The Journal of Experimental Biology 216, 4435-4442

doi:10.1242/jeb.091751

RESEARCH ARTICLE

The response of brain serotonergic and dopaminergic systems to an acute stressor in rainbow trout: a time course study

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SUMMARY

The brain monoaminergic neurotransmitter systems are known to be involved in the integrated response to stress in vertebrates. However, present knowledge about the timing of their actions as well as their specific roles in the regulation of the endocrine axes that drive the stress response is incomplete. This is partly because of the complexity of the reciprocal interactions among the monoaminergic systems and other biochemical effectors of the stress response such as corticotropin-releasing factor (CRF), arginine vasotocin (AVT), adrenocorticotropic hormone (ACTH) and corticosteroids. In this study, we show for the first time in teleost fish (rainbow trout) the short- and mid-term time course of the response of the forebrain serotonergic and dopaminergic activities after exposure to an acute stressor. Other stress markers like the plasma levels of cortisol, glucose and lactate were also monitored, providing a context in which to precisely locate the monoaminergic activation within the fish acute stress response. Our results show that acute stress induced a rapid increase in forebrain serotonergic activity, which became elevated after only 15 s of chasing. Several hours after stress, serotonergic activity recovered its basal levels, in parallel with the recovery of other stress markers such as plasma catecholamines and cortisol. Dopaminergic activity was also increased after stress, but only in the telencephalon and only after 20 min. The increase in serotonergic activity happened before the elevation of plasma catecholamines, suggesting that this monoamine system could have a key role in triggering the initial steps of the activation of not only the hypothalamus–pituitary–inter-renal axis but also the brain–sympathetic–chromaffin axis in fish.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/216/23/4435/DC1

Key words: stress, catecholamines, dynamics, serotonin, dopamine, trout.

Received 29 May 2013; Accepted 27 August 2013

INTRODUCTION

When exposed to a stressor, animals respond with a complex series of behavioral and biochemical mechanisms at different levels in order to be prepared to cope with any potential threat (Barton, 2002). The vertebrate stress response is initiated by the activation of several biochemical pathways leading to an increase in the plasma levels of catecholamines and corticosteroids, which are known as the primary responses to a stress stimulus. Catecholamines and corticosteroids are the respective main end-products of the two major pathways coordinating the stress response in mammals: the brain–sympathetic–adrenal medulla axis and the hypothalamus–pituitary–adrenal (HPA) axis. In teleost fish, the brain–sympathetic–chromaffin cells (BSC) and the hypothalamus–pituitary–inter-renal cells (HPI) axes are equivalent to these mammalian systems (Wendelaar Bonga, 1997).

Several biochemical factors participate in the normal function and regulation of these axes in fish, including, among others, the adrenocorticotropic hormone (ACTH; the main secretagogue of corticosteroids), the corticotropin-releasing factor (CRF; the major regulator of ACTH secretion by the hypothalamus), the hypophyseal nonapeptide arginine vasotocin (AVT) and the brain monoaminergic neurotransmitters, including the catecholamines dopamine (DA) and noradrenaline and the indoleamine serotonin (5-hydroxytryptamine, 5HT) (Balment et al., 2006; Wendelaar Bonga, 1997; Winberg and Nilsson, 1993). The role of most of these elements is well known, whereas the function of some others, such as the brain monoaminergic neurotransmitters, is not yet clear. For instance, the activity of central monoaminergic systems increases after exposure to different types of stressors, such as handling, isolation, predator exposure, pollutant exposure or crowding (Gesto et al., 2008; Gesto et al., 2009; Schjolden et al., 2006; Weber et al., 2012; Winberg and Nilsson, 1993), with the response of the serotonergic activity being especially consistent. However, the causes and consequences of these increased activities within the stress response along with the dynamics of the monoaminergic response to stress are not well known. In this regard, the brain serotonergic system shows complex reciprocal interactions with the HPI axis, affecting and being affected by other elements of the stress response (Chaouloff, 2000; Chaouloff et al., 1999; Heisler et al., 2007; Pottinger, 2008; Winberg et al., 1997). In fact, it seems that the serotonergic system could have a dual role in the stress response, acting as an early signal during the initial steps of the response but also as a late response in situations of maintained stress. For instance, high and sustained levels of serotonergic activity are considered to be output signals in chronic stress situations (Browne et al., 2011; Øverli et al., 2007; Summers et al., 2003). However, very rapid increases in serotonergic activity have been observed in lizards after exposure to a stressor (Emerson et al., 2000; Matter et al., 1998), suggesting that the enhanced serotonergic

neurotransmission could also act as an early signal during the initial steps of the stress response or during the recognition of the stressor by the central nervous system.

Although brain monoaminergic systems are activated by stressors, the temporal sequence of activation of brain monoaminergic activity within the first moments of the stress response is still unknown. Therefore, we carried out the present study to try to obtain a clear picture of the sequential changes in several stress biomarkers during the acute stress response in fish. We paid special attention to the brain monoaminergic response and its temporal relationship with other widely used stress markers such as plasma levels of catecholamines, cortisol, glucose and lactate.

MATERIALS AND METHODS

Animals

Rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792), juveniles were obtained from a local farm (A Estrada, Pontevedra, Spain) and transported to the facilities of the Faculty of Biology of the University of Vigo (Spain). Fish were acclimated for at least 2 weeks before the experiments began in well-aerated 100 l tanks, with a continuous freshwater supply at a stocking density of 20 kg fish m^{-3}. The tanks were maintained under a controlled photoperiod (12 h:12 h day:night) and temperature (13–15°C). Fish were fed daily (1% body mass) with commercial dry pellets (Dibaq-Diprotg SA, Segovia, Spain). The experiments described comply with the Guidelines of the European Union Council (2010/63/UE), and of the Spanish Government (RD 55/2013) for the use of animals in research.

Experimental design

Experiment 1 – short-term acute stress response

A total of 144 trout (80.6±13.9 g body mass) were distributed (12 fish per tank) among 12 experimental tanks (80 l tanks). After an acclimation period of 5 days, each of the following protocols was randomly assigned to two replicate tanks: no chasing (controls) and chasing for 15 s, 2 min, 5 min or 15 min. The fish were chased in their tanks with a small net during the stipulated time in each case. After the chasing protocol, fish were immediately anesthetized in-tank by adding 0.2% 2-phenoxyethanol to the water. After ~1 min of anesthetic exposure, five fish were removed from the tank for sampling, while the remaining fish were immediately transferred to recovery tanks and were not used in the study. During sampling, 1 ml of blood from each fish was obtained by puncturing the caudal peduncle with a 1 ml disposable syringe. After blood extraction, the fish were killed by spinal transection, and the hypothalamus and telencephalon were dissected out and stored in dry ice. Plasma was obtained after centrifugation of blood (6000 ***g***, 10 min, 4°C). All fish were sampled within 3 min of the end of the chasing protocol.

