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Neuronal plasticity as an evolutionary strategy: Implications for neuroprotection in response to hypoxic challenge

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1. Abstract

Phenotypic plasticity has an important place in evolution because in order to survive in a dynamically changing environment, an organism needs to have a well-adapted set of defense responses, which include pre-emptive and retaliatory phenotypic shifts. From previous research it seems that while only a few vertebrates can survive prolonged periods of hypoxia or anoxia, the greatest physiological challenge occurs when severely diminished oxygen levels are encountered

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at tropical temperatures. Until recently all of the neuroprotective strategies examined, have been in hypoxia and anoxia tolerant species that evolved their tolerance at temperatures close to freezing. The discovery of a hypoxia and anoxia tolerant reef shark, the epaulette shark (Hemiscyllium ocellatum), has provided a model in which to examine neuroprotective mechanisms that evolved at tropical temperatures. The most susceptible tissues are the heart and brain because their continued metabolic activity rapidly depletes the energy budget. Successfully hypoxia and anoxia tolerant species of fish and turtles have evolved a set of strategies to forestall cell death. These physiological strategies centre on reversibly reprogramming metabolism by reducing energy consumption and increasing glycolysis. This review will focus on the activation of retaliatory and pre-emptive neuroprotective mechanisms that are elicited in the hypoxia and anoxia tolerant tropical epaulette shark to shut down cerebella activity and conserve brain energy charge during an anoxic challenge and will examine changes in the level of the inhibitory neurotransmitter gamma-aminobutyric acid, gamma-aminobutyric acid receptors, the role adenosine and molecular chaperones in forestalling neuronal death.

2. Introduction

Asphyxia, sleep apnea, stroke and cardiovascular disease are just a few of the conditions that can result in brain damage as a consequence of reduced oxygen levels. A few minutes of oxygen deprivation is enough to cause neuronal death in the mammalian brain. Oxygen levels regulate the pattern of gene expression in health and disease via a master switch, hypoxia inducible factor 1 alpha (HIF-1 alpha) (see review [1]). While HIF-1 alpha acts as a ubiquitous transcription factor to increase cell survival during hypoxia and all organisms studied so far express HIF-1 alpha, there are only a few vertebrates that can survive prolonged periods of hypoxia or anoxia, so the ability to change the level of HIF-1 alpha expression *per se* does not automatically trigger expression of a hypoxia or anoxia tolerant phenotype. The challenge of how to protect vulnerable organs, such as the energetically expensive heart and brain, from hypoxia-induced damage has been solved several times during vertebrate evolution and is retained by some vertebrates. Tolerant species reversibly reprogram gene expression to achieve a “protected phenotype” displaying a suite of retaliatory and pre-emptive mechanisms to forestall cell death. Phenotypic plasticity is defined as the ability of a single genotype to produce a set of specialized phenotypes when exposed to different environmental conditions [2]. Garland and Kelly argue that enhanced phenotypic plasticity result from directional selection imposed by particular environments. Following the August Krough principle, it is important to

choose animal models that most closely parallel the temperature at which hypoxia occurs in humans [3].

Until recently, examination of the neuroprotective strategies used by hypoxia- and anoxia-tolerant fish and turtles were focused on northern hemisphere species that over-winter under frozen lakes and rivers. It is likely that a wider range of molecular coping strategies would be needed by hypoxia- and anoxia-tolerant animals living at high temperatures than by species that live at temperatures close to freezing because their metabolic rate is not depressed by hypothermia. At temperatures close to freezing, successfully hypoxia- and anoxia-tolerant species have evolved a set of strategies to forestall cell death. These physiological and molecular strategies centre on reversibly reprogramming metabolism by simultaneously reducing energy consumption and increasing glycolysis. Extreme fluctuations in the level of oxygen in some aquatic environments have provided the evolutionary drive for a few aquatic vertebrates to develop protective mechanisms to conserve their energy expenditure, reduce protein synthesis, increase glycolysis and prevent excitotoxic glutamate release (see review [4]). A number of specialised physiologically based molecular adaptations have evolved to prolong survival in diminished oxygen levels, [4, 5] yet only a few vertebrates can respond to a marked decline in ambient oxygen levels by rapidly and reversibly reprogramming their metabolism in a highly coordinated manner [4, 6, 7]. The crucian carp (*Carassius carassius L.*), the goldfish (*C. auratus L.*), and the freshwater turtles (*Trachemys scripta* and *Chrysemys picta*) can survive months of hypoxic or anoxic challenge at 0°C [4]. However it is difficult to disentangle the evolution of molecular strategies involved in orchestrating metabolic depression that are triggered by a hibernation-response to cold from those that occur solely in response to low oxygen. While some fish have evolved strategies to survive hypoxia in the intermittently oxygen poor water at tropical temperatures, in the Amazon [8], South Africa [9] and the Great Barrier Reef in Australia [10, 11, 12], the neuroprotective strategies used by tropical fish has only been examined in one species so far, the epaulette shark (*Hemiscyllium ocellatum*).

