J Endocrinol Reprod 18 (2014) 1 : 17-28 **Original Research**

Effect of L-tryptophan feeding on brain mitochondrial ion transport in net-confined climbing perch (*Anabas testudineus* Bloch)

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Summary

Serotonin (5-HT), a neurohormone with many physiological actions, is synthesized from the dietary essential amino acid tryptophan (TRP). However, the effects of TRP on neuronal ion transporters and its role in stress response have not yet been identified in the teleost fish. The effects of varied doses of TRP on the activities of mitochondrial (m) and cytosolic (c) ion transporters were examined in the forebrain (FB), midbrain (MB) and hindbrain (HB) segments of an air-breathing fish *Anabas testudineus* Bloch kept either in non-stressed or in stressed condition. Feeding the fish with varied doses of TRP (1, 2 and 4 mg g^{-1} feed) for seven days produced dose-dependent effects on Na^+, K^+-ATPase and H^+-ATPase activities in different regions of fish brain. A decrease (P<0.001) in Na^+, K^+-ATPase activity was found in FB and MB after seven days of TRP treatment. TRP decreased (P<0.001) H^+-ATPase activity in the FB but increased Na^+, K^+ ATPase activity in all the three regions of the brain. In non-stressed fish, feeding 20 mg g^{-1} TRP for two days produced a substantial rise (P<0.001) in cH^+-ATPase activity in the FB and HB of the fish. But mH^+-ATPase showed a reversed response to TRP feeding. On the contrary, TRP treatment in net-confined fish showed a decrease (P<0.001) in the cH^+-ATPase activity in the FB and HB, whereas it produced an increase (P<0.01) in the MB. In non-stressed fish, cytosolic and mitochondrial Ca^{2+}-ATPase activities in FB and MB decreased (P<0.001) after TRP feeding. Feeding TRP in stressed fish reduced (P<0.001) cCa^{2+} ATPase activity in the MB but produced an increase (P<0.001) in its activity in mitochondria. In non-stressed fish, TRP feeding decreased (P<0.001) mMg^{2+}ATPase activity in the FB and MB segments. TRP treatment, in stressed fish, however decreased (P<0.001) Mg^{2+}-ATPase activity in the MB but not in other brain segments. The data indicate that TRP can regulate brain mitochondrial ion transport, and induction of stress may modify the TRP-induced mitochondrial ion transport response of air-breathing fish.

Key words: L-tryptophan, serotonin, stress, ATPases, brain, feed, fish

Introduction

L-tryptophan (TRP), the precursor of the monoamine neurotransmitter serotonin (5-hydroxytryptamine; 5-HT), is known for its physiologic actions in fishes (Winberg et al., 2001; Lepage et al., 2002, 2005; Costas et al., 2012; Wolkers et al., 2012; Martins et al., 2013). Tryptophan is also needed for the biosynthesis of a number of molecules that have important cellular and neural functions including melatonin (Maitra et al., 2012). The first and rate-limiting step in the biosynthesis of serotonin is the hydroxylation of TRP to 5-hydroxytryptophan, a reaction catalyzed by the enzyme tryptophan hydroxylase (Winberg et al., 2001). Serotonin levels in the brain are highly dependent on the levels of 5-hydroxytryptophan (5-HTP) in the central nervous system (Winberg et al., 1997). In contrast to mammals, 5-HT can pass through the blood-brain barrier in teleost fishes, contributing to the high levels of biogenic amines in the periphery (Khan and Deschaux, 1997).

Fishes are constantly exposed to stressful conditions in the aquatic environment and they respond to stressors by evoking varied patterns of stress responses (Barton et al., 2002; Peter, 2013). Compensatory physiological adaptations thus occur in stressed organisms to combat the disturbed homeostasis (Peter, 2013). Confinement to net induces stress in climbing perch that alters their osmotic and metabolic responses (Peter and Peter, 2011). Brain plays a critical role in the body's perception, and its response to chronic stress can adversely affect the performance of serotonergic system particularly in fish (Mathews, 2001). 5-HT can induce hyperactivity in the metabolism of brain serotonin in mammals (Koopmans et al., 2005; Liu et al., 2013) and in fish (Winberg et al., 1997). In fishes, several ion-dependent

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ATPases are involved in maintaining the osmotic and ionic homeostasis which is sensitive to many intrinsic and extrinsic factors (Peter and Peter, 2011). Many ATPases that transport ions across membrane require energy for this translocation (Peter et al., 2007). These transport systems thus lead key role in intracellular functions including cell volume regulation, osmotic pressure and membrane permeability (Peter et al., 2007).

In fish, activation of catecholamine system (norepinephrine and dopamine) and indoleamine (serotonin) has been found during stressful conditions (Khan et al., 1996). 5-HT plays an important role in brain physiology as it regulates the biological rhythms in vertebrates (Overli et al., 2005). Furthermore, 5-HT has been implicated in stress adaptation in fish (Peter, 2013). This is mainly because of its involvement in the regulation of hypothalamic-pituitary-adrenocortical (HPA) axis in mammals as well as in the control of the hypothalamic-pituitary-interrenal (HPI) axis in teleosts (Winberg et al., 1997; Overli et al., 2005; Hoglund et al., 2007).

Dietary supplementation of TRP has been shown to reduce the physiological stress response in fish as it modulates the activity of the neurotransmitter serotonin (Costas et al., 2012). Elevated dietary intake of TRP has been reported to result in increased brain levels of TRP and elevated rates of 5-HT synthesis and metabolism (Aldegunde et al., 1998, 2000; Winberg et al., 2001), and TRP-rich diets have been shown to modulate the activity of serotonin in farmed animals (Martins et al., 2013). The central 5-HT system is believed to have an inhibitory effect on aggressive behavior in a variety of vertebrates including teleost fish (Edwards and Kravitz, 1997). TRP-supplemented diets have been shown to reduce stress response, particularly cortisol response, after acute stress (Lepage et al., 2002, 2005) and stress-induced anorexia (Hoglund et al., 2007). On the other hand, the action of dietary TRP on mitochondrial ion transport, particularly during stress response, is not yet known in teleost fish brain. In this study, the effect of TRP on transport of ions was examined in the brain of air-breathing fish *Anabas testudineus* Bloch. In addition, the role of TRP in the stress response was also studied in this fish brain.

Materials and methods

Fish holding conditions

Tropical freshwater air-breathing fish, commonly known as climbing perch (*Anabas testudineus* Bloch) belonging to order Perciformes and family Anabantidae, was used as the test species. This native teleost fish inhabiting the backwaters of Kerala in Southern India is an obligate air-breathing fish equipped to live in demanding environmental conditions with their well-defined physiologic and biochemical mechanisms (Peter et al., 2007; Peter and Peter, 2011). These fish in their post-spawning phase were collected from the wild conditions and kept in the laboratory for three weeks under natural photoperiod (12 h L:12 h D) and at water temperature ranging from 28 to 29 ^{0}C with a mean water pH of 6.2. Fish were fed with dry commercial fish feed at 1.5% of body mass and were transferred to 50 L glass tanks. There was no mortality during experimentation and the fish ate their normal food. The regulations of Animal Ethical Committee of the University were followed.

Experimental protocol

The dose and time-responsive effects of dietary TRP on ion transport were studied in the brain of climbing perch.