Experiment 2 – recovery after acute stress

A total of 144 fish (90.7±13.5 g body mass) were distributed (12 fish per tank) among 12 experimental tanks. After an acclimation period of 5 days, each of the following chasing protocols was assigned to two replicate tanks: no chasing (controls) or chasing for 5 min followed by a recovery period of 15 min, 45 min, 2 h, 4 h or 8 h. After this recovery period, fish were anesthetized, killed and sampled as described for experiment 1.

To avoid strong interactions of social hierarchies with the stress response, all fish tanks were inspected several times a day during the acclimation period. The tanks contained more fish than required for reducing the possible development of strong dominance hierarchies, which are known to occur more often in this species when the stocking density is low. Two tanks of fish where a clear dominant specimen was present (usually swimming in the center of the tank, with other fish swimming near the surface) were not used in the experiment. As we had extra tanks before beginning the experiments, we could avoid using these tanks without affecting the final number of experimental fish.

Biochemical analyses

Plasma glucose, lactate and cortisol

The plasma levels of cortisol were analyzed using an enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical Company, Ann Arbor, MI, USA). The plasma glucose and lactate levels were measured using commercial kits from Spinreact (Girona, Spain).

Plasma catecholamines

Plasma adrenaline and noradrenaline levels were quantified by high performance liquid chromatography (HPLC) with electrochemical detection after purification of plasma by deproteinization followed by solid-phase extraction (SPE). Samples were processed as follows: a 100 µl plasma aliquot was deproteinized with 25 µl 0.6 mol l^{-1} perchloric acid ($HClO_4$; Merck, Darmstadt, Germany). After centrifugation (14,000 ***g***, 4 min, 4°C), the supernatant was neutralized with 25 µl of 1 mol l^{-1} $KHCO_3$ (Merck). Following centrifugation (14,000 ***g***, 1 min, 4°C), the supernatant was diluted to 1 ml in ultrapure water for use in the SPE procedure. The SPE cartridges (1 ml–100 mg tubes, Discovery DSC-WCX, Supelco, Bellefonte, PA, USA) were conditioned with 1.5 ml of ultrapure water at a flow rate of 5 ml min^{-1}. The samples were then applied to the conditioned columns at 1 ml min^{-1}, after which the columns were washed twice with 1 ml of ultrapure water (5 ml min^{-1}). Finally, the catecholamines were eluted from the columns with two 400 µl aliquots of 0.3 mol l^{-1} $HClO_4$ at 1 ml min^{-1}. Recoveries for noradrenaline and adrenaline were above 97%. Aliquots (20 µl) of these eluates were directly injected into the HPLC system, which was equipped with a Jasco PU-2080 Plus pump, a 5 µm analytical column (Nucleosil C18, 150 mm length×4.6 mm diameter; Phenomenex, Macclesfield, Cheshire, UK) and an ESA Coulochem II detector (Chelmsford, MA, USA). The detection system included a double analytical cell (M5011) with oxidation potentials set at +40 mV (first electrode) and +400 mV (second electrode). The mobile phase was composed of 25 mmol l^{-1} citric acid (Panreac, Barcelona, Spain), 25 mmol l^{-1} Na_2HPO_4 (Merck), 25 µmol l^{-1} Na_2EDTA (Sigma, St Louis, MO, USA), 0.21 mmol l^{-1} sodium 1-octanesulfonate (Fluka, Sigma) and 1% (v/v) methanol (Panreac); pH was adjusted to 3.4 with ortho-phosphoric acid (before the addition of methanol) and it was filtered (0.20 µm filter, Millipore, Bedford, MA, USA) and degassed by vacuum before use. Analytical run time was 10 min at an isocratic flow rate of 1.3 ml min^{-1} at room temperature. The sample peaks were quantified by comparing peak areas to those of appropriate external standards. The detection limits for the catecholamines were 3 pg of noradrenaline and 5 pg of adrenaline per injection, with a signal-to-noise ratio of 3. Acquisition and integration of chromatograms were performed using ChromNAV version 1.12 software (Jasco Corp., Tokyo, Japan).

Telencephalic and hypothalamic monoamines

The hypothalamus and telencephalon were weighed and then homogenized by ultrasonic disruption in 0.5 ml of HPLC mobile phase. The homogenates were then centrifuged (16,000 ***g***, 10 min) and supernatants further diluted 1:2 (supernatant:mobile phase) prior to HPLC analysis. Data were normalized by the protein content of the tissues, which was measured with the bicinchoninic acid method (Smith et al., 1985). The noradrenaline, DA, 3,4-

dihydroxyphenylacetic acid (DOPAC, a major DA oxidative metabolite), 5HT, 5-hydroxyindole-3-acetic acid (5HIAA, a major 5HT oxidative metabolite) and 5-hydroxytryptophan (5HTP, immediate 5HT precursor) content of the hypothalamus and telencephalon was analyzed by HPLC with electrochemical detection (HPLC-EC), as previously described (Gesto et al., 2006), with some modifications. The HPLC system was equipped with a Jasco PU-2080 Plus pump, a 5 µm analytical column (Nucleosil C18, 150 mm length×4.6 mm diameter; Phenomenex), a Jasco AS-2057 autosampler and an ESA Coulochem II detector. The detection system included a double analytical cell (M5011) with oxidation potentials set at +40 mV (first electrode) and +340 mV (second electrode). The mobile phase was composed of 63.9 mmol l^{-1} NaH_2PO_4, 0.1 mmol l^{-1} Na_2EDTA, 0.80 mmol l^{-1} sodium 1-octanesulfonate and 15.3% (v/v) methanol; pH was adjusted to 2.95 with ortho-phosphoric acid and it was filtered (0.20 µm filter, Millipore) and degassed by vacuum before use. Analytical run time was 15 min at an isocratic flow rate of 1.0 ml min^{-1} at room temperature. The detection limit for the amines and their metabolites was between 0.5 and 1.5 pg per injection, with a signal-to-noise ratio of 3. Acquisition and integration of chromatograms were performed using ChromNAV version 1.12 software (Jasco Corp.).

Statistics

Data were analyzed by ANOVA followed by Tukey's *post hoc* test. The data corresponding to fish from replicate tanks were pooled together after confirming that there were no statistical differences between replicates for any of the parameters assessed. Differences were considered statistically significant at $P \leq 0.05$.

RESULTS

Plasma catecholamines

Catecholamine levels increased rapidly in response to stress, with fish chased for 15 s showing increased levels of both adrenaline and noradrenaline (Fig. 1A). Both catecholamines then further increased until reaching a plateau 2 min after the start of chasing. In experiment 2, both catecholamines showed signs of recovery at 45 min after chasing (Fig. 1B). At the next sampling point, 2 h after the stress stimulus, both adrenaline and noradrenaline had returned to unstressed levels. Although the two plasma catecholamines showed very similar trends of variation after the acute stress, adrenaline was clearly more important quantitatively than noradrenaline.

