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In Vivo Action of Nitric Oxide Donor Sodium Nitroprusside (SNP) on Mitochondrial Ion Transporter Function in Brain Segments of Immersion-Stressed Air-Breathing Fish (*Anabas testudineus* Bloch)

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Abstract

The neuronal circuitries of brain and the corresponding ion transporters contribute to the maintenance of ion homeostasis in fish brain. The sensitivity of these neuronal clusters in response to environmental clues brings neural plasticity and subsequent regulation of stress acclimation. Nitric oxide (NO), a gasotransmitter, is involved in ion transport in many peripheral tissues of fishes including air-breathing fish. However, the role of NO in mitochondrial ion transporter activity has not yet been investigated in fish brain. We, therefore, investigated the short-term *in vivo* action of a NO donor, Sodium Nitro-Prusside (SNP), on mitochondrial ion transporters such as H^+- Ca^{2+}- and Mg^{2+}-dependent ATPases in brain segments such as Prosen-Cephalon (PC), Mesen-Cephalon (MC) and Meten-Cephalon (MeC) of immersion-stressed *Anabas testudineus*. Intraperitoneal injection of SNP for 30 min lowered the activities of bafilomycin-sensitive H^+ATPase and vanadate sensitive Ca^{2+}ATPase in PC, whereas in MeC, these transporters showed significant rise in activities after SNP treatment. The oligomycin-sensitive Mg^{2+}ATPase activity showed a significant decrease in PC and MC of brain after SNP treatment in non-stressed fish. Induction of stress by water immersion altered the activities of these ion transporter activities. However, the treatment of SNP in immersed fish showed recovery of the immersion-induced modulation in the activities of these mitochondrial ion transporters. Our data, thus, provide evidence for a decisive role of NO in the recovery process of mitochondrial ion transporters function during immersion stress, confirming a direct differential role of NO in mitochondrial ion homeostasis in teleost brain.

Keywords: Immersion-Stress, Nitric Oxide, Sodium Nitroprusside, Ca^{2+}-ATPase, H^+-ATPase, Mg^{2+}-ATPase

1. Introduction

Teleosts respond to environmental stressors by evoking physiological responses that enable them to cope up with disturbance and maintain the homeostatic state. The stress response primarily consists of adaptive mechanisms that constitute a network of neuro-endocrine and physiological interactions[1-4]. As in all vertebrates, teleosts possess Central Nervous System (CNS), as the primary chemical centre that coordinates the body activities against the stressors. Fishes cope with stressful challenges by adapting their physiology and behavior and these adaptations are supported by neural plasticity[5,6]. Pre-

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and post-synaptic transmission, membrane trafficking, cytoskeletal remodeling, gene transcription and protein synthesis are the basic mechanisms that deliver the neural plasticity at the cellular and molecular levels[7-9]. Another important factor which is involved in the regulation of the complex processes of synaptic plasticity is mitochondria[10]. By generating energy in the form of ATP and NAD^+ and regulating Ca^{2+} homeostasis and redox homeostasis, mitochondria act as essential components in controlling fundamental processes in neuroplasticity[11-13]. Recently, Picard *et al*.[14] reviewed mitochondria as a central organelle of stress adaptation emphasizing the mode of operation of mitochondria at multiple levels within the stress response cascade. Mitochondria can also play a key role in adaptive signaling processes and stress-induced neuroendocrine mechanisms[14]. Moreover, mitochondria sense changes in energy demand associated with stress and rapidly respond to it. Mitochondria are thus considered as mediators and targets of the main stress axes.

Neuronal mitochondria provide energy for electrochemical gradient across their membranes that demand an intact ion distribution. The disturbed ion homeostasis in neurons during stress can affect synaptic potentials, since excitatory and inhibitory synaptic events are driven by ionic gradients, which demand a fine tuning of ionic balance. Active participation of ion transporters is essential for the ion homeostasis that contributes to the normal functioning of brain segments. Previous studies imply that the induction of stress by immersion altered the functions of the ion transporters during stress response and later these transporters regain its basal activity level during recovery or ease response by way of modifying the disturbed cellular and extracellular ion gradients[15].