Dose-responsive effects of dietary TRP

Laboratory-acclimated freshwater climbing perch were held as four groups of six each. Varied doses (1, 2 and 4 mg g^{-1}) of L-tryptophan (TRP) (Sigma-Aldrich) were mixed with powdered fish feed and made into pellets again. Control feed, which lacked TRP, was also made in the same manner and fed to a group of control fish for seven days. The fish in groups-2, 3 and 4 were fed 1, 2, and 4 mg g^{-1} TRP, respectively, for seven days.

In another experiment a selected dose 2 mg g^{-1} of TRP feed was fed to a group of fish for two days. The parallel control group received the control feed.

Effects of dietary TRP in stressed and non-stressed fish

This experiment tested the effects of dietary TRP on the brain ion transport during stress response in fish. Acclimated fish were divided into two sets and each set comprised two groups of six each. In the non-stressed fish set, the first group was given control feed for two days. The second group fish were fed with a selected dose of TRP (2 mg g^{-1} feed) for two days. In the stressed fish set, the first group fish was given control feed and the second group fish received 2 mg g^{-1} TRP feed for two days. All these groups of fish were then kept for 1 hr in net confinement before sampling.

Sampling and analysis

After the specific time intervals, all fish were anaesthetized in 0.1% 2-phenoxyethanol (Sigma, St. Louis,

MO) solution and blood was collected from the caudal vessels, using a heparinized syringe. Following blood sampling, the fish were then sacrificed by spinal transsection and the whole brain was carefully excised within 1-2 min. The brain was then sliced into three portions based on the morphological and functional properties. The first portion, the forebrain, (FB) included olfactory bulbs and telencephalon; second portion, the midbrain (MB), included optic tectum, pituitary and hypothalamus; and the third portion, the hindbrain (HB), comprised cerebellum, medulla and a part of the spinal cord. These three brain segments were blotted, weighed and kept in ice-cold BME buffer (pH 7.4) and stored at -80 ºC for further analysis.

Isolation of brain mitochondria and cytosol

Mitochondria were isolated from the three different segments of fish brain namely FB, MB, and HB following the method of Lee et al. (1993) and Veauvy et al. (2002) with modifications. Briefly, each segment of brain was kept in brain mitochondrial extraction (BME) buffer containing 0.25 mM sucrose, 10 mM HEPES, 0.5 mM EDTA, and 0.5 mM EGTA (pH 7.4). The brain tissue was chopped and homogenized (8-10 strokes) using a glass homogenizer. The collected homogenate was first centrifuged (Plastocrafts-Superspin-R) at 2000 xg for 3 min at 4ºC to separate the membrane constituents from mitochondria and synapses. A portion of this supernatant was used for analyzing the Na^+, K^+ ATPase activity. The other portion was then centrifuged at 12,000 xg for 8 min at 4^0C. The supernatant was collected and transferred to an eppendorf tube for analyzing the cytosolic ion transporters such as H^+- and Ca^{2+}-ATPases. The pellets were then washed in the isolation buffer with BSA and centrifuged at 12,000 xg for 10 min. The pellets were then re-suspended in a 0.25 M sucrose solution and centrifuged again for 10 min. These final pellets were then resuspended in the sucrose medium which served as the enzyme source.

Determination of Na^+, K^+-ATPase-specific activity

The ouabain-sensitive Na^+, K^+-ATPase-specific activity was quantified in the brain membrane preparation adopting the method of Peter et al. (2000). Saponin (0.2 mg protein^{-1}) was added to the membrane preparation to optimize substrate accessibility. The samples in duplicates were added to a 96-well microplate containing 100 mmol L^{-1} NaCl, 30 mmol L^{-1} immidazole (pH 7.4), 0.1 mmol L^{-1} EDTA and 5 mmol L^{-1} $MgCl_2$ with or without ouabain and incubated at 37^0C. The reaction was initiated by the addition of ATP and the reaction was terminated with addition of 8.6% TCA. The liberated inorganic phosphate content was determined in Autoreader 4011 (Spam Diagnostics Ltd., Surat, India) at 700 nm and expressed in µmol Pi h^{-1} mg protein^{-1}. The protein content of the tissues was measured using modified Biuret Assay (Alexander and Ingram, 1980) with bovine serum albumin as standard.

Determination of H^+-ATPase-specific activity

The baffilomycin-sensitive H^+-ATPase activity in the brain mitochondria was measured as described for Na^+, K^+-ATPase using an inhibitor baffilomycin A. The mitochondrial and cytosolic samples in duplicate were added to a 96-well microplate containing baffilomycin A and the reaction was initiated by the addition of ATP and incubated for 15 min at 37^0C. The reaction was terminated by adding 8.6% TCA and the inorganic phosphate content was determined as above and expressed in µmol Pi h^{-1} mg protein^{-1}.

Determination of Ca^{2+}-ATPase-specific activity

The vanadate-dependent Ca^{2+}-ATPase activity in the brain cytosol or mitochondria was determined as described for Na^+, K^+-ATPase using an inhibitor vanadate. Samples in duplicate were added to a 96-well microplate containing either $CaCl_2$ or vanadate. The assay mixture was incubated with ATP for 15 min at 37^0C. The inorganic phosphate content released was determined as above and expressed in µmol Pi h^{-1} mg protein^{-1}.

Determination of of Mg^{2+}-ATPase-specific activity

The specific activity of oligomycin-sensitive Mg^{2+}-ATPase in brain mitochondria was estimated as described for Na^+, K^+ATPase but using an inhibitor oligomycin. Mitochondrial samples in duplicate were added to a 96-well microplate with or without oligomycin. The assay mixture was incubated with ATP for 15 min at 37^0C. The inorganic phosphate content released was measured and expressed in µmol Pi h^{-1} mg protein^{-1}.

Statistical analysis

Data were collected from six animals in each group. Statistical difference among groups was tested by means of one-way analysis of variance (ANOVA) followed by SNK comparison test. Significance between the groups was analyzed with the help of Graphpad Software (Graphpad Instat-3, San Diego) and the level of significance was accepted if $P < 0.05$.

Results

Dose-responsive effects of dietary TRP

Administration of varied doses (1, 2 and 4 mg g^{-1} feed) of TRP for seven days produced decline (P<0.001)