Plasma cortisol, glucose and lactate

Cortisol levels showed a steep increase with time after stress (Fig. 2). The maximum cortisol level was observed in the group exposed to 15 min of chasing, in which cortisol levels were about 16 times higher than in control group. A smaller (1.9 times) significant increase in cortisol levels was observed after only 2 min of chasing. In experiment 2, cortisol levels showed a maximum at 45 min after the stress stimulus and decayed after that, returning to control values 8 h after the chasing protocol (Fig. 2B).

At early sampling times, the plasma levels of glucose increased with time after stress, reaching a maximum in the group exposed to 15 min of chasing (Fig. 2). Similar to cortisol, glucose levels were already increased after 2 min of stress, with respect to control fish. In the second experiment, the glucose levels reached maximum values at 2 and 4 h after stress, and returned to control values 8 h after chasing (Fig. 2B).

Lactate levels showed a pattern of changes quite similar to that of cortisol (Fig. 2), increasing with chasing duration until the end of the first experiment (15 min chasing). However, in this case the increase was statistically significant even in the group exposed to 15 s of chasing. In experiment 2, the lactate levels reached a maximum 15 min after chasing and returned to control levels after 4 h (Fig. 2).

Brain serotonergic and dopaminergic activity

Serotonergic activity rapidly increased in both hypothalamus and telencephalon, as shown by the 5HIAA/5HT ratio (Fig. 3A). The increase was obvious after only 15 s of stress. After that initial increase, serotonergic activity remained above control levels without significant changes until the end of experiment in both brain regions. In experiment 2, serotonergic activity showed a maximum in both regions 45 min after stress. The values subsequently started to decrease, returning to control values after 2 h in the hypothalamus and 8 h in the telencephalon (Fig. 3B). In both regions, the increases in the serotonergic ratio were parallel to those in the levels of the metabolite 5HIAA whereas 5HT levels remained unaltered in all groups (supplementary material Figs S1–S4). Also, stress did not induce any effect on the levels of the precursor 5HTP (supplementary material Figs S1–S4).

The alterations in the dopaminergic system were minor. In experiment 1, no significant changes in the dopaminergic activity index (the DOPAC/DA ratio) were observed in the hypothalamus or in the telencephalon (Fig. 4A), although the DOPAC levels were increased after 15 min of chasing in the hypothalamus (supplementary material Fig. S1) and after 2 min of chasing in the telencephalon (supplementary material Fig. S3). In experiment 2, no changes were detected in the hypothalamus (Fig. 4B; supplementary material Fig. S2), whereas the telencephalic dopaminergic activity was increased (Fig. 4B), with no changes in the levels of DA or DOPAC (supplementary material Fig. S4). A maximum was reached in the telencephalic dopaminergic ratio 45 min after stress, and dopaminergic activity returned to control levels 4 h after chasing (Fig. 4B).

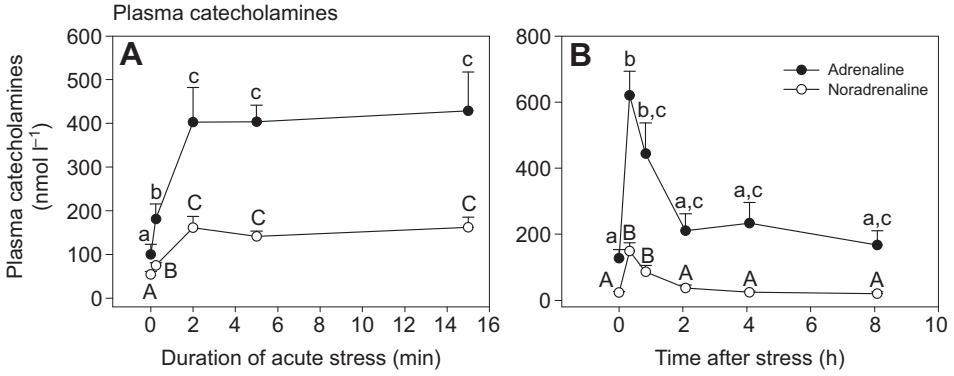

Fig. 1. Changes in the plasma levels of the catecholamines noradrenaline and adrenaline in rainbow trout. (A) Fish exposed to acute handling stress for 15 s, 2 min, 5 min or 15 min and sampled immediately afterwards. (B) Fish exposed to 5 min of handling stress and sampled 15 min, 45 min, 2 h, 4 h or 8 h later. Values are means ± s.e.m. (*N*=10). Different letters indicate statistically significant differences among groups.

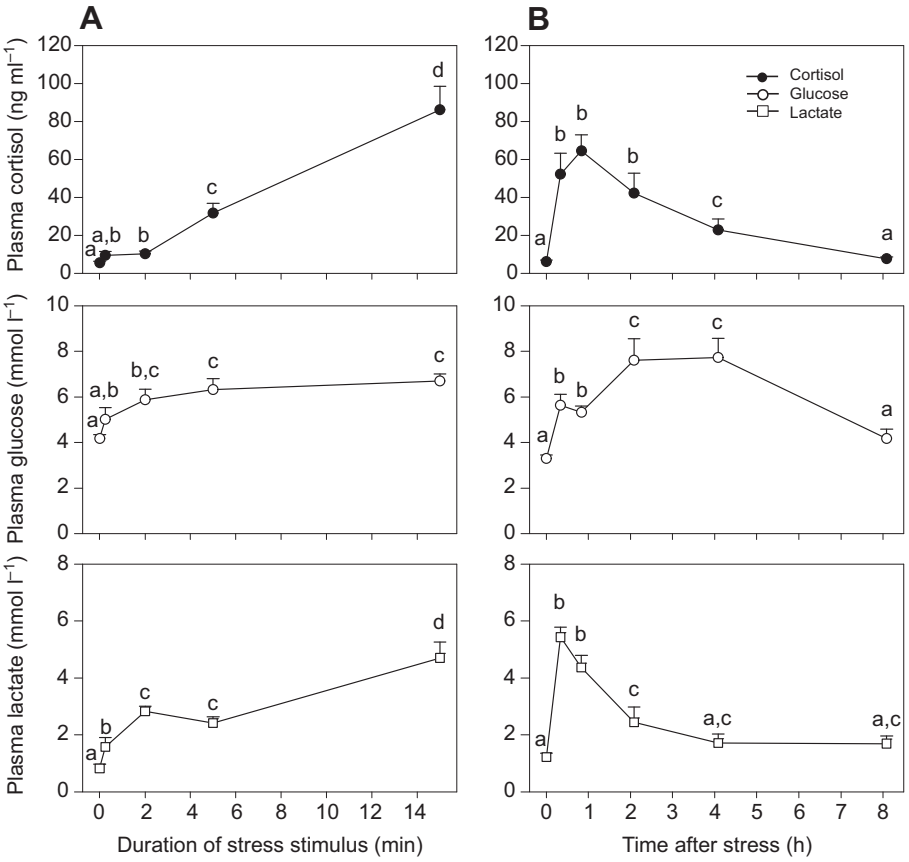

Fig. 2. Changes in the plasma levels of cortisol, glucose and lactate in rainbow trout. (A) Fish exposed to acute handling stress for 15 s, 2 min, 5 min or 15 min and sampled immediately afterwards. (B) Fish exposed to 5 min of handling stress and sampled 15 min, 45 min, 2 h, 4 h or 8 h later. Values are means ± s.e.m. (*N*=10). Different letters indicate statistically significant differences among groups.