The contribution of NO to the regulation of stress response has long been debated since NO is capable of exerting either beneficial or deleterious effects[16]. In mammals, NO has been implicated as a mediator of neuronal disruption during stress[17,18]. However, in some studies, it is shown that NO has a neuroprotective action[19,20]. Numerous studies claim that mitochondrial activity can be modulated by NO on mitochondrial ion transporter function particularly in brain is inadequate. In view of understanding the short-term *in vivo* action of NO on brain mitochondrial ion transport during immersion-stress, the pattern of mitochondrial ion-dependent transporter ATPases such as H^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase after treatment of NO donor, Sodium Nitro-Prusside (SNP) in different brain regions was quantified in immersion-stressed climbing perch.

2. Materials and Methods

2.1 Experimental Animals

Tropical freshwater air-breathing fish, commonly known as climbing perch (*Anabas testudineus* Bloch), belonging to order Perciformes and family Anabatidae, was used as the test species. This native teleost fish inhabiting the backwaters of Kerala is an obligate air-breathing fish equipped to live in demanding environmental conditions with their well-defined physiologic and biochemical mechanisms[3,24]. These fish in their post-spawning phase were collected from the wild conditions and kept in the laboratory for three weeks under natural photoperiod (12h L: 12h D) and at water temperature ranging from 28±1^{0}C with a mean pH of 6.2. Fish were fed with dry commercial fish feed at 1.5% of body mass and were transferred to 60 L glass tanks. There was no mortality during experimentation and the regulations of Animal Ethical Committee of the University were followed.

2.2 Experimental Set Up

Two experiments were carried out. The first one addressed the dose-responsive *in vivo* action of NO donor, SNP, and the second one analyzed the action of SNP in immersion-stressed fish.

2.2.1 Dose-Responsive Effects of SNP

In this experiment, laboratory acclimated fish (n=24) were held as four groups of six each and kept in separate glass tanks (60×30×30 cm). The first group, which served as the control fish, was administered intraperitoneal injections of 0.65% saline. The second, third and fourth groups were administered varied doses of SNP (2.5, 5 and 10 µg g^{-1}) (Sigma Aldrich) as intraperitoneal injections and kept for 30 min. All treatments were made between 9.00 and 11.00 a.m. and the volume of vehicle was kept as 100 µL.

2.2.2 Action of SNP in Immersion-Stressed Fish

Laboratory-acclimated freshwater climbing perch were divided into two sets and each set comprised two groups of six each. In the non-stressed set, the first group was administered 0.65% saline intraperitoneally

and served as non-stressed control while the second group was administered a selected dose of SNP (5 µg g^{-1}) intraperitoneally and kept for 30 min. In the stressed set, fish in first group were administered 0.65% saline and those in the second group received 5 µg g^{-1} SNP intraperitoneally and both these groups were then subjected to immersion stress for 30 min after injection. In this experiment, water immersion was selected as the mode of stress and was accomplished by keeping the fish under wire mesh just below the water surface that prevented them from gulping air. Feeding was stopped for 24 h prior to sampling to ensure optimum experimental conditions. Both control and treated groups were handled in the same manner.

2.3 Sampling and Analysis

After the specific time intervals, all fish were anesthetized in 0.1% 2-phenoxyethanol solution (SRL, Mumbai) and blood was collected from the caudal vessels, using a heparinized syringe. The fish were then sacrificed by spinal trans-section 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 PC, included olfactory bulbs and telencephalon; second portion, the Mesen-Cephalon (MC) included optic tectum, pituitary and hypothalamus; and the third portion, the Meten-Cephalon (MeC) comprised cerebellum, medulla and a part of the spinal cord. These three segments were blotted and kept in ice-cold Brain Mitochondrial Extraction (BME) buffer (pH 7.4) for the mitochondrial ATPase-specific activities and stored at -80^{0}C for further analysis.