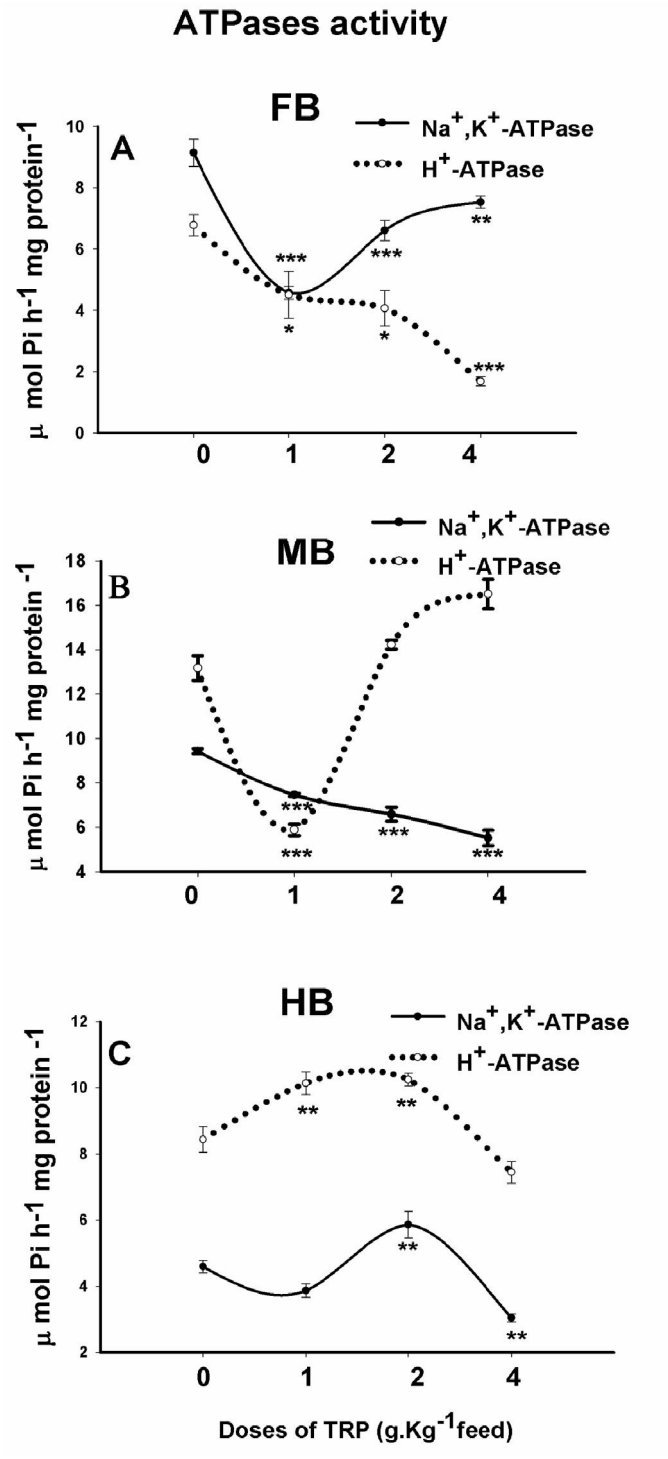

Fig. 1. Dose-responsive effects of dietary TRP on the activity of ion-dependent-ATPases in the brain regions, FB (A), MB (B), and HB (C) of climbing perch. Varied doses of TRP (1, 2 and 4 mg g^{-1}) were given to fish for seven days. Each data point represents mean ± SEM of six fish. *(*P*< 0.05), ** (*P*< 0.01), *** (*P*< 0.001) denote significance when compared to control.

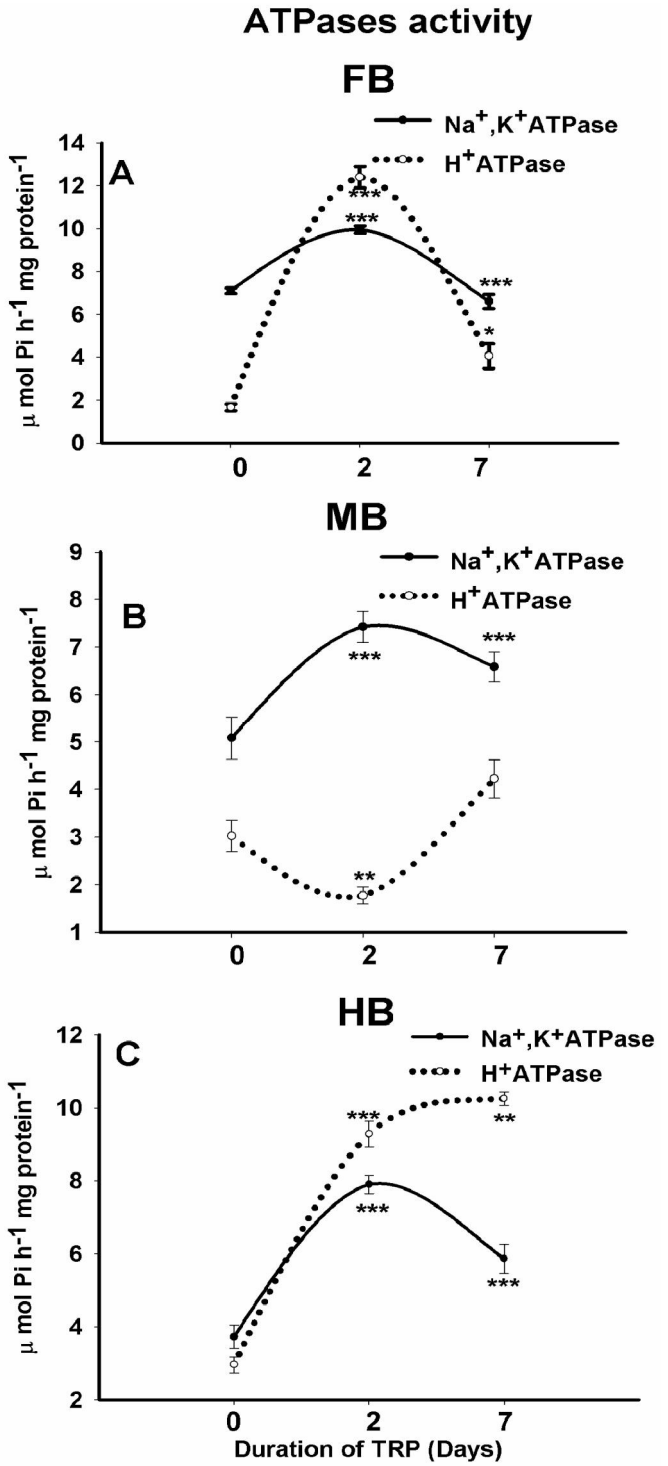

Fig. 2. Time-responsive effects of dietary TRP on the activity of ion-dependent-ATPases in the brain regions, FB (A), MB (B), and HB (C) of climbing perch. TRP (2 mg g^{-1}) was supplemented for two and seven days and sampled. Each data point represents mean ± SEM of six fish. *(*P*< 0.05), ** (*P*< 0.01), *** (*P*< 0.001) denote significance when compared to control.