The discovery of a hypoxia- and anoxia-tolerant reef shark, the epaulette shark has been used as a “test model” in which to examine neuroprotective mechanisms that evolved at tropical temperatures. It is expected that the examination of neuroprotective mechanisms in tropical and temperate species will not only provide a valuable addition to the comparative physiology of hypoxia- and anoxia-tolerance but also a greater understanding of the repertoire of protective molecular strategies that can be used to change from a vulnerable to a protected phenotype. Some reef platforms on the Great Barrier Reef in Australia can be subject to extreme fluctuations in dissolved oxygen levels. Heron Island reef platform ($23^{o}27$’S, $151^{o}55$”E) is surrounded by a

fringing reef and dissolved oxygen levels range from over 150% saturation at midday to 30% saturation on some nocturnal low tides [13]. During nocturnal low tides when the water on the reef platform is cut off from the surrounding ocean water by a fringing reef and the prevailing wind conditions do not provide mixing and re-oxygenation of the surface waters, the dissolved oxygen levels can fall to 19-20% of O_2 saturation ([14], Renshaw unpublished observations). This intermittently extreme environment provides cycles of nocturnal low tides that could potentially pre-condition its inhabitants to hypoxia. The epaulette shark is a nocturnal feeder that benefits from the molecular switches, involved in protective neuronal plasticity, that confer an adaptive advantage over non-hypoxia tolerant species and allow it to successfully exploit this extreme habitat. However, the full repertoire of reversible phenotypic change that underlies this tropical vertebrate's neuronal plasticity in response to diminished oxygen levels is not yet known.

The epaulette shark has exhibited remarkable hypoxia- and anoxia-tolerance at tropical temperatures [10, 15]. In previous studies we have shown that the epaulette survives severe hypoxia, 0.39mg O_2 l^{-1} for 2 hours, without delayed neuronal apoptosis [11] and at least one hour of anoxia [15] without a deleterious decrease in brain energy charge. More recent experiments have established that epaulette sharks recover from 5-6 hours of anoxia (Chapman and Renshaw, unpublished results). The metabolic and ventilatory depression that occurs in response to hypoxia [16] and anoxia [15] may serve to match energy consumption to reduced ATP generation in the epaulette shark. Interestingly, the constituent level of hypoxia- or anoxia-tolerance can be increased further in this shark after exposure to a series of sub-lethal hypoxic or anoxic challenges (preconditioning) [15, 16].

The preconditioning phenomenon was first described in goldfish [17] and has since been reported to also increase the hypoxia tolerance of non-hypoxia tolerant vertebrates [18-20]. Evidence suggests that preconditioning elicits a protective response by affecting gene transcription as well as gene translation [7] so hypoxic or anoxic preconditioning serve as useful research tools with which to identify and examine the underlying molecular mechanisms of phenotypic plasticity. The identities and functions of novel genes involved in adaptive anoxia-tolerance in a strategic model system of normothermic hypoxia-tolerance, at tropical temperatures, could provide important new insights into the molecular mechanisms of cytoprotection in general. As exemplified by the translation of fundamental research findings from ischemic preconditioning in rodents and dogs to new therapies for humans in the IONA study [21] demonstrates that there can be a high level of homology in cytoprotective pathways. While the molecular strategies used to switch on the protected phenotype in the epaulette shark are still being examined, experiments so far, have shown that neuronal plasticity in response to hypoxia