Throughout the experiments there were no stress effects on the levels of noradrenaline, either in the hypothalamus or in the telencephalon (supplementary material Figs S1–S4).

DISCUSSION

When fish are exposed to an acute stressor, both the BSC and HPI axes become activated. As a result, large quantities of the catecholamines noradrenaline and adrenaline, and then cortisol, are released into plasma (Wendelaar Bonga, 1997). Both plasma catecholamines and cortisol act on multiple targets within the fish, enabling them to cope with stress. Catecholamines enhance respiration and cardiac output as well as the mobilization of energy substrates such as glucose (mainly by increased liver glycogenolysis) or free fatty acids (Wendelaar Bonga, 1997). Cortisol contributes to the increase in the plasma availability of glucose (mainly by raised gluconeogenesis in the liver) and free fatty acids (Mommsen et al., 1999; Sheridan, 1994), and also induces inhibition of reproductive and immune functions (Mommsen et al., 1999). In this context, the role and timing of the action of brain monoaminergic neurotransmission is not fully understood. Therefore, we carried out our study to get a view of the pattern of activity of brain monoamines in the context of the simultaneous activation of both the BSC and HPI axes during an acute stress response.

The dynamics of the response of several stress markers such as plasma cortisol or glucose after exposure to an acute stressor have been studied in fish (Aluru and Vijayan, 2006; Arends et al., 1999; Cockrem, 2013; Fast et al., 2008; Pickering et al., 1982; Saera-Vila et al., 2009; Small, 2004; Wilson et al., 1998). However, the first sampling time was usually performed 15 min or more post-stress. Studies monitoring the activation of those markers in a more detailed fashion during the first minutes of the stress response are very scarce (Biron and Benfey, 1994; Pepels et al., 2004). In agreement with the study of Biron and Benfey (Biron and Benfey, 1994), we demonstrate here that the typical increase of plasma cortisol, glucose and lactate in response to a handling challenge takes place rapidly in rainbow trout, with all three parameters showing an increase within the initial 2 min of handling. In the available studies, these factors usually returned to control levels in a few hours, although recovery times strongly depend on species, the kind of stressor and exposure time (Aluru and Vijayan, 2006; Arends et al., 1999; Biron and Benfey, 1994; Fast et al., 2008; Pickering et al., 1982; Small, 2004; Wilson et al., 1998). In this study, plasma lactate returned to control levels within 4 h of acute handling, whereas plasma cortisol and glucose returned to control levels 8 h after exposure. In both cases, the time periods were similar to those observed in other studies in salmonid fish (Fast et al., 2008; Pickering et al., 1982; Wilson et al., 1998). It is noteworthy that plasma glucose levels increased in two phases. An initial fast increase occurred in less than 2 min, and is most probably the result of increased glycogenolytic potential in liver elicited by enhanced levels of plasma catecholamines. A further increase was observed between 45 min and 2 h, which could be related to the secondary effects of cortisol promoting gluconeogenesis in the liver (Mommsen et al., 1999). Plasma cortisol levels increased slowly at first, with an acute increase occurring between 2 and 15 min after stress, when the plasma levels of glucose were already high. These changes are in agreement with previous findings suggesting that plasma glucose could have a role in promoting the release of cortisol by the inter-renal cells in fish under ACTH stimulation (Conde-Sieira et al., 2013).

The time course of the response of plasma catecholamines to acute stress has not been well studied to date because of the difficulty of

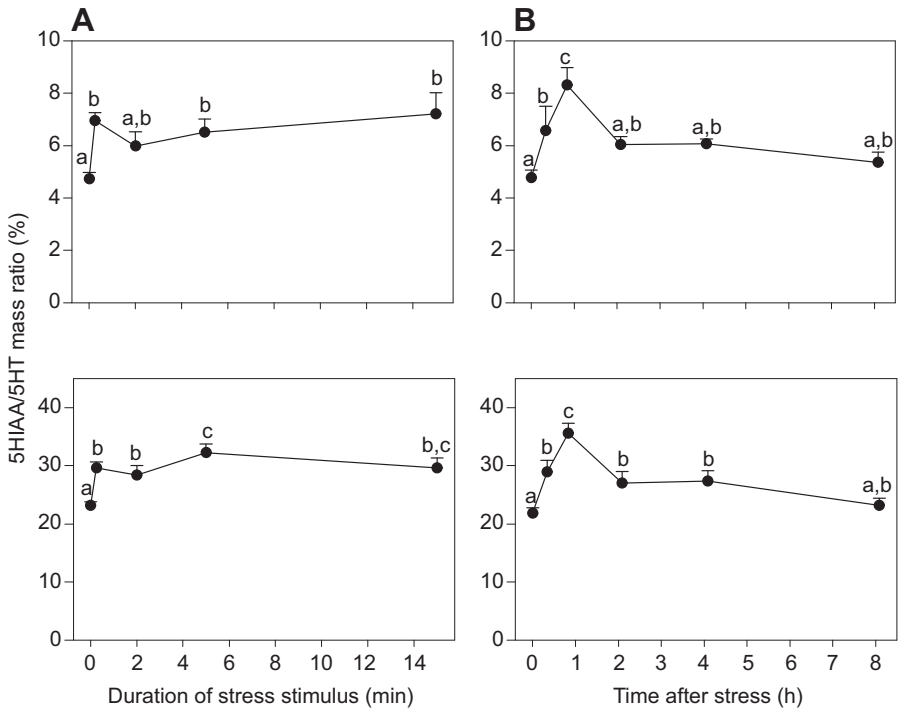

Fig. 3. Changes in serotonergic activity [estimated by the 5-hydroxyindole-3-acetic acid (5HIAA)/5-hydroxytryptamine (5HT) ratio] in the hypothalamus (top panels) and the telencephalon (bottom panels) of rainbow trout. (A) Fish exposed to acute handling stress for 15 s, 2 min, 5 min or 15 min and sampled immediately afterwards. (B) Fish exposed to 5 min of handling stress and sampled 15 min, 45 min, 2 h, 4 h or 8 h later. Values are means ± s.e.m. (*N*=10). Different letters indicate statistically significant differences among groups.

sampling and measuring basal levels of catecholamines in unstressed fish. In this study, the anesthetic was administered surreptitiously to the fish home tanks. Following this method, the first contact with the fish took place when they were already anesthetized, thus highly reducing the impact of capturing them from the tanks (Caamaño-Tubío et al., 2010; Gerwick et al., 1999), and therefore minimizing sympathetic activation. Plasma catecholamines increased very fast as expected, and both noradrenaline and adrenaline went back to control levels within 2 h following the stress stimulus. The recovery time is in agreement with previous studies in rainbow trout, which described recovery times for plasma catecholamines of between 1 and 4 h post-stress (Milligan and Wood, 1987; Wood, 1991; Wood et al., 1990).