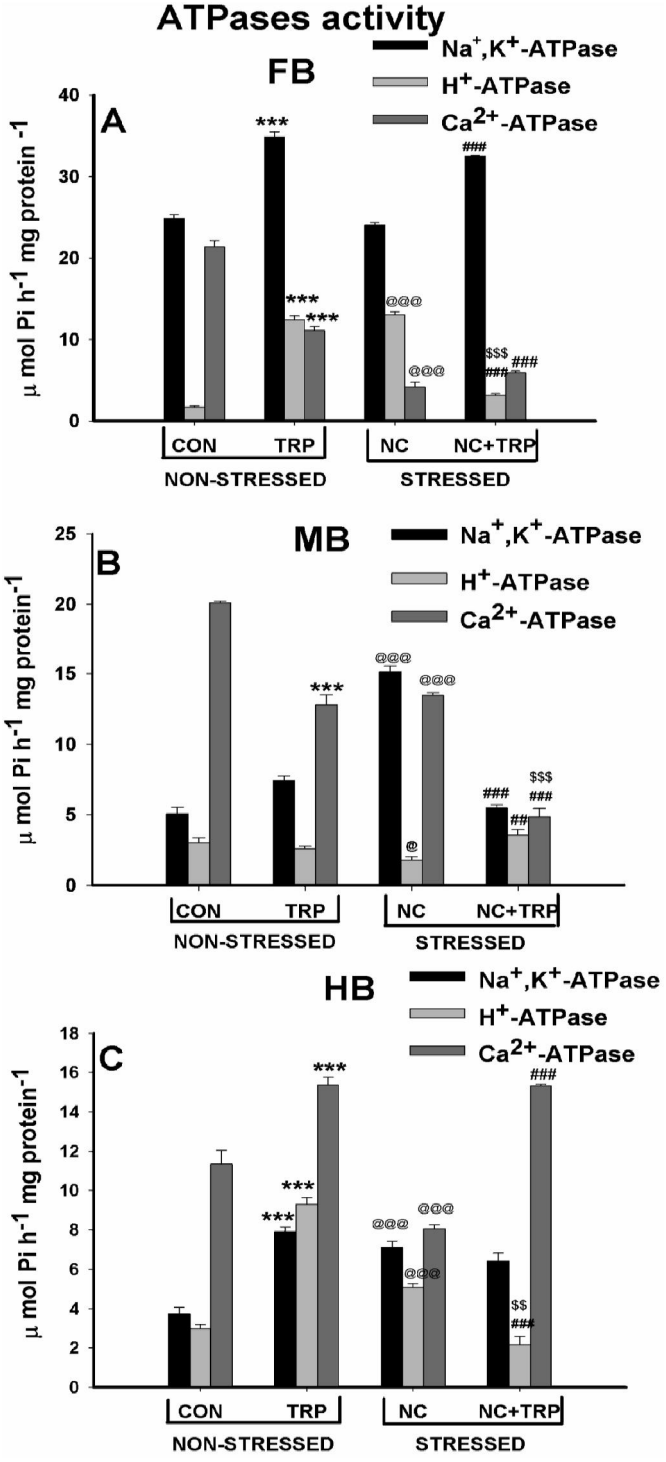

Fig.3. Effects of ion-dependent ATPases such as Na^+, K^+-ATPase, H^+-ATPase and Ca^{2+}ATPase activities in the brain regions, FB (A), MB (B), HB (C) of climbing perch either fed TRP for 2 days or kept for 1 hr in net-confinement. Each column is mean ± SEM for eight fish. *** ($P< 0.001$) denotes significant difference with non-stressed control fish. @@@ ($P < 0.001$) denotes significant difference with stressed control (NC) fish group. $$$ ($P<0.001$) denotes significant difference with stressed fish.

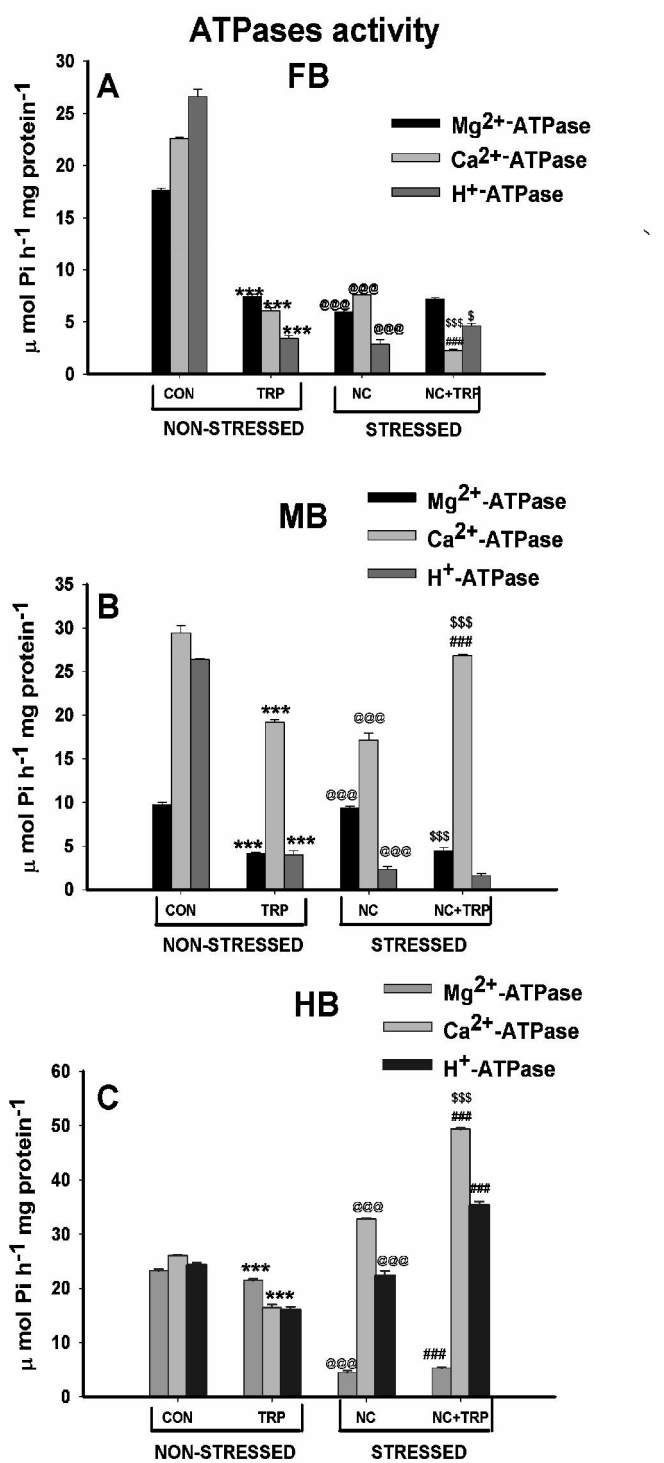

Fig.4. Effects of mitochondrial ion-dependent ATPases such as Mg^{2+}-ATPase, Ca^{2+} ATPase and H^+-ATPase activities in the brain regions, FB (A), MB (B), HB (C) of climbing perch either fed TRP for (2 days) and kept for 1 hr in net-confinement or both. Each column is mean ± SEM for eight fish. *** ($P< 0.001$) denotes significant difference from non-stressed control fish. @@@ ($P< 0.001$) denotes significant difference from stressed control (NC) fish group. $$$ ($P<0.001$) denotes significant difference from stressed fish.

in the Na^+, K^+-ATPase activity in the FB and MB (Fig. 1). A dose-responsive effect of TRP on Na^+, K^+-ATPase was found in the HB where medium dose of TRP produced a significant rise (P<0.001) but high dose produced reduction in the activity (Fig. 1). The H^+-ATPase activity in the FB decreased (P<0.001) after varied doses of TRP feeding (Fig.1). In the MB, a lower dose of TRP decreased H^{+-} ATPase activity whereas the higher doses produced no effect (Fig. 1). Significant rise (P<0.01) in the H^+-ATPase activity was found in the HB after low and medium doses of TRP feeding, whereas high dose had little effect on this H^+ transporter activity (Fig. 1).