and anoxia is mediated by the action of the retaliatory metabolite adenosine and the inhibitory neurotransmitter, GABA. There is also evidence that the molecular chaperone, heat shock protein 70 (Hsp70) responds to changes in both oxygen levels and energy levels. This review examines the evolutionary strategy of phenotypic plasticity as a means of facilitating prolonged survival in a naturally hypoxic or anoxic environment, on a tropical reef platform. Examining the neuroprotective strategies that are turned on in response to diminished oxygen at tropical temperatures allows us to identify the activation of retaliatory and pre-emptive neuroprotective mechanisms that are elicited without the concurrent trigger of hypothermia. The strategic neuroprotective advantages that evolved in an evolutionary ancient vertebrate are discussed with particular emphasis on the effect that adenosine, gamma-aminobutyric acid (GABA) and molecular chaperones could have in forestalling neuronal death. Since there is considerable genomic and proteomic homology between sharks and higher vertebrates [22], the mechanisms involved in achieving a protected phenotype in an early vertebrate may provide a deeper understanding of evolutionary processes involved in neuroprotection *per se*. Moreover these may also prove to be useful for the development of novel intervention strategies in clinical settings to minimize ischemia-reperfusion injury following stroke or to counter a variety of artificial environmental stressors associated with eutrophication and pollution.

3. The retaliatory and pre-emptive effects of adenosine receptor mediated tissue protection

Sub-lethal hypoxic preconditioning increases hypoxic and ischemic tolerance in the epaulette shark [16] and it is generally accepted that preconditioning is a polygenic response. The adenosine receptor serves as a molecular switch during preconditioning in one of the most hypoxia vulnerable tissues, the mammalian myocardium [19]. In some but not all systems, adenosine appears to be a major retaliatory molecule which pre-empts energy failure and increases natural repair systems and reduces cell damage [4]. When the oxygen supply is diminished, high energy purines such as ATP, ADP and AMP can not be re-synthesised at a rate to match their usage, with the result that increased levels of adenosine are formed from the dephosphorylation of AMP or via IMP and inosine pathways in what Lutz *et al.* [4] term the “energetically compromised brain.” The rising adenosine level can be both friend and foe. While a rise in adenosine can signal imminent destruction of tissue via necrosis or apoptosis, in vulnerable tissues such as the mammalian brain and heart, an elevated level of adenosine can act as a molecular switch to conserve energy in hypoxia- and anoxia-tolerant animals by reducing metabolic rate and neuronal activity, as

well as stimulating glycolysis to increase available energy and ultimately delaying the onset of tissue damage [4] thereby serving retaliatory functions as energy reserves are depleted. In the brain of tolerant animals, the action of adenosine on its receptor conserves neuronal energy because it clamps the resting membrane potential and inhibits transmitter release making it less likely that the neuron will respond to or generate an action potential and thereby pre-empting energy failure.

There are a number of lines of evidence that adenosine elevation has multi-system effects to pre-empt the deleterious effects of reduced oxidative phosphorylation during hypoxia or anoxia. On a cellular level, adenosine stimulates glucose uptake [23] and activates potassium-sensitive adenosine triphosphate (K_{ATP}) channels. Adenosine sensitive mitochondrial K_{ATP} channel activation protects cardiomyocytes by inhibiting the opening of the mitochondrial permeability transition pore (MPTP) at reperfusion [24], which in turn would delay onset of cardiomyocyte cell death via apoptosis. The transient opening of MPTP can trigger the onset of apoptosis via the mitochondrially released cell death signal cytochrome c while prolonged opening of the MPTP signalling mitochondrial uncoupling results in death by necrosis (see review [25]). MPTP opening can be prevented by hypoxic preconditioning prior to ischemia [26] or by hypoxic post conditioning after ischemia [27] by activating common prosurvival kinases (see review [28]). In addition, adenosine receptors are involved in increasing hypoxia- and anoxia-tolerance by triggering metabolic depression in hypoxia- and anoxia-tolerant vertebrates that evolved their tolerance at 0^{o}C (see review [4]). Furthermore, adenosine mediates neuroprotective preconditioning in the mammalian brain [29] indicating that adenosine may act as a pre-emptive molecular strategy in both tolerant and non-tolerant species. However the nature molecular cascades activated by adenosine may have diverged during evolution because most vertebrates including mammals have lost the ability to withstand prolonged hypoxia or anoxia.