Central 5HT seems to be an output signal of the integrated stress response, as chronically stressed fish, including socially subordinate individuals, usually display elevated serotonergic activity (Øverli et al., 2005; Winberg et al., 1992). Moreover, at the same time, 5HT activity seems to have a key role in the activation of the hormonal stress response and it is known that serotonergic activity acts as a stimulator of the HPA/HPI axis in both mammals and fish (Dinan,

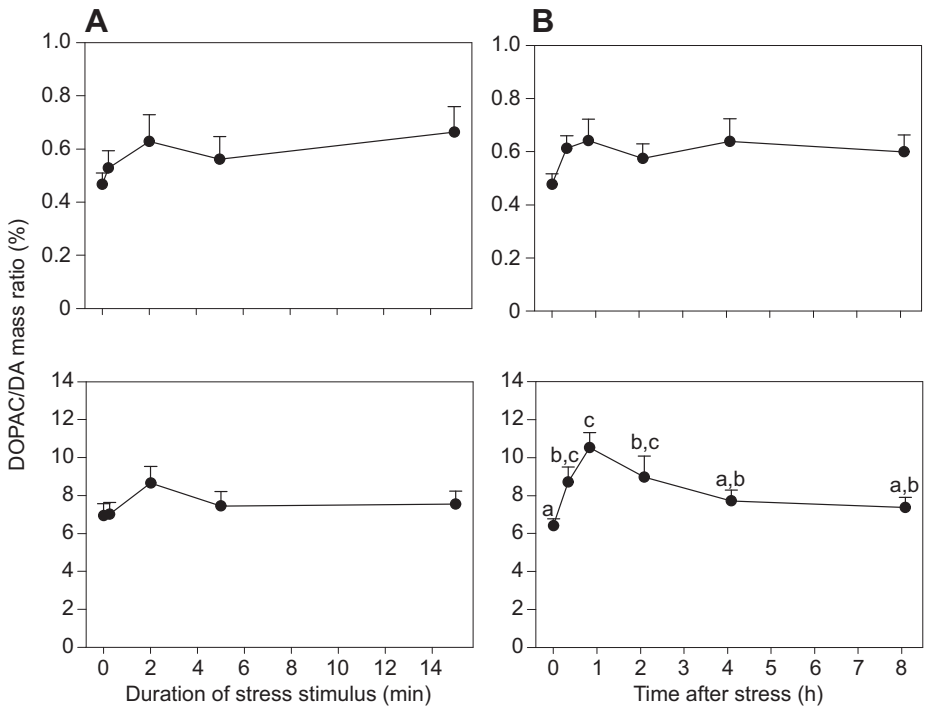

Fig. 4. Changes in dopaminergic activity [estimated by the 3,4-dihydroxyphenylacetic acid (DOPAC)/dopamine (DA) ratio] in the hypothalamus (top panels) and the telencephalon (bottom panels) of rainbow trout. (A) Fish exposed to acute handling stress for 15 s, 2 min, 5 min or 15 min and sampled immediately afterwards. (B) Fish exposed to 5 min of handling stress and sampled 15 min, 45 min, 2 h, 4 h or 8 h later. Values are means ± s.e.m. (*N*=10). Different letters indicate statistically significant differences among groups.

1996; Le Feuvre et al., 1991; Winberg et al., 1997). In fact, the involvement of serotonergic activity in the stress response is very complex. For instance, it has been shown in mammals that 5HT stimulates CRF release by the hypothalamus (Boisvert et al., 2011; Calogero et al., 1989) and also ACTH release by the pituitary (Calogero et al., 1993). Additionally, serotonergic activity is affected by other elements of the stress response such as cortisol, CRF or AVT (DiBattista et al., 2005; Dinan, 1996; Price and Lucki, 2001; Sangiao-Alvarellos et al., 2004; Summers et al., 2005). Therefore, we consider that studying the temporal pattern of activation of serotonergic signaling after exposure to a stressor is of vital importance to understanding the participation of the brain serotonergic system in the integrated stress response. However, such studies are very infrequent in the literature. Several studies about the dynamics of the monoaminergic response have been carried out with lizards (Summers, 2002; Summers et al., 2003) after social interaction, which is known to be a potent stressor. Those studies show that the central serotonergic response to social stress seems to be biphasic, with an early increase in all animals and a second increase that is delayed in time in subordinated fish with respect to dominant individuals (Summers et al., 2003). In our study, the acute stress protocol induced a very rapid change in the hypothalamic and telencephalic 5HIAA/5HT ratio, an indicator of serotonergic activity, which increased in seconds, something that has been observed before in lizards, which displayed elevated serotonergic activity only 30 s after a stressful social confrontation (Emerson et al., 2000; Matter et al., 1998). The serotonergic ratio reached a plateau very rapidly, and showed a further increase over time, reaching a maximum around 45 min post-stress. The promoting effect of acute stress on serotonergic activity was transient, and serotonergic ratios went back to control levels in a few hours in a very similar way to plasma cortisol and plasma catecholamines. Our results suggest that the response of 5HT activity occurs before or at least concomitantly with the rise of catecholamine levels in plasma. Therefore, central 5HT activity could play a role in triggering the BSC axis, leading to the massive release of chromaffin cell catecholamines to the blood. The initial activation of the brain monoaminergic neurons could be part of the stressor recognition mechanisms, taking part in the central integration of the stress signals to initiate the neuroendocrine response. The observed peaks in both hypothalamic and telencephalic serotonergic activities 45 min after stress could be the result of the indirect effects of other participants in the stress response such as cortisol, which is known to promote serotonergic activity in fish (Summers and Winberg, 2006; Weber et al., 2012). It is known that the regulation of the serotonergic neuronal system is different in cases of chronic stress, where the activation seems to be more permanent (Øverli et al., 2005; Øverli et al., 2007). Furthermore, a reorganization of brain serotonergic receptors seems to take place after stress and is probably different in acute than in chronic stress (Chaouloff et al., 1999; McKittrick et al., 1995). The differential regulation of the serotonergic response after acute or chronic stress deserves further investigation.

The central dopaminergic system seems to be involved in the regulation of activities like aggression, social status, motor activity, learning and motivation/reward (Summers and Winberg, 2006). It has been reported that serotonergic and dopaminergic activities are antagonistically involved in the neural circuitry controlling aggression, which is known to overlap with that regulating stress responses (Summers and Winberg, 2006). In this respect, it has been shown in lizards (and also in fish) that, after social interactions, dominant individuals have higher levels of dopaminergic activity than subordinated fish, the opposite to what occurs with serotonergic activity (Winberg and Nilsson, 1993). As increased serotonergic activity is considered to be a behavioral inhibitor, Höglund and colleagues (Höglund et al., 2001) suggested that an initial activation of brain DA systems could serve to counteract the effects of a stress-induced elevation in brain 5HT activity in dominant fish. Despite the above, the involvement of the central dopaminergic activity in the regulation of the HPA/HPI axis is unclear. In mammals, both stimulatory and inhibitory effects of stress on dopaminergic activity have been reported (Höglund et al., 2001; Waters et al., 2005). In fish, although several studies have reported a stimulatory action of different kinds of stressors on the central dopaminergic system (Backström et al., 2011; Gesto et al., 2008; Øverli et al., 1999; Weber et al., 2012), such stimulation did not always take place (M.G. and J.M.M., unpublished observations). The very limited data on the central dopaminergic response to stress deserves further attention. In our study, dopaminergic activity remained unaffected in the hypothalamus and increased in the telencephalon after the acute stress protocol, supporting the idea that the activation of the dopaminergic tone is strongly dependent on stressor type and severity, and on the brain region considered.

