question of its participation in ALB can be raised. Our unpublished experiments with octopamine injections (done in the same conditions as with 5-HT or DA in this study) did not change crayfish behavior in the dark-light maze, which seems to reject the hypothesis of octopamine control of ALB induction.

Bioamine levels after anxiolytic treatment

Our previous observations in crayfish have shown that treatments with anxiolytics after stress are able to suppress ALB without affecting the stress response. Indeed, after CDZ injection, crayfish behavior in the dark/light plus maze returns to normal, but blood glucose remains at high levels (Fossat et al., 2014). In addition, our present study shows that CDZ does not alter 5-HT and DA concentrations in the brain while inhibiting ALB, which confirms that the mode of action of this anxiolytic occurs independent of changes of bioamine levels in crayfish. In vertebrates, in spite of several reports having shown that benzodiazepine compounds can alter 5-HT or DA levels (Wise et al., 1972; Finlay et al., 1995), it is now accepted that CDZ acts as a GABA-A receptor agonist (Lopez-Munoz et al., 2011). Of course, our results in crayfish do not exclude the possibility that CDZ could alter stress response and bioamine levels in other conditions, i.e. if injected before rather than after applying our acute stress protocol or if injected during a chronic stressful stimulation, but they strongly suggest that ALB, once elicited, is maintained, or can be suppressed, independent of the other stress responses controlled by 5-HT.

The primordial role of 5-HT

As in mammals, the response to stress in crustaceans involves at least two closely interlinked facets: a behavioral response that is due to changes in nervous system regulations and a metabolic response that is mainly controlled by a neuroendocrinological axis (Fig. 8). The finding that CDZ alters neither 5-HT levels nor the elevated levels of blood glucose clearly indicates that 5-HT plays a pivotal role in crayfish upstream of the neuroendocrinological and behavioral responses to stress. Indeed, in addition to the numerous changes in behavioral responses (Fossat et al., 2014, and this study), 5-HT has been shown to directly control the release of CHHs (reviewed by Fingerman, 1997; Saenz et al., 1997; Lorenzon et al., 2005). These peptidic hormones, produced in the X-organ and released from the sinus gland of eyestalks, control the stress response in peripheral organs, particularly leading to energy mobilization by an increase in blood glucose (reviewed by Fingerman, 1997; Fanjul-Moles, 2006). Moreover, the link between 5-HT and glucose has been previously highlighted using 5-HT antagonists, which are able to block the increase in glucose concentrations in crayfish (Lee et al., 2000). Various stressors elicit the release of CHHs and the subsequent increase in blood glucose in crustaceans, such as hypoxia and emersion, disease, thermal stress, heavy metals exposure or social interactions (Webster, 1996; Chang et al., 1998; Stentiford et al., 2001; Wilcockson et al., 2002; Lorenzon et al., 2004; Elwood, 2011; Aquiloni et al., 2012). Thus, it would be interesting to further study the consequences of such stressors in terms of ALB and of 5-HT accumulation in the brain.

The finding that 5-HT antagonists prevent the behavioral and neuroendocrine responses induced by 5-HT supports this scenario (Fossat et al., 2014). Moreover, we have also demonstrated during the present study that the injection of a mixture of 5-HT antagonists was able to prevent the induction of ALB by 5-HT. This observation reinforces our interpretation on the primordial role played by 5-HT in the control of stress response and ALB (Fig. 8).

In vertebrates, 5-HT is also clearly acknowledged as an important factor in anxiety. Indeed, numerous drugs targeting 5-HT receptors