We demonstrated that hypoxic preconditioning in the hypoxia- and anoxia-tolerant epaulette shark significantly lowered its resting metabolic rate and level of oxygen consumption during progressive hypoxia (Fig. 1) displaying the hallmarks of compensatory metabolic depression in response to hypoxia which would conserve lowering energy reserves and pre-empt energy failure [16]. The critical oxygen concentration at which routine metabolic rate can no longer be sustained was 2.16mg l^{-1} for control and 1.42mg l^{-1} for preconditioned epaulette sharks. While the role of adenosine in hypoxic preconditioning has not been specifically tested in this shark, it is clear that adenosine switches on potentially protective mechanisms in response to anoxia at tropical temperatures and makes an appreciable difference in conserving brain energy charge [15].

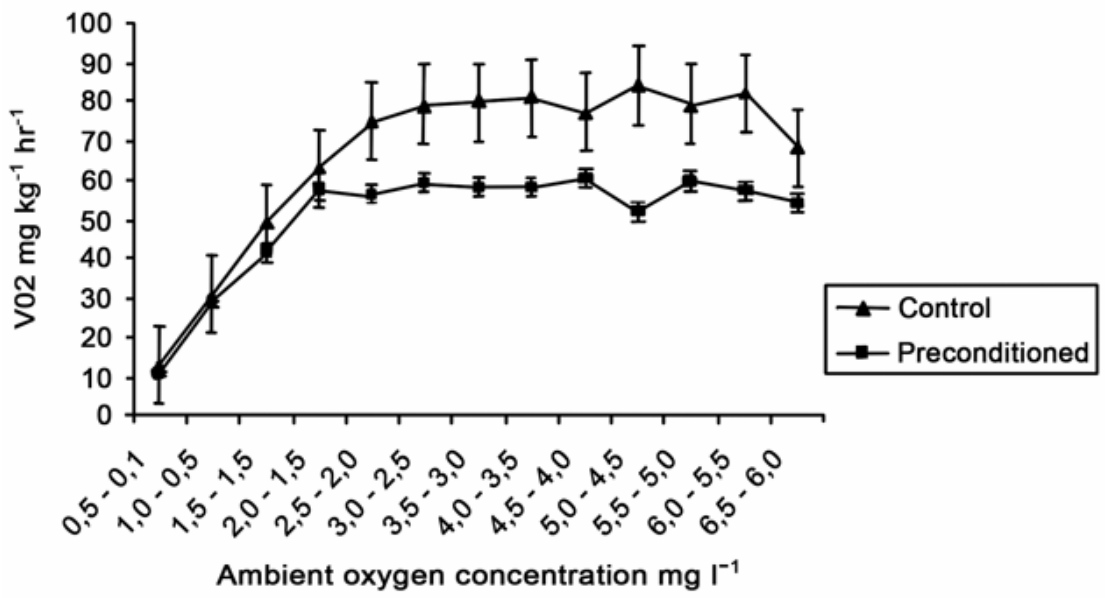

Figure 1. The mean rate of oxygen consumption is reported during progressive hypoxia for epaulette sharks (control) and for hypoxia preconditioned epaulette sharks. There was a significantly lower ($p<0.05$) resting metabolic rate and critical oxygen concentration $[O_2]_{crit}$ after preconditioning. The P_{crit} was 48.5 Torr for non-preconditioned sharks and 26.3Torr for preconditioned sharks. Adapted from [16].

4. The role of adenosine receptor activation in eliciting a protected phenotype

Aminophylline, a non-specific adenosine receptor antagonist, was used to examine the potential neuroprotective role of adenosine in response to anoxia [15]. Briefly, when epaulette sharks were exposed to an anoxic environment (<0.02mg O_2 l^{-1}) for the first time they lost their righting reflex after 46.3 ± 2.8 min (episode 1) and regained it immediately after they were returned to normoxic conditions. Sharks were pair matched for their time to loss of righting reflex in episode 1. After 24 hours, one member of each pair was injected with either saline alone (controls) or aminophylline (30mg kg^{-1}) in saline and re-exposed to anoxia for a 50-minute anoxic challenge (episode 2). In this second anoxic episode, saline treated controls sharks shut down brain areas involved in the righting reflex 56% earlier than in episode 1 (Fig. 2). While aminophylline-treated epaulette sharks took 46% longer to shut down brain activity (loss of righting reflex), this extended responsiveness was at the expense of a significant decrease in brain adenylate energy charge compared to their saline treated controls. The adenylate energy charge [30] is a ‘metabolic control parameter” which ranges from 0 when all of the adenylate is in the form of AMP to 1.0 when all of the adenylate is phosporylated, it represents metabolic potential and is calculated using the following formula:

$$EC = ([ATP] + ½ [ADP]) / ([ATP] + [ADP] + [AMP].$$

The mean brain energy charge (adenylate ratio) in the aminophylline treated sharks was 0.687 ± 0.018 s.e., which was significantly lower than the energy charge in both the saline-treated anoxic group ($p<0.05$) and the normoxic group ($p<0.001$). It was suggested that the loss of righting reflex was a mechanism associated with an energy sparing brain shut down because brain energy charge had been was prevented from acting on its receptor [15]. Since anoxia also caused a significant 3.5-fold increase in brain adenosine levels in response to 50 minutes of anoxia in both saline ($p < 0.05$) and aminophylline ($p <0.05$) treated sharks compared to their normoxic controls, it seems likely that the build up of adenosine acted as a retaliatory signal to initiate metabolic depression (including the loss of righting reflex) in the epaulette shark as it does in anoxia tolerant turtles [31]. Metabolic depression would in turn reduce ATP consumption and maintain brain ATP levels.

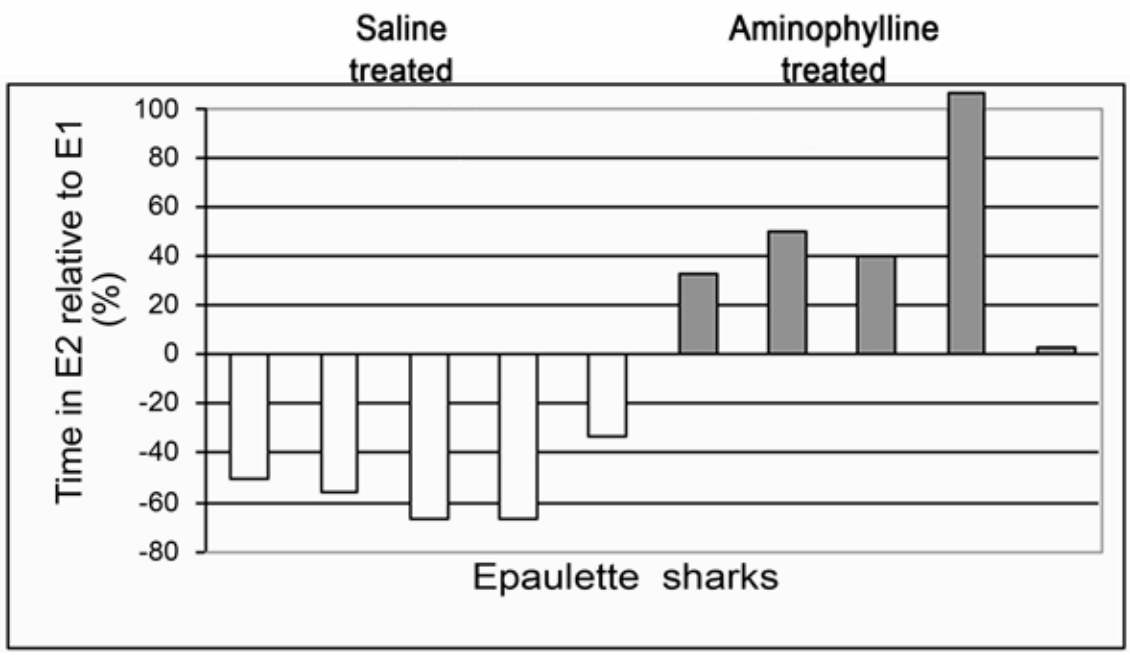

Figure 2. The percentage change in the time to loss of righting reflex in aminophylline and saline treated control epaulette sharks. Each histogram represents the percentage of time to loss of righting reflex in E2 relative to episode 1 (E1) shown as the baseline. Sharks were pair matched for their time to loss of righting reflex in E1, allowed to recover in a normoxic holding tank then 24 hours later one animal from each pair was injected with aminophylline (30mg kg^{-1} in saline) and the other with saline alone. Animals were moved to sudden anoxia 15 minutes after injection and the time to loss of right reflex was measured. Adapted from [15].