The brain noradrenergic system also seems to be involved in the stress response in mammals (Dunn et al., 2004) and probably in fish (Øverli et al., 2001). Noradrenaline could have a role in triggering the release of CRF, leading to the activation of the HPA axis in mammals (Dunn et al., 2004). In our experiments, we did not observe any alteration of noradrenaline levels in the hypothalamus or telencephalon but with no data on the noradrenaline metabolization the effects on noradrenaline levels alone are difficult to interpret.

The simultaneous assessment of different stress markers allowed us to temporally locate the increase in serotonergic activity within the context of an integrated stress response. The transient increase in forebrain serotonergic activity after an acute stress took place very rapidly and its initial increase occurred prior to or at least concomitant with the increase in plasma catecholamines. Thus, besides its role in the regulation of the HPI axis, it is also possible that an increase in forebrain serotonergic activity participates in the activation of the BSC axis, leading to the massive release of noradrenaline and adrenaline into the circulation. In this regard, it is already known that peripheral serotonin promotes the release of catecholamines in mammals and fish through a cholinergic innervation-independent mechanism (Winberg et al., 1997; Reid et al., 1998). The rapid serotonergic response after exposure to an acute stressor suggests that serotonergic tone could be important in the recognition of potential stressors by the central nervous system and may act as a mediator between the perception of the stressor by the sensory organs and the primary release of catecholamines and corticosteroids into the plasma. Furthermore, the activity of the serotonergic system after stress seems to be tightly linked to other elements of the BSC or HPI axis, as the transient increase observed after stress is driven back to resting levels in a very similar fashion to other elements of the stress response such as catecholamines or cortisol. Whether or not there is a direct cause–effect relationship between forebrain serotonergic activity and other stress markers should be confirmed in future studies.

LIST OF ABBREVIATIONS

5HIAA	5-hydroxyindole-3-acetic acid
5HT	5-hydroxytryptamine (serotonin)
5HTP	5-hydroxytryptophan
ACTH	adrenocorticotropic hormone
AVT	arginine vasotocin
BSC	brain–sympathetic–chromaffin axis
CRF	corticotropin-releasing factor

DA	dopamine
DOPAC	3,4-dihydroxyphenylacetic acid
HPA	hypothalamus–pituitary–adrenal axis
HPI	hypothalamus–pituitary–inter-renal axis
HPLC	high performance liquid chromatography
SPE	solid-phase extraction

ACKNOWLEDGEMENTS

Special thanks to Linda Manrique for her valuable corrections on the manuscript.

AUTHOR CONTRIBUTIONS

Conception and experiment design: M.G., J.L.S. and J.M.M. Execution and analysis: M.G., M.A.L.-P. and J.H. Data interpretation: M.G., M.A.L.-P. and J.M.M. Writing and revisions: M.G., J.L.S. and J.M.M.

COMPETING INTERESTS

No competing interests declared.

FUNDING

This study was supported by the Spanish Ministerio de Educación y Ciencia and European Fund for Regional Development [AGL2010-22247-C03-03] and Xunta de Galicia (Consolidación e estructuración de unidades de investigación competitivas do sistema universitario de Galicia) [CN2012/004]. M.A.L.-P. was recipient of an Isidro Parga Pondal Researcher [P.P. 0000 300S 14008] scholarship from Xunta de Galicia. M.G. was recipient of a post-doctoral fellowship from Xunta de Galicia (Spain, Ángeles Alvariño program).

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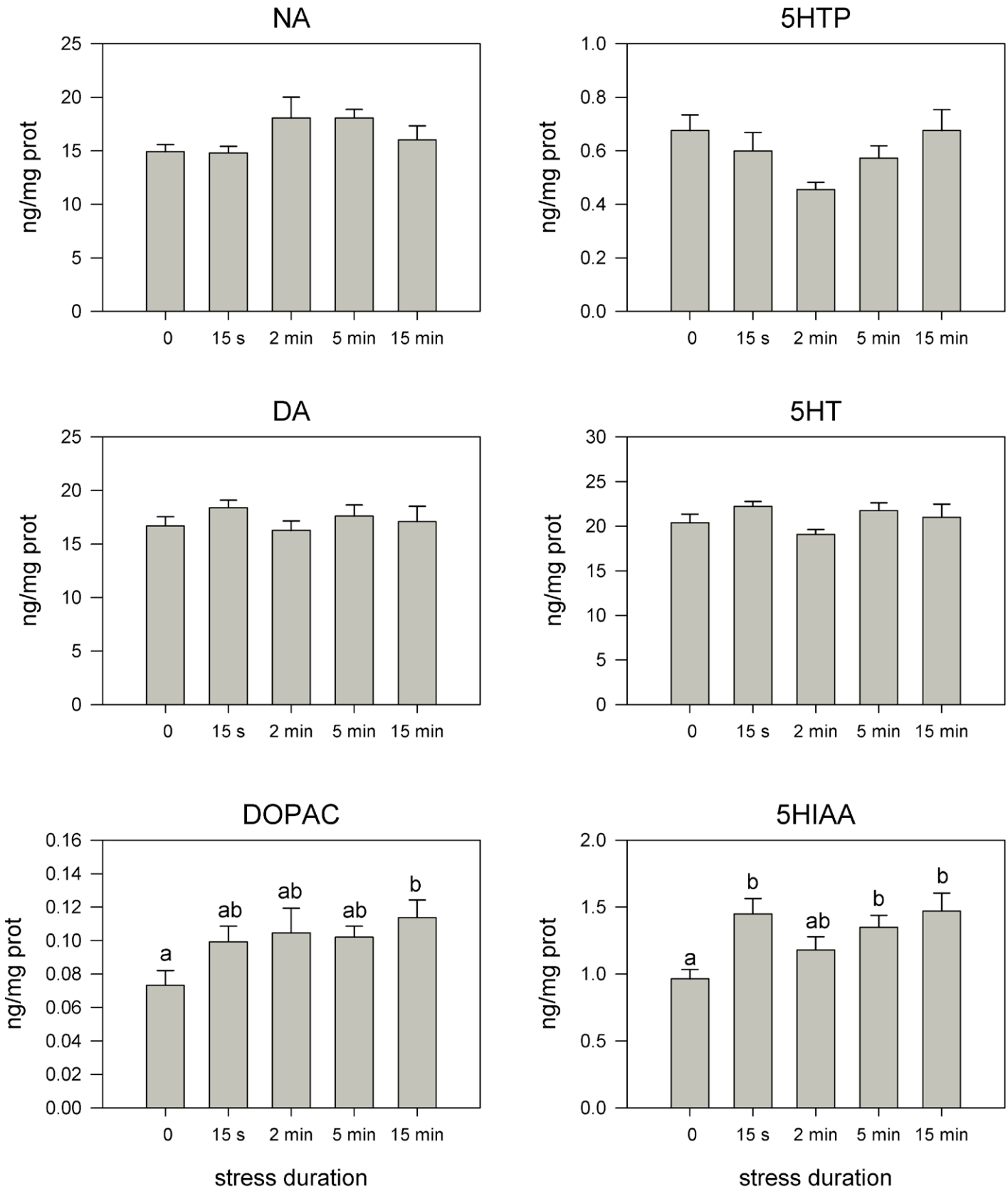