Feeding of a selected dose (2 mg g^{-1}) of TRP for two days increased (P<0.001) the specific activity of Na^+, K^+-ATPase in FB and HB (Fig. 2). However, its activity remained unresponsive to TRP feeding in MB (Fig. 2). Likewise, treatment of TRP for two days produced substantial rise (P<0.001) in H^+-ATPase activity in FB and HB of fish, though its activity did not respond to TRP in the FB (Fig. 2).

Effects of TRP in non-stressed and stressed fish

1. Effect of TRP on Na^+, K^{+-}ATPase activity

Administration of TRP (2 mg g^{-1}) in non-stressed fish for two days produced a significant rise (P<0.001) in the Na^+, K^+-ATPase activity in FB and HB of fish (Fig. 3). A significant (P<0.001) rise in its activity was found in the FB of stressed fish supplemented with TRP (Fig. 3). The specific activity of Na^+, K^+-ATPase in the MB of fish showed a decrease (P<0.001) in the stressed fish fed TRP (Fig. 3). TRP feeding increased (P<0.001) the Na^+, K^+-ATPase activity in the HB of non-stressed fish but TRP had no effect on Na^+, K^+ -ATPase activity in this brain region of stressed fish (Fig. 3).

2. Effect of TRP on H^+-ATPase activity

Treatment of non-stressed fish with 2 mg g^{-1} TRP for two days produced substantial rise (P<0.001) in H^+-ATPase activity in the FB and HB of fish (Fig. 3). On the contrary, mitochondrial H^+-ATPase showed a reversed response in all the three regions (FB, MB and HB) of fish brain (Fig. 4). TRP treatment in stressed fish decreased (P<0.001) cytosolic H^+-ATPase activity in the FB and HB, whereas in the MB TRP feeding produced an increase (P<0.01) in its activity (Fig. 3). In the stressed fish, the mitochondrial H^+-ATPase activity increased in FB (P<0.05) and HB (P<0.001) after TRP treatment but its activity remained unresponsive in MB (Fig. 4).

3. Effect of TRP on Ca^{2+}-ATPase activity

In non-stressed fish the cytosolic (Fig. 3) and mitochondrial (Fig. 4) Ca^{2+}-ATPase activity in the FB and MB decreased (P<0.001) after two days of TRP feeding. Substantial decline (P<0.001) in the mitochondrial Ca^{2+}ATPase activity was found in the HB (Fig. 4), whereas a rise (P<0.01) in cytosolic Ca^{2+}ATPase activity occurred after TRP treatment in these fish. In stressed fish the specific activity of mitochondrial (Fig. 4) Ca^{2+}-ATPase decreased significantly (P<0.001) in the FB after TRP treatment whereas an increase in its activity occurred (P<0.001) in the HB. Feeding TRP in stressed fish produced a decrease (P<0.001) in cytosolic Ca^{2+}-ATPase activity in the HB (Fig. 3) but a significant rise (P<0.001) in mitochondrial Ca^{2+} ATPase activity occurred in this region (Fig. 4).

4. Effect of TRP on Mg^{2+}-ATPase activity

In the FB and MB of non-stressed fish, TRP feeding produced a reduction (P<0.001) in mitochondrial Mg^{2+}ATPase activity (Fig. 4). Similarly, TRP treatment in stressed fish produced significant decrease in (P<0.001) Mg^{2+}-ATPase activity in the MB without affecting its activity in other regions of the brain (Fig. 4).

Discussion

A pronounced action of dietary TRP on brain ion transporter was found in this study. The electric activity of neurons makes them one of the most energy-consuming cell types. The endless firing of action potentials requires a very high activity of Na^+, K^+-ATPase in order to maintain the neuronal ion gradients (Nilsson et al,. 2000). Na^+, K^+-ATPase represents the sodium pump activity and is responsible for the maintenance of ionic gradients across epithelial membranes (George et al., 2013) and also an index to determine the energetic cost of the brain (Erecinska and Silver, 1989). The responsiveness of brain Na^+, K^+-ATPase to different doses of TRP in our fish brain model emphasizes its role in Na^+/ K^+ transport across neuronal membrane. The reduction in Na^+, K^+-ATPase activity by the varied doses of TRP in the three regions of fish brain point to the disturbances in membrane potential and its osmotic balance. In several fish species, the requirement of dietary amino acid TRP showed varied quantitative requirement, and in rainbow trout, as in mammals, altered dietary levels of TRP resulted in alterations of plasma and brain TRP, brain serotonin, and brain 5-hydroxyindoleacetic acid (Johnston et al., 1990). In our study, supplementing 2 mg g^{-1} L-TRP feed could lead to an elevation of brain 5-HT level as we observed consistent response to this dose of TRP as evident in the specific activity of Na^+, K^+- and H^+-ATPass. This is consistent with an earlier report on the sensitivity of TRP

to Na^+, K^+-ATPase and the activation of ion pumps (Lingrel and Kuntzweiler, 1994).

Na^+, K^+-ATPase plays a key role in the active transport of monovalent cations like Na^+ and K^+ across the membrane. Na^+, K^+-ATPase is responsible for establishing the electrochemical gradient of Na^+ and K^+ across the plasma membrane, which is necessary for maintaining resting membrane potential and electrical activity of neurons for neurotransmitter uptake and osmotic balance of neuronal cells (Vornanen and Paajanen, 2006). The dose-dependent sensitivity of TRP to Na^+, K^+-ATPase and H^+ATPase activity found in all the three regions of brain indicate that dietary TRP can lower the osmotic competence of neuronal cells. This impairment of sodium pump by TRP reflects the direct action of TRP on maintaining resting membrane potential as this pump has a key role in restoring ion-gradients across plasma membranes in many electrically excitable tissues (Greger, 1996). On the other hand, feeding fish with selected dose (2 mg g^{-1}) of TRP for two days increased the specific activity of Na^+, K^+ -ATPase in FB and HB though its activity remained unresponsive in MB. Likewise, treatment of TRP for two days produced substantial increase in H^+-ATPase activity in FB and HB though its activity did not respond to TRP in the FB. These stimulatory effects of dietary TRP clearly provide evidence for short-term activation of Na-pump by TRP. It is likely that the short-term enhanced neuronal ion transporter activity and its inhibition during long-term exposure reflects a differential response to TRP. Furthermore, the inhibition of Na pump activity during the longer exposure of TRP may indicate desensitization and channel closure as suggested earlier (Hylland et al., 1997).

Similarly, H^+-ATPase activity showed a differential response to varied doses of TRP. Significant rise in the H^+-ATPase activity in the HB and its decrease in FB indicate that TRP has both specific and spatial actions on brain ion transporters. This differential pattern of H^+-ATPase activity further points to an ATP-dependent proton pump activity that establishes a proton-motive force which is vital for the acid-base equivalents in the neuronal cells.

Furthermore, the sensitivity of TRP to the activity of ion transporters in the brain points to the availability of TRP or even in the form of 5-HTP levels in all the three regions of brain. Winberg et al. (2001) have reported that dietary supplementation of TRP (8.38 mg $TRPg^{-1}$ dry feed) can elicit a response in rainbow trout. In the present study, short-term feeding of dietary TRP for two days showed maximum response than the long-term administration of seven days in our test species. Martins et al. (2013) observed a rise in serotonin metabolite in the brain of Nile tilapia after 7 days of TRP administration. Symptoms of excess tryptophan intake included reduced food intake and growth rate (Moehn et al., 2012). Our results, thus, clearly indicate dose-dependent and time-dependent differential sensitivity of TRP to Na^+, K^+-ATPase and H^+-ATPase system in the varied regions of fish brain.