Because, neuronal metabolic demand shows a high level of plasticity (see review [32]), the ability of an animal to alter metabolic coupling is highly adaptive in an extreme environment with large fluctuations in ambient oxygen levels. Adenosine appears to be one of the retaliatory molecular triggers that rapidly and reversibly changes neuronal phenotype in response to diminished oxygen levels not only in vertebrates that over-winter in anoxic lakes [4, 31], but also in the anoxia-tolerance of a tropical reef shark [15]. In this model system of hypoxia- and anoxia-tolerance at tropical temperatures, tolerance appears to be an inducible adenosine-dependent process because brain energy charge was only conserved if adenosine could act at its receptor to cause metabolic depression, which occurred earlier in the second anoxic episode than in the first. In rats exposure to sub-lethal anoxic episodes also had an adenosine dependent preconditioning effect, which protected the rat brain from subsequent ischemic insults [33]. Continued investigation of the effects preconditioning on the initiation of molecular neuro- and cardio-protective strategies in evolutionarily ancient hypoxia- and anoxia-tolerant vertebrate that survives extreme changes in ambient oxygen is expected to have relevant implications for increasing the tolerance of tolerant mammalian and other non-tolerant species to diminished oxygen availability.

4.1 The molecular basis of adenosine in mediating tissue protection

Increased hypoxia tolerance through hypoxic preconditioning is thought to protect tissues via “survival kinase” mediated cell protection pathways [34] which can be also be triggered by the activation of G protein-coupled adenosine receptors [35]. Survival kinases are up regulated by adenosine receptor agonists and the effect of adenosine can be blocked by administration of specific adenosine receptor antagonists, however details of the signaling pathways mediating protection remain unclear [36]. Nevertheless, it is known that the effect of adenosine during preconditioning acts on A1 and A3 but not A2A adenosine receptors and that preconditioning promotes the expression of anti-apoptotic factors via MEK1/ERK1/2 pathway in neonatal rats [37]. In addition, a PI-3K dependent pathway is involved in A3-induced preconditioning [38]. Since kinase mediated cell protection is triggered by the activation of adenosine receptors via G protein-coupled mechanisms, the manipulation of adenosine receptors and/or its down stream effectors could be used to mimic preconditioning and initiate a protected phenotype. The responsiveness of the epaulette shark to adenosine reveals that the hypoxia- and/or anoxia-tolerant phenotype arose earlier in evolution than previously thought yet the gene cluster responsive to adenosine and/or preconditioning, in hypoxia- and anoxia-tolerant species, has not been identified for any tolerant species.

5. Role of the inhibitory neurotransmitter GABA in conserving energy budget

Adenosine is not the only neuroprotective mechanism promoting hypoxia- and anoxia-tolerance in the epaulette shark brain. Evidence suggests that the inhibitory neurotransmitter GABA is involved in metabolic depression in both crucian carp and turtles [39]. GABA may act in concert with adenosine to preserve energy charge during anoxia in the epaulette shark. Recent experiments in our lab, on the epaulette shark, indicate that the stimulus of hypoxic preconditioning induces a phenotypic shift involving a significant increase in both the number of GABA receptors and in the level of GABA in the cerebellum immunoreactivity compared to normoxic controls. Such strategies involving neuronal plasticity may contribute to the neuroprotection of the cerebellum, which is extremely vulnerable to hypoxic damage in most vertebrate species. The evolution of neuronal plasticity could have provided an evolutionary strategy to enable these animals to take advantage of the shelter, safety and food resources on reef platforms, which undergoes extreme fluctuations in ambient oxygen levels.

The effect of a neurotransmitter on its target synapses is dependent on receptor density and affinity. Increases in GABA receptor numbers and/or GABA release could potentially provide a powerful means of protecting neurons from a hypoxic challenge by reducing neuronal energy expenditure which would delay the onset of what has been described as the “anoxic brain catastrophe” [4]. Unfortunately in non-tolerant mammalian species both GABA receptor density and affinity are diminished by hypoxic exposure [40]. While it is widely accepted that the brains of anoxia-tolerant turtles at 0°C respond to diminished oxygen levels by increasing the number of $GABA_A$ receptors [41], as well as by increasing GABA release [42], it was not known whether vertebrates that evolved hypoxia- and anoxia-tolerance at tropical temperatures also used such a strategy.