2.3.1 Isolation of Mitochondria from Fish Brain

Mitochondria were isolated from the three different segments of fish brain namely PC, MC and MeC following the method as described previously[25]. Briefly, each segment of brain was kept in BME buffer containing 0.25 mM sucrose, 10 mM HEPES, 0.5 mM EDTA and 0.5 mM EGTA (pH 7.4). The brain tissues were chopped and homogenized using a glass homogenizer. The collected homogenate was first centrifuged (5430R, Eppendorf, Germany) at 2000 × g for 3 min at 4^{0}C to separate the membrane constituents from synapses. The collected supernatant was then centrifuged at 12,000 × g for 8 min at 4^{0}C. The pellets were then washed in the isolation buffer with BSA and centrifuged at 12,000 × g for 10 min at 4^{0}C and then re-suspended in 0.25 M sucrose solution and centrifuged again for 10 min at 12,000 × g. These final pellets were then re-suspended in the sucrose solution and served as the isolated mitochondrial fraction.

2.3.2 Determination of Baffilomycin-Sensitive H^+ATPase-Specific Activity

The baffilomycin-sensitive V-H^+ATPase activity was measured as described by Anupriya *et al.*[25]. The samples in duplicates were added to a 96-well microplate along with buffering medium (containing 100 mM NaCl, 0.1 mM EDTA, 5 mM MgCl and 30 mM immidazole, pH 7.4). Baffilomycin A (32 nM) was used as an inhibitor and the reaction was initiated by the addition of 0.3 mM ATP and incubated at 37^{0}C for 15 min. The liberated inorganic phosphate content was determined when the reaction was terminated with the addition of 8.6% TCA measured using a microplate reader (Synergy HT) at 700 nm and expressed in µmol Pi h^{-1} mg $protein^{-1}$. The protein content of the mitochondrial fraction of tissues was measured using modified Biuret assay with bovine serum albumin as standard.

2.3.3 Determination of Vanadate-Sensitive Ca^{2+}ATPase –Specific Activity

The vanadate-sensitive Ca^{2+}ATPase activity in mitochondrial fraction of brain regions were measured as described earlier[25]. The samples in duplicates were added to a 96-well micro-plate along with assay buffer (containing 60 mM imidazole, 0.2 mM EGTA and 75 mM KCl). About 10 mM $CaCl_2$ was used as the promoter and 2µM vanadate was used as the inhibitor and the reaction was initiated by the addition of 0.3 mM ATP. After 15 min incubation at 37^{0}C, 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 $protein^{-1}$.

2.3.4 Determination of Oligomycin-Sensitive Mg^{2+}-ATPase Activity

The oligomycin-sensitive Mg^{2+}-ATPase activity in brain mitochondria was estimated as described earlier using an inhibitor oligomycin[25]. Mitochondrial samples in duplicate were added to a 96- well microplate with or without oligomycin. The assay mixture (containing 60 mM imidazole, 10 mM $MgCl_2$, 0.2 mM EDTA and

75 mM KCl) was then incubated with 0.3 mM ATP for 15 min at 37^{0}C. The reaction was terminated by adding 8.6% TCA and the inorganic phosphate content released was determined as above and expressed in µmol Pi h^{-1} mg $protein^{-1}$.

2.4 Statistical Analysis

Data were collected from six animals in each group for the ATPase activities. Before statistical analysis, data were checked for normal distribution and variance homogeneity. 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 Graph Pad software (Instat-3, San Diego) and the level of significance was accepted if $p< 0.05$.

3. Result

3.1 Action of SNP on Baffilomycin-Sensitive H^+ATPase Specific Activity in Brain Mitochondria

In vivo administration of varied doses of SNP (2.5, 5 and 10 µg g^{-1}) for 30 min significantly decreased the baffilomycin-sensitive H^+ATPase-specific activity in PC and MC of climbing perch (Figure 1A), whereas the lower and higher doses of SNP (2.5 and 10 µg g^{-1}) produced a significant rise in the MeC (Figure 1A). Immersion stress for 30 min produced a rise in the activity of H^+ATPase in PC of *Anabas* (Figure 1B), and the activity showed a significant decline in MC and MeC (Figure 1C & D).