As in mammals, the density of serotonergic receptors is higher in the telencephalon than in the diencephalon of fish brain (Khan et al., 1996) and also the dorsal and ventral parts of the dorsal area of the teleostean telencephalon are a putative homologue of the mammalian hippocampus (Northcutt and Davis, 1983). Inhibition of the Na^+, K^+-ATPase activity by TRP leading to disturbance of ion balance may be one of the reasons of enrichment of serotonergic soma in hypothalamus and medulla as reported earlier (Aldegunde et al., 2000).

ATPases are integral membrane proteins which exist in all cell membranes and regulate the ionic concentration inside the cells. Inhibition of ATPases that leads to decreased ATP breakdown and the reduced availability of free energy may affect several physiological processes and also result in the breakdown of active transport mechanisms needed for the absorption of ions and metabolites (Skou and Esmann, 1992).

One of the earliest physiological responses of fish to physical stress is that the neurotransmitter, serotonin, is released deep in the brain, triggering a neurological cascade that culminates several minutes later in the release of the stress hormone, cortisol, into the blood (Winberg et al., 1997; Overli et al., 2005). Animals that suffer continually high levels of stress live with high levels of cortisol all the time, but the beneficial effects of the hormone begin to fail after several days, and more sinister side effects of the hormone begin to appear (Winberg et al., 1997). A relief of the negative aspects of social stress may be obtained by dietary measures. It has been documented that dietary tryptophan (TRP) can affect brain serotonin neurotransmission and, as such, can temper abnormal and aggressive behavior and stress susceptibility (Tejpal et al., 2009; Wolkers et al., 2012). The mechanism behind this phenomenon is that an increase in brain TRP availability will enhance brain serotonin levels because TRP serves as the immediate precursor for serotonin synthesis.

TRP feeding increased the Na^+, K^+ATPase activity in the HB of non-stressed fish but it had no effect

on Na^+, K^+ATPase activity in this region of stressed fish brain. This specific antagonistic action of the Na^+, K^+-ATPase activity in these regions of brain may be due to the action of 'channel arrest' or 'channel closure' as reported earlier (Hylland et al., 1997). It is likely that the decrease in Na^+, K^+-ATPase activities could be related to the strong depression of brain electric and metabolic activities utilized as a survival strategy by this species where brain Na^+, K^+-ATPase activities may lead effectively through these physiological actions (Vornanen and Paajanen, 2006).

Recently, it became apparent that V-ATPases function not only in internal organelles but also on the plasma membrane (Nelson, 1991). It was also observed that neurotransmitter uptake into synaptic vesicles is driven by an electrochemical proton gradient generated by the vacuolar H^+-ATPase (V-ATPase) in the vesicle membrane (Wang and Floor, 1998). Treatment of non-stressed fish with 2 mg g^{-1} TRP for two days produced substantial rise in H^+-ATPase activity in the FB and HB regions of fish brain. On the contrary, mitochondrial H^+-ATPase showed a reverse response in all the three regions (FB, MB and HB). TRP treatment in stressed fish reduced cytosolic H^+-ATPase activity in the FB and HB, whereas in the MB TRP feeding produced an increase in its activity. In the stressed fish, on the contrary, the mitochondrial H^+-ATPase activity was increased in the FB and HB after the TRP treatment. This indicates a modulatory role of TRP in the brain proton pump activity during stress response in fish. ATP-dependent proton pump provides abundant energy in the form of proton-motive force while preventing over-acidification in the interior of the organelle and it acts as a driving force for catecholamine uptake (Moriyama et al., 1990). The sensitivity of mitochondrial and cytosolic H^+-ATPases activity to TRP in brain regions indicates a role of TRP in regulating the proton gradient across neuronal membranes as this pump activity may lead to intracellular acidosis and provide energy for a massive uptake of neurotransmitters (Nelson, 1991).

The inactivation of P-type pumps, like Na^+, K^+-ATPase, leads to partial membrane depolarization allowing excessive Ca^{2+} entry inside neurons with resultant excitotoxic events. In non-stressed fish, the cytosolic and mitochondrial Ca^{2+}-ATPase activity in the FB and MB decreased after the treatment of TRP feeding. Substantial decrease in the mitochondrial Ca^{2+}ATPase activity was found in the HB, whereas an increase in cytosolic Ca^{2+}ATPase activity occurred after TRP treatment in this fish. In stressed fish, the specific activity of mitochondrial Ca^{2+}-ATPase went down in the FB, while increasing its activity in the HB after TRP feeding. TRP feeding in stressed fish, on the contrary, produced a decrease in cytosolic Ca^{2+} ATPase activity but produced a rise in mitochondrial Ca^{2+} ATPase activity in HB. This clearly indicates the decisive role of Ca^{2+} transporter in fish brain and its sensitivity to TRP and stress condition. The calcium ions that enter the neuronal terminal during an action potential are removed by calcium pumps, which restore the intracellular calcium concentration to its normal low level (Kostyuk and Verkhratsky, 1994).

An increase in intracellular Na^+ in turn increases intracellular concentration of Ca^{2+} by stimulating Na^+/Ca^{2+} exchanger. The regulation of Na^+, K^+-ATPase activity and Ca^{2+}-ATPase activity by TRP may point to the Na^+/Ca^{2+} pump dynamics and the transport of these ions in the neuronal cells. Cytosolic Ca^{2+} which is responsible for receptor activation at the cell surface controls many cellular functions (Rasmussen and Barret, 1984). Under physiological resting conditions, the cytosolic Ca^{2+} is kept at low levels (around 10^{-7}M) by the Ca^{2+} homeostasis systems involved in extrusion and compartmentalization activities (Carafoli, 1987). Ca^{2+} plays an important role in the regulation of mitochondrial oxidative metabolism, and its gradient in the inner mitochondrial membrane is regulated by Ca^{2+}- ATPase (Rubin, 1970). The decrease in Ca pump activity caused by TRP in the brain cytosol and its elevated activity in mitochondria of stressed fish further point to direct control of TRP on neuronal Ca^{2+} signaling, a major target during stress response. It is likely that TRP may facilitate a higher calcium uptake by mitochondria during stress response. Furthermore, it appears that dietary TRP, which provides serotonin, may result in Ca^{2+} imbalance and that could upregulate the Ca^{2+}-ATPase activity in the neuronal cells.