We examined the effect of hypoxic preconditioning on both $GABA_A$ receptor number and binding affinity to determine whether altered $GABA_A$ receptor binding could be responsible for exerting a neuroprotective effect during hypoxic exposure. The cerebellum of the epaulette shark was targeted for this study because in several other species, cerebellar neurons are extremely sensitive to hypoxia-induced damage [43]. Exposure to eight 2 hour-cycles of hypoxia 12 hours apart elicited a change in neuronal phenotype that involved a 257% increase in the maximum binding capacity (B_{max}) of the $GABA_A$ agonist [^{3}H] Ro 15-1788 to membranes from experimental animals without a decrease in receptor affinity (K_D) (Table 1). Furthermore, Scatchard analysis and Hill plots confirmed that [^{3}H] Ro 15-1788 bound non-co-operatively to a single receptor population and that there was no evidence of low-affinity binding sites. We noted that $GABA_A$ receptors were ^{3}[H] flunitrazepam

Table 1. Changes in binding characteristics of [^{3}H] Ro15-1788 to $GABA_A$ subunits in membranes isolated from epaulette shark brains.

	Control	***Hypoxia Preconditioned***
B_{max}	97.10 fmol/mg protein	249.26fmol/mg protein*
K_D	$3.77 \pm 4.66 \times 10^{-3}$	$2.46 \pm 9.46 \times 10^{-3}$
Hill coefficient	0.87	0.76
Scatchard analysis	-0.26	-0.38

insensitive in the epaulette shark (Wise, Renshaw and Dodd, unpublished observations). It can be argued that the impact of a given concentration of GABA would be greater as a result of the marked increase in receptor number with unchanged receptor sensitivity. This plasticity in phenotype could increase neuronal inhibition and result in reduced neuronal energy expenditure during hypoxia when ATP production is impaired due to the progressive slowing of oxidative phosphorylation.

In the mammalian brain, $GABA_A$ receptors also play a role in mediating the effects of neuroactive steroids which bind to intracellular receptors/transcription factors regulating gene expression [44, 45] which in turn cause both rapid non-genomic changes and slow genomic changes in brain function. These interactions highlight the potential role of $GABA_A$ receptors in achieving neuroprotection (see review [45]). It is not known if $GABA_A$ receptors bind neuroactive steroids in fish or if so whether they also play a role in neuroprotection.

In a separate immunochemical study we used the hypoxic preconditioning regimen, described above, to examine changes in the level of GABA in cerebellum of the epaulette shark (Wise and Renshaw, unpublished results). Optical density analysis of GABA-like immunoreactivity (GABA-IR), of transverse sections through the cerebellum, revealed that staining intensity was $20 \pm 4\%$ higher ($p < 0.01$) in sections from experimental animals than those from controls. More detailed examination of the neuroanatomy of the cerebellum revealed that the GABA-IR could be localised (Fig. 3) to white matter axon tracts as well as terminal boutons and neurons in the granular layer.