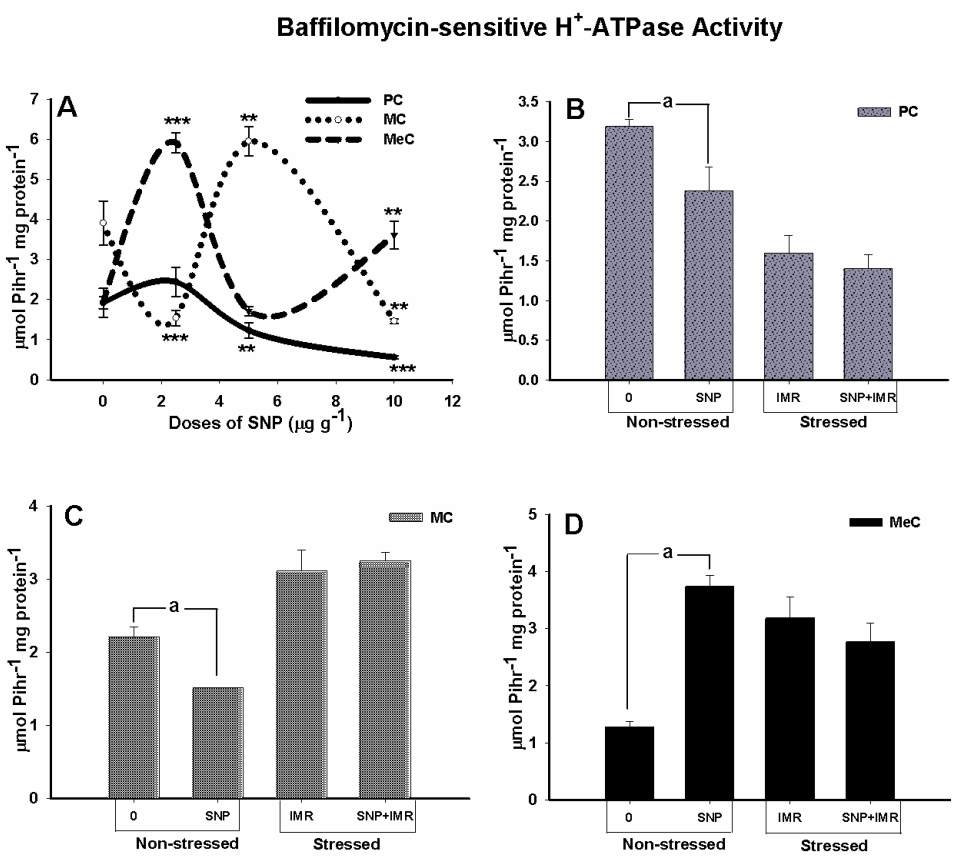

Figure 1. Dose-responsive action of sodium nitroprusside (SNP; 2.5, 5 and 10 µg g^{-1}) treatment for 30 min on bafilomycin-sensitive H^+-ATPase activity in the prosencephalon (PC), mesencephalon (MC) and metencephalon (MeC) segments of *Anabas testudineus* **(A)**. The activity pattern of H^+-ATPase in the prosencephalon (PC) is presented in "**(B)**", in mesencephalon (MC) is presented in **"(C)"** and in metencephalon (MeC) is presented in **"(D)"**. These brain segments were obtained from non-stressed and immersion-stressed fish after SNP treatment (5 µg g^{-1}). Each bar is mean ± SE for 6 fish. In figure 1A, the significant levels are represented as "**" (p<0.01) and "***" (p<0.001) when compared with control fish (0 µg g^{-1}). The significance levels of figure 1B to D are represented as "**a**" when compared between control and SNP-treated fish (SNP) and "**b**" represents significance between immersed control fish (IMR) and SNP-treated stressed (SNP+IMR) fish.

However, its activity remained unresponsive to the treatment with selected dose of SNP (5 µg g^{-1}) in stressed fish (Figure 1B, C & D).