In the vertebrate brain, about 50-80% of the total energy is used to maintain cellular ion gradients and the contribution of Ca^{2+} and Mg^{2+}-dependent ATPases found in neural cells is vital for maintaining brain mitochondrial function (Snelling and Nicholls, 1985). The lowered response of oligomycin-sensitive Mg^{2+}-ATPase activity to TRP, particularly in stressed fish brain, may indicate a tight regulation of Mg transport in neuronal cells. This view is consistent with the report which showed that Mg^{2+}-ATPase is not uniformly distributed and differs in respect to affinity for ATP in rat brain regions (Nedeljkovic et al., 1998). In addition, the effects of GABA-aminobutyric acid and ethanol on Mg^{2+}-ATPase from mitochondrial and microsomal fractions were studied in fish brain and it was

found that ethanol-sensitive Mg^{2+}-ATPase is located in the area of synaptic junctions and is bound to plasma, vesicular, and smooth endoplasmic reticulum membranes (Menzikov et al., 2000). Mg^{2+}-ATPase is involved in the control of passive permeability and oxidative phosphorylation, and its inhibition may result in altered energy metabolism and respiration (Torlinska and Grochowalska, 2004). The decreased mitochondrial Mg^{2+}ATPase activity in the FB and MB of non-stressed fish as caused by feeding of TRP shows an inhibitory action of Mg pump, probably related to slower metabolic demands. Similarly, the decreased Mg^{2+}ATPase activity by TRP in the MB of stressed fish supports this view. Furthermore, this decrease points to sensitivity of brain mitochondrial Mg^{2+} transport to dietary TRP and its relationship with stress response. A role of Mg in ionic homeostasis of neuronal cells particularly in association with other ion transporter activity has been reported (Matsuda, 1991). The decrease in Mg^{2+}ATPase activity also indicates a high demand for this ion-dependent ATPase activity in brain mitochondria and the subsequent demand for energy.

It appears that the magnitude of disturbance in ion homeostasis in the neuronal cells during stress could affect the TRP-induced serotonin availability which may finally modify the rate of neuronal ion transporters. Furthermore, the results also show a functional interaction of transporter activity following TRP supplementation as evident in its responses in the varied regions of stressed fish brain. This view concurs with the earlier reports on the behavioral effects of dietary TRP supplementation in several teleost fishes in which decreased aggression and attenuation of stress-induced disturbance could be seen due to the changes in 5-HT signaling (Winberg et al., 2001; Hseu et al., 2003; Hoglund et al., 2005; Wolkers et al., 2012) and in other vertebrate species (Lepage et al., 2003; Koopmans et al., 2005; Guzik et al., 2006; Tejpal et al., 2009; Leopoldo et al., 2010; Liu et al., 2013; Basic et al., 2013; Martins et al., 2013; Costas et al., 2012; Hosseini and Hosseini, 2013).

It is concluded that for a successful adaptation to stress, at least in part, fish may depend on indoleamine secretion including serotonin. The results suggest that dietary TRP can induce brain serotonergic activity which can modify the energy-transducing pumps in both cytosol and mitochondria of varied regions of fish brain. The results also point to the dose- and time-responsive actions of TRP on ion transporters and their sensitivity in fish brain.

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References

Aldegunde M, Garcia J, Soengas JL, Rozas G. (1998) Uptake of tryptophan into brain of rainbow trout (*Oncorhynchus mykiss*). *J Exp Zool.* **282**: 285-289.

Aldegunde M, Soengas JL, Rozas G. (2000) Acute effects of L-tryptophan on tryptophan hydroxylation rate in brain regions (hypothalamus and medulla) of rainbow trout (*Oncorhynchus mykiss*). *J Exp. Zool.* **286**: 131–135.

Alexander JB, Ingram GA. (1980) A comparison of five methods commonly used to measure protein concentration of fish sera. *J Fish Biol.* **16**: 115-122.

Barton BA, Morgan JD, Vijayan MM. (2002) Physiological and condition-related indicators of environmental stress in fish. In: Adams SM (Ed) *Biological Indicators of Stress in Fish*, 2nd ed. pp 111-148. Bethesda, Maryland: American Fisheries Society.

Basic D, Krogdahl A, Schjolden J, et al. (2013) Short- and long-term effects of dietary L-tryptophan supplementation on the neuroendocrine stress response in seawater-reared Atlantic salmon (*Salmo salar*). *Aquaculture* **388-391**: 8–13.

Carafoli E (1987) Intracellular calcium homeostasis. Ann Rev Biochem. **56**: 395-433.

Costas B, Aragao C, Soengas JL, et al. (2012) Effects of dietary amino acids and repeated handling on stress response and brain monoaminergic neurotransmitters in Senegalese sole (*Solea senegalensis* Kaup, 1858) juveniles. *Comp Biochem Physiol*. A **161**: 18–26.

Edwards DH, Kravitz EA. (1997) Serotonin, social status and aggression. *Curr Opin Neurobiol.* **7**: 812-819.

Erecinska M, Silver IA (1989) ATP and brain function. *J Cereb Blood-Flow Metab*. **9**: 2-19.

George N, Peter VS, Peter MCS (2013) Physiologic implications of inter-hormonal interference in fish: Lessons from the interaction of adrenaline with cortisol and thyroid hormones in climbing perch (*Anabas testudineus* Bloch) *Gen Comp Endocrinol.* **181**:122-129.

Greger R (1996) Cellular membrane transport mechanism. In: Greger WR, Windhorst U (Eds). *Comprehensive Human Physiology*. pp 149-171. Heidelberg, Berlin: Springer Verlag.

Guzik, AC, Matthews JO, Kerr BJ, et al. (2006) Dietary tryptophan effects on plasma and salivary cortisol and meat quality in pigs. *J Anim Sci.* **84**: 2251–2259.

Hoglund E, Sorensen C, Bakke MJ, et al. (2007) Attenuation of stress-induced anorexia in brown trout (*Salmo trutta*) by pre-treatment with dietary L-tryptophan. *Br J Nutr.* **97**: 786–789.

Hoglund E, Jorgensen MB, Overli O, et al. (2005) Suppression of aggressive behavior in juvenile Atlantic cod (*Gadus morhua*) by tryptophan supplementation. *Aquaculture* **249**: 525–531.

Hosseini SA, Hosseini SM. (2013) Effect of dietary tryptophan on stress response of wild common carp *Cyprinus carpio* L. *World J Fish & Marine Sci.* **5:** 49-55.

Hseu JR, Lu FI, Su HM, et al. (2003) Effect of exogenous tryptophan on cannibalism, survival and growth in juvenile grouper, *Epinephelus coioides*. *Aquaculture* **218**: 251-263.

Hylland P, Milton S, Pek M, et al. (1997) Brain Na^+/K^+-ATPase activity in two anoxia tolerant vertebrates: Crucian carp and freshwater turtle. *Neurosci Lett*. **235**:89-92.

Johnston WL, Atkinson JL, Hilton JW, Were KE. (1990) Effect of dietary tryptophan on plasma and brain tryptophan, brain serotonin, and brain 5-hydroxyindoleacetic acid in rainbow trout. *J Nutr Biochem.* **1**: 49-54.

Khan NA, Deschaux P. (1997) Role of serotonin in fish immunomodulation. *J Exp Biol.* **200**: 1833-1838.

Khan NA, Troutaud D, Moulinoux J-PH, Deschaux P. (1996) Characterisation of serotonin receptors in fish brain: modulation by polyamines. *Neurosci Res Commun*. **18**: 97-105.

Koopmans SJ, Ruis M, Dekker R. (2005) Surplus dietary tryptophan reduces plasma cortisol and noradrenaline concentrations and enhances recovery after social stress in pigs. *Physiol Behav.* **85**: 469 – 478.

Kostyuk P, Verkhratsky A. (1994) Calcium stores in neurons and glia. *Neuroscience* (Oxford) **63**: 381-404.

Lee CP, Sciamanna M, Peterson PL. (1993) Intact rat brain mitochondria from a single animal: preparation and properties. *Methods Toxicol.* **2**: 41-50.