Fig. S1. Changes in the hypothalamic levels of NA, DA, DOPAC, 5HTP, 5HT and 5HIAA in rainbow trout exposed to acute handling stress for 15 s, 2 min, 5 min or 15 min and killed immediately afterwards. Values are means ± s.e.m. (*N*=10). Different letters indicate statistically significant differences among groups.

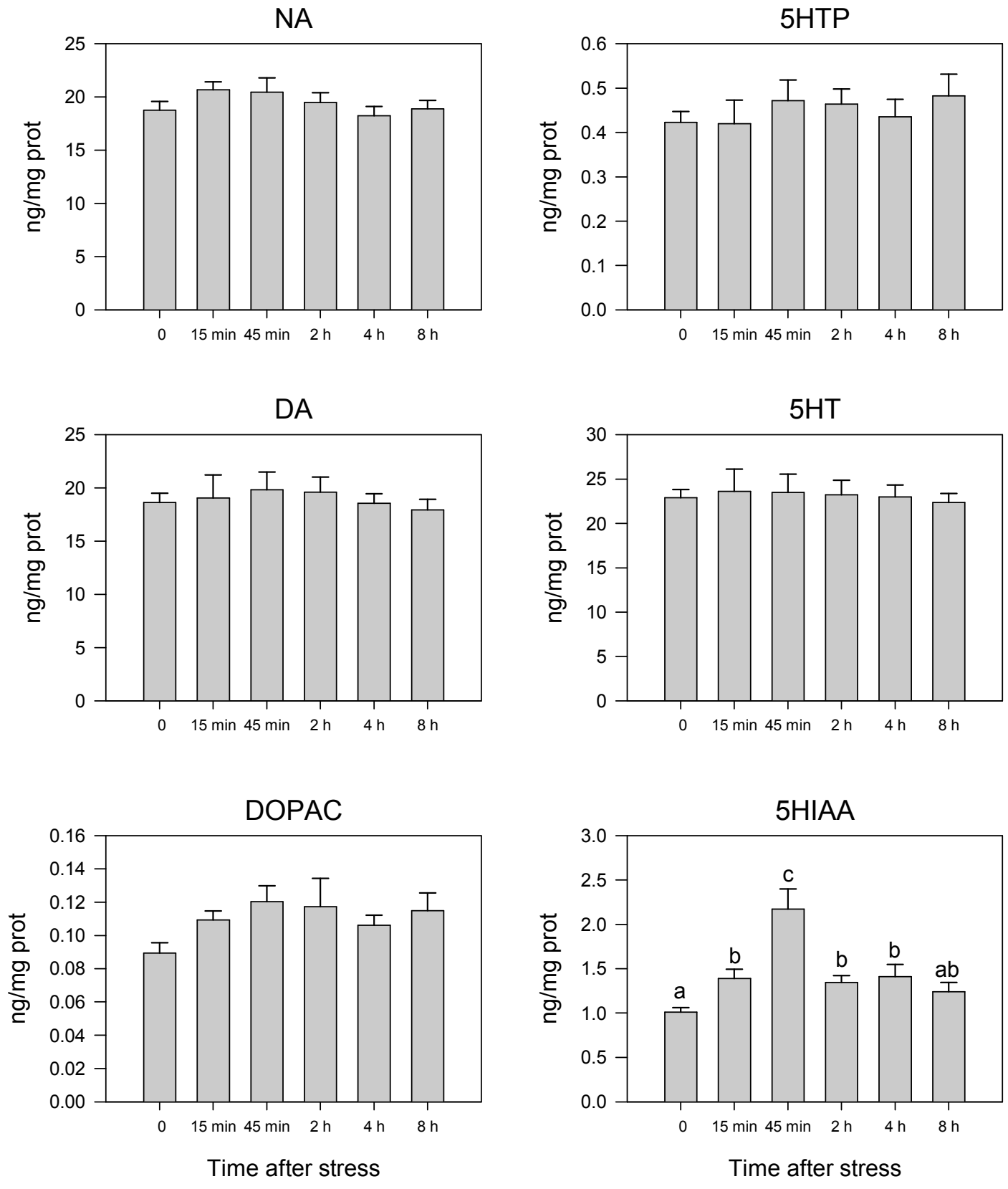

Fig. S2. Changes in the hypothalamic levels of NA, DA, DOPAC, 5HTP, 5HT and 5HIAA in rainbow trout exposed to 5 min of handling stress and killed 15 min, 45 min, 2 h, 4 h or 8 h later. Values are means ± s.e.m. (*N*=10). Different letters indicate statistically significant differences among groups.