3.2 Action of SNP on Vanadate-Sensitive Ca^{2+}ATPase Specific Activity in Brain Mitochondria

Vanadate-sensitive Ca^{2+}ATPase specific activity increased in PC and MC of *Anabas* after lower dose of SNP (2.5 µg g^{-1}), whereas in MeC, the activity decreased significantly (Figure 2A). However, higher doses of SNP (5 and 10 µg g^{-1}) increased the Ca^{2+}ATPase specific activity in MeC (Figure 2A). Immersion stress produced a significant reduction in the activity of Ca^{2+}ATPase in PC of *Anabas* brain and the SNP treatment during stress further lowered its activity (Figure 2B). In contrast, the Ca^{2+}ATPase activity in mitochondria of MC and MeC of brain did not respond to immersion stress, whereas SNP treatment in immersion-stressed fish showed a significant rise in its activity in these regions (Figure 2C & D).

3.3 Action of SNP on Oligomycin-Sensitive Mg^{2+}ATPase Activity in Brain Mitochondria

A dose-dependent decrease in the activity of oligomycin-sensitive Mg^{2+}-ATPase after SNP treatment (2.5, 5 and

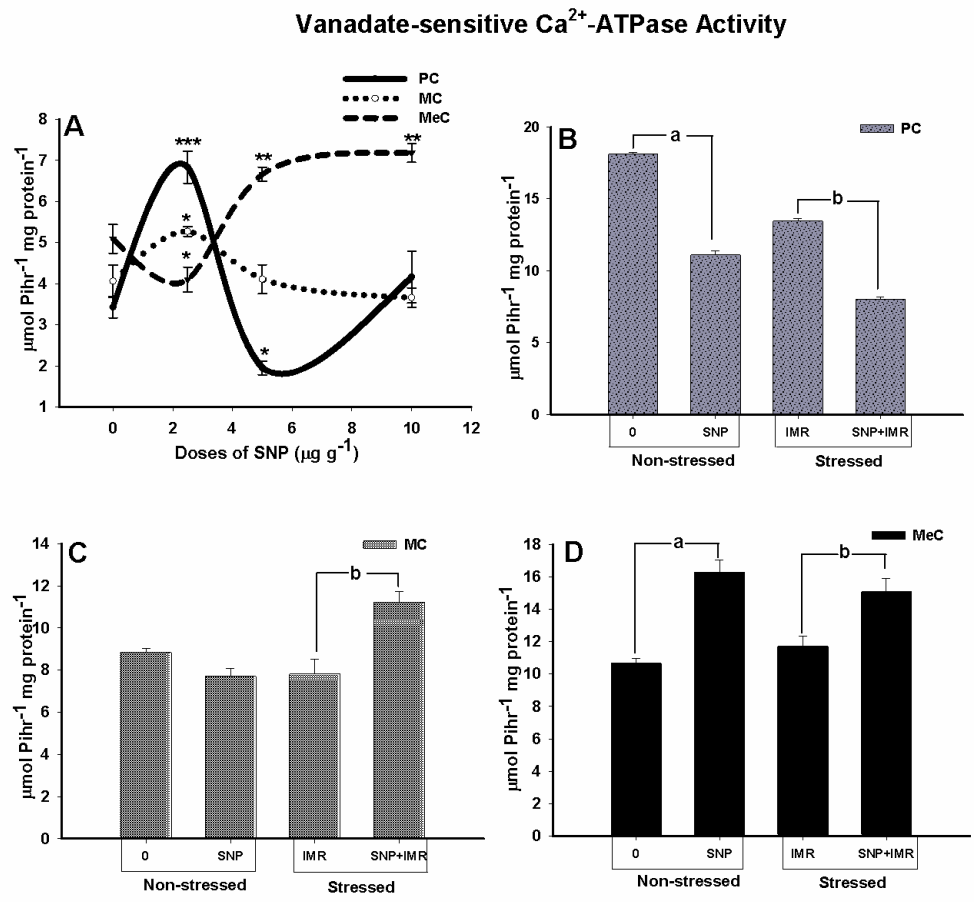

Figure 2. Dose-responsive action of sodium nitroprusside (SNP; 2.5, 5 and 10 µg g^{-1}) treatment for 30 min on vanadate-sensitive Ca^{2+}-ATPase activity in the prosencephalon (PC), mesencephalon (MC) and metencephalon (MeC) segments of *Anabas testudineus* **(A)**. The activity pattern of Ca^{2+}-ATPase in the prosencephalon (PC) is presented in **"(B)"**, in mesencephalon (MC) is presented in **"(C)"** and in metencephalon (MeC) is presented in **"(D)"**. These brain segments were obtained from non-stressed and immersion-stressed fish after SNP treatment (5 µg g^{-1}). Each bar is mean ± SE for 6 fish. In figure 2A, the significant levels are represented as "*" (p<0.05), "**" (p<0.01) and "***" (p<0.001) when compared with control fish (0 µg g^{-1}). The significance levels of figure 2B to D are represented as "**a**" when compared between control and SNP-treated fish (SNP) and "**b**" represents significance between immersed control fish (IMR) and SNP-treated stressed (SNP+IMR) fish.

10 µg g^{-1}) was found in PC and MC of brain, whereas in MeC, the activity showed a significant decrease after treatment of high dose of SNP (10 µg g^{-1}) (Figure 3A). Immersion stress increased the Mg^{2+}-ATPase activity in the mitochondria of PC, whereas its activity showed a significant decline in MC (Figure 3B & C). The administration of a selected dose of SNP (5 µg g^{-1}) during immersion stress produced a decline in its activity in PC (Figure 3B), while the activity in MC and MeC showed a significant rise (Figure 3C & D).

4. Discussion

Exposure of fish to stressors elicit many physiological response that demands interference from neuroendocrines and CNS[1,2]. Fishes cope with stressful challenges by adapting their physiology and behavior and these adaptations are supported by neural plasticity[6]. Neuromodulators have a critical role in neural plasticity particularly during stress response[26]. Since NO is a well-known neuromodulator of CNS[27], it is likely that it plays a role in stress response in