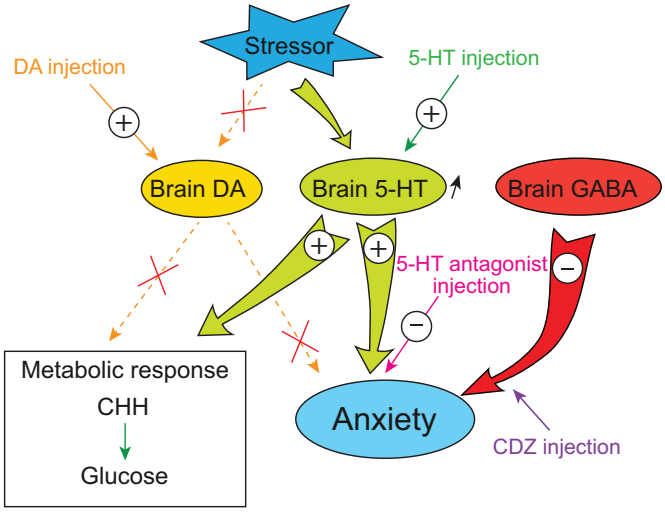

Fig. 8. Schematic diagram summarizing the control of stress and ALB in crayfish. After experiencing a stressful situation (stressor), 5-HT increases in crayfish brain, whereas dopamine remains almost stable. The increase in 5-HT is responsible for triggering: (1) a metabolic response characterized by hemolymph glucose increase, probably via the control of hormones as CHHs; and (2) anxiety-like behavior (Anxiety). Whereas 5-HT systemic injection elicits elevation of brain 5-HT, and thereby activates these two responses, neither of them was elicited by elevation of brain DA after DA systemic injection. The injection of 5-HT antagonists prevents the effect of 5-HT injection on ALB. The injection of CDZ, an anxiolytic known to potentiate the effects of GABA on GABA-A receptors, suppresses ALB after stress, but has no effect on the other levels of this scheme.

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or transporters possess anxiolytic properties, in spite of the apparent paradox that drugs that are enhancing 5-HT extracellular levels may also have anxiolytic properties (Millan, 2006). However, the actions of 5-HT in the anxiety of vertebrates are diverse, probably because various loci are involved, which receive dense 5-HT innervation and have numerous 5-HT receptors (Soubrié, 1986; Barnes and Sharp, 1999; Millan, 2006). Although 5-HT also acts on neuroendocrine responses, the activation of the 5-HT system can also be under the control of corticotropin-releasing factor (CRF), which acts as the first step in the hypothalamo–pituitary–adrenal axis of vertebrates (Leonard, 2005). CRF-like peptides also exist in arthropods, in which they are generally considered diuretic hormones (Gade, 2004), but their involvement in the control of stress and ALB remains to be investigated.

A possible mechanism for 5-HT activation

Our previous and present observations in crayfish were only focused on a single and relatively short periods of stress, during which we used tail flips as a cue for aversion: electric fields below the threshold for tail flipping did not trigger ALB, whereas maximal stimulation generated repeated tail flips. Thus, neurons underlying this escape behavior might be directly sensitive to electric field stimulation and/or necessary for the induction of the stress response and ALB. This hypothesis is reinforced by previous observations showing that the activation of such networks, more particularly lateral giant neuron networks, is able to directly stimulate 5-HT neurons in lobster abdominal ganglia (Horner et al., 1997). Although an increase in the 5-HT level could be a direct consequence of tail flip network activation, crayfish may also experience many other stressful situations that are not directly related to tail flips (Elwood et al., 2009), and that may also lead to ALB (our unpublished observations). It is thus possible that several other networks involved in the stress response could similarly stimulate an increase in 5-HT and lead to ALB.

MATERIALS AND METHODS

Animals

We used a total of 130 male crayfish (*Procambarus clarkii* Girard 1852), averaging 8.7±0.2 cm in length and 22±1 g in mass. Crayfish were fished in swamps close to the laboratory (Réserve naturelle de Bruges), and stored in individual tanks (50×30×30 cm) equipped with recirculating water, inside a specific animal house at 20°C with a 12 h:12 h light:dark cycle. They were fed pellets *ad libitum*. Each experimental animal was isolated for at least 3 weeks before any experiment in order to erase past life histories and to avoid social interactions. All experiments were performed in accordance with the CNRS guidelines for animal care.

Induction of stress

Crayfish were placed in a specific tank (30×20×10 cm, light intensity: 15 lx). Two electrodes placed on opposite sides of the tank were connected to a stimulator (AMPI) that delivered repetitive trains of 0.1 s duration, consisting of ten 5 ms pulses at 100 Hz, each separated by a 5 s interval for a total time of 30 min (Fossat et al., 2015). Various electric field intensities were used in the experiments: 0 (control), 2 mA, 4 mA, 6 mA, 8 mA, and 10 mA. For the smallest intensities, tail flips were not elicited for each stimulus. For the largest intensities, tail flips were more systematically elicited, but the occurrence and intensity of tail flips generally decreased with time, probably as a result of habituation.

Measurement of stress response

Glucose, considered as a circulating stress marker, was measured as previously described (Fossat et al., 2014) from 25 µl samples of hemolymph, extracted with 100 µl of 3 mmol l^{-1} trichloracetic acid, placed in 500 µl of reactive solution (Glucose RTU, BioMérieux SA, France) and incubated at 37°C for 10 min. Then, optical densities were measured with a spectrophotometer at 505 nm.