Leopoldo QJ , Emilia TQ, Mae RC, Relicardo MC. (2010) Effects of dietary L-tryptophan on the agonistic behavior, growth and survival of juvenile mud crab *Scylla serrata*. *Aquaculture* **310**: 84–90.

Lepage O, Tottmar O, Winberg S. (2002) Elevated dietary intake of L-tryptophan counteracts the stress-induced elevation of plasma cortisol in rainbow trout (*Oncorhynchus mykiss*). *J Exp Biol.* **205**: 3679–3687.

Lepage O, Vilchez IM, Pottinger TG., Winberg S. (2003) Time-course of the effect of dietary L-tryptophan on plasma cortisol levels in rainbow trout *Oncorhynchus mykiss*. *J Exp Biol.* **206**: 3589–3599.

Lepage O, Larson ET, Mayer I, Winberg S. (2005) Tryptophan affects both gastrointestinal melatonin production and interrenal activity in stressed and non-stressed rainbow trout. *J Pineal Res.* **38**: 264–271.

Lingrel JB, Kuntzweiler T. (1994) Na^+,K^+-ATPase. *J Biol Chem.* **269**: 19659–19662.

Liu HW, Shi BM, Liu DS, Shan AS. (2013) Supplementary dietary tryptophan modifies behavior, concentrations of salivary cortisol, plasma epinephrine, norepinephrine and hypothalamic 5-hydroxytryptamine in weaning piglets. *Livestock Sci.* **151**; 213–218.

Maitra SK, Chattoraj A, Mukherjee S, Moniruzzaman M. (2012) Melatonin: a potent candidate in the regulation of fish oocyte growth and maturation. *Gen Comp Endocrinol.* **181**: 215-222.

Martins CIM, Silva PIM, Costas B, et al. (2013) The effect of tryptophan supplemented diets on brain serotonergic activity and plasma cortisol under undisturbed and stressed conditions in grouped-housed Nile tilapia *Oreochromis niloticus*. *Aquaculture* **400-401**:129-134.

Mathews GG. (2001) *Neurobiology*, Ed 2. Massachusetts: Blackwell Science, Inc.

Matsuda H. (1991) Magnesium gating of the inwardly rectifying K^+ channel. *Ann Rev Physiol.* **53**: 289-298.

Menzikov SA, Ruzhinskaya NN, Menzikova OV. (2000) Mg^{2+}-ATPase in the fish brain and its ultrastructural localization. *J Evol Biochem Physiol*. **36**: 346-352.

Moehn S, Pencharz PB, Ball RO. (2012) Lessons learned regarding symptoms of tryptophan deficiency and excess from animal requirement studies. *J Nutr.* **142**: 2231S-2235S.

Moriyama Y, Futai M. (1990) H^+-ATPase, a primary pump for accumulation of neurotransmitters, is a major constituent of brain synaptic vesicles. *Biochem Biophys Res Commun.* **173**: 443-446.

Nedeljkovic N, Nikezic G, Horvat A, et al. (1998) Properties of Mg^{2+}-ATPase rat brain synaptic plasma membranes. *Gen Physiol Biophys*. **17**: 3 – 13.

Nelson N. (1991) Structure and pharmacology of the proton-ATPases. *Trends Pharmacol Sci.* **12**: 71-75.

Nilsson GE, Routley MH, Renshaw GMC. (2000) Low mass-specific brain Na^+/K^+-ATPase activity in elasmobranch compared to teleost fishes: implications for the large brain size of elasmobranchs. *Proc Biol Sci.* 267:1335-1339.

Northcutt RG, Davis RE. (1983) Telencephalic organization in ray-finned fishes. In: Northcutt RG, Davis RE (Eds) Fish Neurobiology. 2:203-236. Ann Arbor: University of Michigan Press.

Overli O, Winberg S, Pottinger TG. (2005) Behavioral and neuroendocrine correlates of selection for stress responsiveness in rainbow trout- A review. *Integr Comp Biol.* **45**:463–474.

Peter MCS. (2013) Understanding the adaptive response in vertebrates: The phenomenon of ease and ease response during post-stress acclimation. *Gen Comp Endocrinol.* **181**: 59-64.

Peter VS, Peter MCS. (2011) The interruption of thyroid and interrenal and the inter-hormonal interference in fish: does it promote physiologic adaptation or maladaptation? *Gen Comp Endocrinol.* **174**: 249-258.

Peter MCS, Lock RAC, Wendelaar Bonga SE. (2000) Evidence for the osmoregulatory role of thyroid hormones in the Mozambique tilapia *Oreochromis mossambicus*. *Gen Comp Endocrinol.* **120**: 157–167.

Peter VS, Joshua EK, Wendelaar Bonga SE, Peter MCS. (2007) Metabolic and thyroidal response in air-breathing perch (*Anabas testudineus*) to water-borne kerosene. *Gen Comp Endocrinol.* **152**: 198–205.

Rasmussen H, Barret PQ. (1984) Calcium messenger system: an integrated view. *Physiol Rev.* **64***:* 938-984.

Rubin RP. (1970) The role of calcium in the release of neurotransmitter substances and hormones. *Pharmacol Rev.* **22**: 389-428.

Skou JC, Esmann M. (1992) The Na+/ K+ - ATPase. *J Bioenerg Biomembr*. **24**: 249-261.

Snelling R, Nicholls D. (1985) Calcium efflux and cycling across the synaptosomal plasma membrane. *J Biochem.* **226**: 225-231.

Tejpal CS, Pal AK, Sahu NP, et al. (2009) Dietary supplementation of L-tryptophan mitigates crowding stress and augments the growth in *Cirrhinus mrigala* fingerlings. *Aquaculture* **293**: 272–277.

Torlinska T, Grochowalska A. (2004) Age-related changes of Na^+, K^+, Ca^{2+}-ATPase and Mg^{2+}-ATPase activities in rat brain synaptosomes. *J Physiol Pharmacol.* **55**: 457-465.

Veauvy CM, Wang Y, Walsh PJ, Perez-Pinson MA. (2002) Comparison of the effects of ammonia on brain mitochondrial function in rats and gulf toadfish. *Am J Physiol Regul Integr Comp Physiol.* **283**:R598-R603.

Vornanen M, Paajanen V. (2006) Seasonal changes in glycogen content and Na^+/K^+-ATPase activity in the brain of crucian carp. *Am J Physiol.* **291**: R1482-R1489.

Wang Y, Floor E. (1998) Hydrogen peroxide inhibits the vacuolar H^+-ATPase in brain synaptic vesicles at micromolar concentration. *J Neurochem*. **70**: 646–652.

Winberg S, Nilsson A, Hylland P, et al. (1997) Serotonin as a regulator of hypothalamic–pituitary–interrenal activity in teleost fish. *Neurosci Lett.* **230**: 113–116.

Winberg S, Overli O, Lepage O. (2001) Suppression of aggression in rainbow trout (*Oncorhynchus mykiss*) by dietary L-tryptophan. *J Exp Biol*. **204**: 3867–3876.

Wolkers CP, Serra M, Hoshiba MA, Urbinati EC. (2012) Dietary L-tryptophan alters aggression in juvenile matrinxa *Brycon amazonicus*. *Fish Physiol Biochem*. **38**: 819–827.