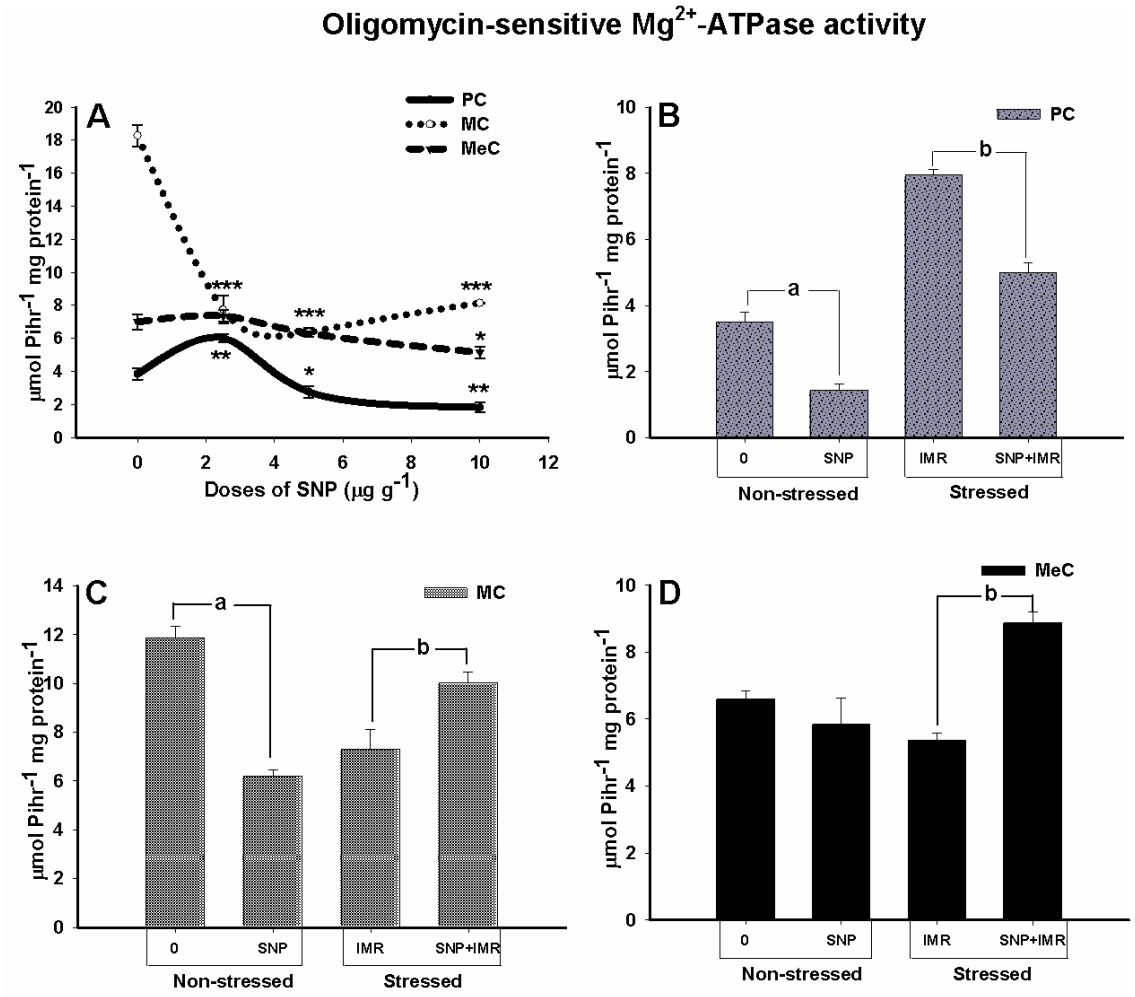

Figure 3. Dose-responsive action of sodium nitroprusside (SNP; 2.5, 5 and 10 µg g^{-1}) treatment for 30 min on oligomycin-sensitive Mg^{2+}-ATPase activity in the prosencephalon (PC), mesencephalon (MC) and metencephalon (MeC) segments of *Anabas testudineus* **(A)**. The activity pattern of Mg^{2+}-ATPase in the prosencephalon (PC) is presented in **"(B)"**, in mesencephalon (MC) is presented in **"(C)"** and in metencephalon (MeC) is presented in **"(D)"**. These brain segments were obtained from non-stressed and immersion-stressed fish after SNP treatment (5 µg g^{-1}). Each bar is mean ± SE for 6 fish. In figure 3A, the significant levels are represented as "*" (p<0.05), "**" (p<0.01) and "***" (p<0.001) when compared with control fish (0 µg g^{-1}). The significance levels of figure 3B to D are represented as "**a**" when compared between control and SNP-treated fish (SNP) and "**b**" represents significance between immersed control fish (IMR) and SNP-treated stressed (SNP+IMR) fish.

teleosts. For normal brain function, neurons must maintain differential electrical gradients across their membranes which demand an intact ion distribution. The disturbed ion homeostasis in neurons during stress response can affect synaptic potentials, since excitatory and inhibitory synaptic events are driven by ionic gradients that demand a fine-tuning of ionic balance. Active participation of ion transporters is thus essential for the ion homeostasis that contributes to the normal functioning of brain segments. In the present study, we found that SNP, as NO donor modulates the transporters of H^+, Ca^{2+} and Mg^{2+} in different neuronal clusters.