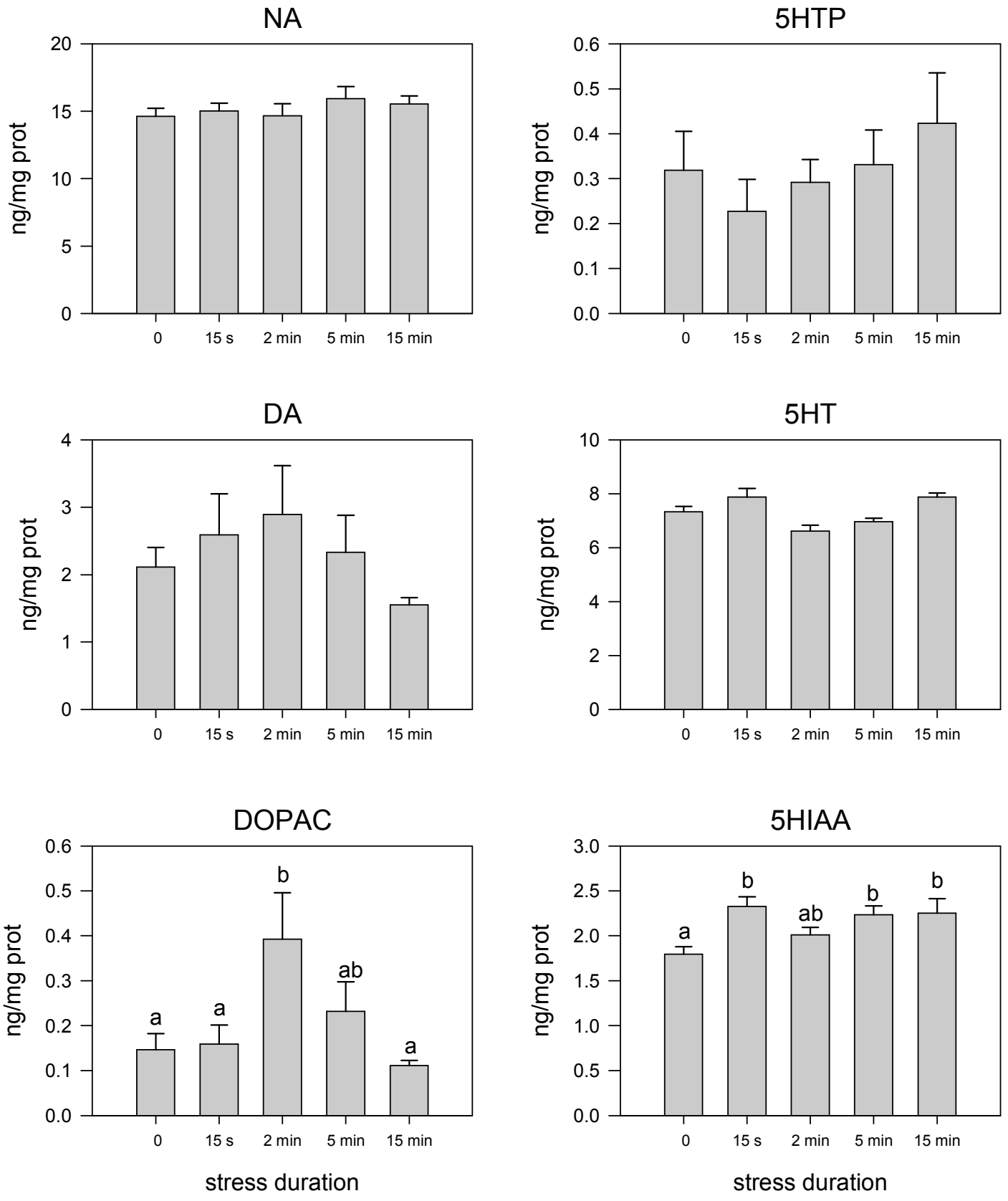

Fig. S3. Changes in the telencephalic levels of NA, DA, DOPAC, 5HTP, 5HT and 5HIAA in rainbow trout exposed to acute handling stress for 15 s, 2 min, 5 min or 15 min and killed immediately afterwards. Values are means ± s.e.m. (*N*=10). Different letters indicate statistically significant differences among groups.

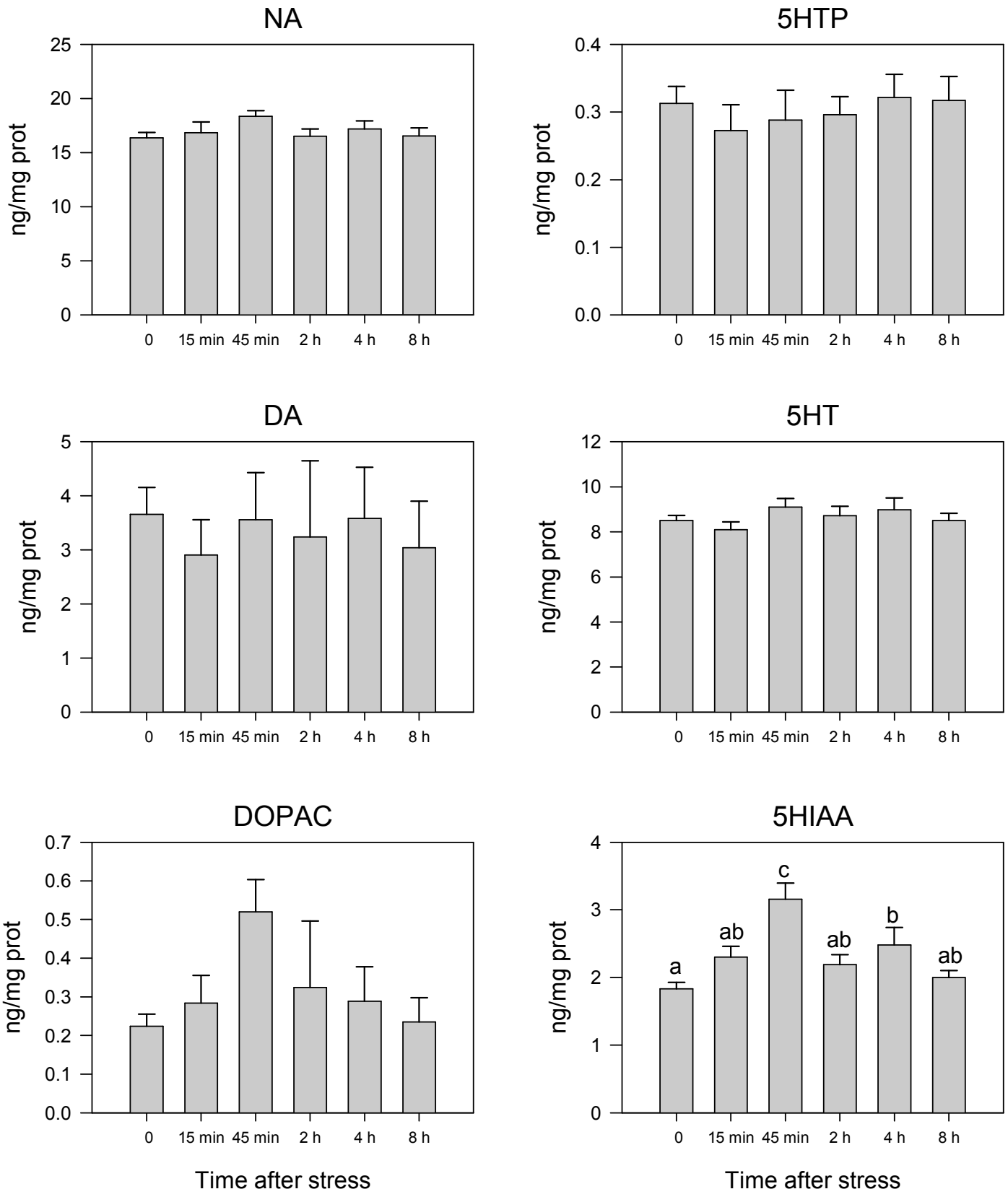

Fig. S4. Changes in the telencephalic levels of NA, DA, DOPAC, 5HTP, 5HT and 5HIAA in rainbow trout exposed to 5 min of handling stress and killed 15 min, 45 min, 2 h, 4 h or 8 h later. Values are means ± s.e.m. (*N*=10). Different letters indicate statistically significant differences among groups.