Drug treatments

Serotonin, dopamine and chlordiazepoxide hydrochloride (CDZ), purchased from Sigma-Aldrich (St Louis, MO, USA), were dissolved in crayfish saline (195 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 13 mmol l^{-1} $CaCl_2$, 2 mmol l^{-1} $MgCl_2$ and 3 mmol l^{-1} HEPES, pH 7.65). Crayfish were injected intramuscularly, between two abdominal segments. DA and 5-HT were used at 5 µg g^{-1} and CDZ at 15 µg g^{-1} fresh weight. A mix of mianserin hydrochloride (5-HT2 blocker, Sigma-Aldrich) and methysergide maleate salt (5-HT1 and 2 blocker, Sigma-Aldrich) was injected at 1 nmol g^{-1}, 5 to 10 min prior to 5-HT injection.

Behavior analysis: the dark/light plus maze protocol

The objective of this protocol was to analyze the spontaneous exploration behavior of crayfish confronted with a novel environment (see Fossat et al., 2015). The dark/light plus maze (total dimensions, 60×60 cm) comprises two dark arms (light intensity, 10 lx) and two illuminated arms (light intensity, 50 lx). Each arm was 25 cm in length and 10 cm in width.

Trial

Animals were tested only once in the maze. Each tested animal was first placed in the center of the arena and confined for 1 min under a small opaque chamber. After this delay, the crayfish was released, and exploratory behavior was recorded with a video camera (Sony) placed above the arena.

Tracking

We used Ethovision software XT8 (Noldus, NL) to detect and track crayfish in the arena.

Bioamine level measurements

DA and 5-HT were measured using reverse phase high performance liquid chromatography with electrochemical detection (RP-HPLC-ECD) as previously described (Fossat et al., 2014). Briefly, crayfish brains were rapidly extirpated and weighed, then separately homogenized in 200 µl of 0.1 N $HClO_4$ by sonication and centrifuged at 13,000 rpm for 30 min at 4°C. Aliquots of 10 µl of the supernatants were injected into RP-HPLC column (Chromasyl Stability C8, 150×4.6 mm). The mobile phase (70 mmol l^{-1} NaH_2PO_4, 0.1 mmol l^{-1} disodium EDTA, 2 mmol l^{-1} sodium octane-1-sulfonate monohydrate, 7% methanol, pH 3.9) was delivered at 1 ml min^{-1} by a Beckman 128 pump. A coulometric detector (Coulochem II, ESA), equipped with a dual-electrode analytical cell (5011 analytical cell, ESA; potentials set at +350 and −270 mV, respectively), enabled detection of DA and 5-HT. Chromatograms were acquired and analyzed using Spike 2 software (Cambridge Electronic Design Ltd, Cambridge, UK) on a computer connected to a CED1401 interface (Cambridge Electronic Design). Known quantities (5–1000 pg) of reference compounds allowed detector calibration and bioamine measurements in brain extracts. Linearity of the detector was verified from 5 to 1000 pg. Sensibility of detection in biological samples was equal or inferior to 10 pg, but aliquots containing at least 50 pg were generally injected. Each individual extract was analyzed at least in duplicate to check for the reproducibility of results. Because of the very simple extraction procedure and the convenient stability (over months) of our colorimetric detector, the use of an internal standard, which could also interfere with endogenous unknown compounds, was not retained in our protocol. Results were expressed as pg or pmol per mg brain fresh weight, but also as 5-HT/DA ratios (such ratios are not altered by possible errors in brain weight measurements or possible losses during extraction procedure).

Statistical analyses

Comparisons of means±s.e.m. were obtained and plotted using Prism software (GraphPad v5). Non-parametric Mann–Whitney tests were used to compare two groups, and Kruskal–Wallis tests, followed by Dunn's multiple comparison tests, were used for more than two groups. A value of $P<0.05$ was considered significant (*$P<0.05$, **$P<0.01$ and ***$P<0.001$).

Principal component analysis (PCA) was also performed using R software (Ade4 package) based on several behavioral variables (see Fossat et al., 2014). The separation between pairs of groups was evaluated by calculating the inertia, which was defined as the ratio of the between-group variance to the global variance. The statistical significance of inertia for group separation was estimated using a Monte Carlo permutation test (1000 runs) and fixed to $P<0.05$.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
P.F. and J.B.-C. performed behavioral experiments. P.D.D. and J.P.D. made amine measurements. D.C. performed PCA. The manuscript was written by P.F., J.P.D. and D.C. This work was co-directed by D.C. and J.P.D.

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