Mitochondria play a key role in overall cell physiology in response to physiological and environmental changes or stressors. NO modulates many physiological functions at the cellular level including synaptic transmission and synaptic plasticity. Brain mitochondria as a major target of NO can thus regulate many mitochondrial functions including ion transport in the neuronal tissues. In vertebrate brain, about 50-80% of the total energy is used to maintain cellular ion gradients and the contribution of Ca^{2+}ATPase in the neuronal cells is vital for maintaining brain mitochondrial function[28]. Regulation of intracellular Ca^{2+} is crucial for the proper processes of neurons ranging from electrical excitability to neurotransmitter release[29,30]. It is noted that NO donors like SNP can increase the intracellular Ca^{2+} influx in neurons[31]. The lowered response of Ca^{2+}ATPase in mitochondria of PC region to SNP and its increased activity in MeC, may indicate a tight regulation of Ca^{2+} transport in these fish brain segments. The activity of vanadate-sensitive Ca^{2+}ATPase in the PC and MeC of SNP treated fish point to NO-mediated regulation of mitochondrial ion homeostasis in fish brain segments. SNP treatment elevated its activity significantly in MC and MeC of brain in stressed fish and this further confirms a direct control of NO on neuronal Ca^{2+} signaling during stress response. During stress, the activation of N-methyl D-aspartate (NMDA) receptor subtype of the glutamate receptor leads to the subsequent release of Ca^{2+} from intracellular stores[31,32]. The decreased activity of Ca^{2+}ATPase activity in the glutamergic enriched PC of brain in our study is an indication of the tight regulation of Ca^{2+} release from the mitochondria which acts as intracellular calcium buffers[33].

Similarly, the baffilomycin-sensitive H^+-ATPase-specific activity in brain mitochondria showed a differential response to varied doses of SNP. Significant decline in the activity of H^+-ATPase in PC and MC and its rise in MeC indicate that SNP has both specific and spatial inhibitory actions H^+ transport in brain clusters of *Anabas*. In brain, stimulation of presynaptic terminals promotes the intracellular accumulation of H^+ ions and is loaded into the presynaptic neurotransmitter containing vesicles by V- H^+ATPase[34], creating an electrochemical gradient that powers vesicle uptake of neurotransmitters including glutamate, GABA and acetylcholine[35]. Previously it has been reported that NO inhibits glutamate uptake by inactivation of the V-H^+ATPase in the synaptic vesicles[36]. It is likely that an interaction of NO with synaptic vesicles occurs in these brain segments that would direct the multiple or differential action on neurotransmission. The present study, thus, points to direct involvement of NO in regulation of synaptic transmission in the brain segments of teleosts. The differential response of V-H^+ATPase in the brain regions during immersion stress further points to the modulation of ion homeostasis in these segments. H^+-ATPase provides energy in the form of proton-motive force while preventing over-acidification in the interior of the organelle and it acts as a primary pump for accumulation of neurotransmitters[37]. The data that explain the pattern of changes after SNP during stress further indicate that SNP-driven NO release could mitigate the immersion-induced H^+ ionic disturbance. The sensitivity of mitochondrial H^+-ATPase activity to SNP in varied brain regions clearly provide evidence for a role of NO in maintaining proton gradient across neuronal membranes as this activity could lead to intracellular acidosis providing energy for a massive uptake of neurotransmitters in synaptic vesicles[38].

The lowered response of oligomycin-sensitive Mg^{2+}ATPase activity in PC, MC and MeC of *Anabas* brain to varied doses of SNP may also indicate a tight regulation of Mg^{2+} transport in neuronal tissues. 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[39]. The decreased mitochondrial Mg^{2+}ATPase activity in brain regions of non-stressed fish after SNP treatment shows an inhibitory action of this pump, which is probably related to the slower metabolic demand. This observation is consistent with the previous reports in rats, where increased production of NO is associated with the decreased activity of Mg^{2+}-ATPase in brain tissues[40]. However, increased activity of Mg^{2+}-ATPase in MC and MeC of brain after SNP treatment in immersion-stressed fish clearly points to the sensitivity of brain mitochondria to stress and the subsequent reallocation of mitochondrial

ion transport. This, further, points to the regulatory role of exogenous NO in ionic homeostasis via regulating Mg^{2+} ion transport in neuronal cells of fish. A role for Mg^{2+} in ionic homeostasis of neuronal tissues particularly in association with other ion transporter activity has been reported earlier[41]. NO has been proposed as the retrograde messenger which coordinates the enhancement of both pre- and post-synaptic mechanisms[42].

Taken together, the tested ion-dependent ATPases in mitochondria of brain segments of *Anabas* clearly illustrate that NO has a direct differential action on these ion transporters in neuronal clusters that are essential for triggering synchronized activity at various regions of teleost brain. The data also point to a decisive role of NO on mitochondria during adaptation to stress as this gasotransmitter plays a direct mitochondrial ion transporter function that could contribute to the normal functioning of brain segments in this air-breathing fish.

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