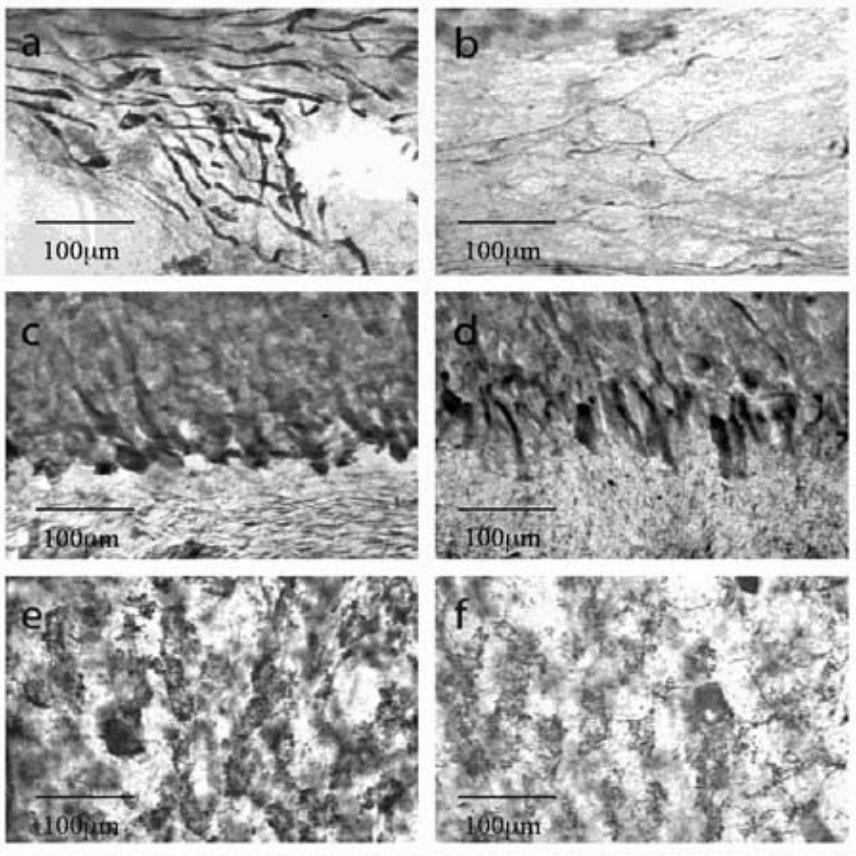

Figure 3. GABA-IR in the white matter tracts in transverse sections through the cerebellum of preconditioned (a) and control (b) epaulette sharks with greater intensity in preconditioned animals than in controls. There was no difference in the intensity of GABA-IR in the neurons of the Purkinje layer of (c) preconditioned and (d) control animals. The GABA-IR of neurons in the granular layer was greater in the preconditioned (e) than in the control (f) treated animals. The intensity of GABA was increased not only in GABA positive neuronal perikarya but also in the terminal boutons surrounding GABA negative neurons in this layer. (Wise and Renshaw, unpublished results).

Detailed examination of the optical density of each layer of the cerebellum revealed that there was a significant increase in GABA-IR in axons in the white mater layer of preconditioned animals, which was 50% greater than in the pair matched controls ($p<0.01$). The intensity of GABA-IR in the perikarya of stellate neurons in the molecular layer, was approximately 110% greater in the preconditioned animals than in the controls ($p<0.001$) and in Golgi cells and punctate terminal boutons the optical density of GABA-IR was approximately 30% greater in preconditioned than control animals ($p<0.01$) (Wise and Renshaw, unpublished results). While it is possible that elevated GABA-IR, in the cerebellum, may represent an increase in the inhibitory action of GABA in the molecular layer leading to a hypoxia-mediated reinforcement of

the refractory period of Purkinje neurons (Wise and Renshaw, unpublished results). Alternatively, it is possible that GABA may simply have accumulated because oxygen is required for its breakdown. However, if this were so there would have been a uniform elevation in GABA in all of the GABAergic nuclei examined in the other areas of the brain. Measurements of GABA-IR in the brainstem revealed a heterogeneous pattern of GABA-IR, rather than a uniform increase, (Mulvey and Renshaw, unpublished observations) that closely mirrored the heterogeneous pattern neuronal hypometabolism observed in brainstem nuclei [46]. In the brainstem, sensory nuclei such as the nucleus dorsalis maintained neuronal activity levels and the level of GABA-IR after hypoxic exposure was not different to that of controls. Conversely nuclei with significantly reduced levels of oxidative neuronal metabolism, such as the nucleus nervi vagi, had significantly increased levels of GABA-IR. Either, GABA accumulated in the hypometabolic nuclei or they had an elevated synthesis of GABA. Microdialysis and HPLC studies are needed to determine whether there is a change in the release and/or synthesis of GABA in the brainstem in response to hypoxic or anoxic challenge.

The up regulation of $GABA_A$ receptors in response to anoxia has reported in the brain of anoxia-tolerant turtles at 0°C [41, 42]. In the epaulette shark, we showed that the levels of GABA-IR rose without a compensatory increase in the level of the excitatory neurotransmitter glutamate in response to hypoxic preconditioning (Wise and Renshaw, unpublished results). However the medical interventions based on increasing GABA concentrations has had mixed levels of success. Some GABAmimetic agents such as benzodiazepines and barbiturates are not neuroprotective while the administration of GABA uptake inhibitors (see reviews [47, 48]) and the $GABA_A$ inhibitor clomethiazole were neuroprotective [47, 49]. New advances may arise from a functional analysis of GABA binding sites in hypoxia- and anoxia-tolerant species to aid effective drug screening to target neuroprotection.

In contrast, to all of the hypoxia- and anoxia-tolerant vertebrate brains studied to date, the brains of hypoxia-intolerant mammals appear to have a built in regulatory switch to prevent increased GABA inhibition because as exogenous GABA levels increase, neocortical neurons induced a compensatory reduction in $GABA_A$ receptor number [50, 51]. Recent studies have provided evidence that, in the mammalian brain, an increased GABA release may provide a second line of defense. Elevated adenosine receptor stimulation via the administration of adenosine agonists prevent the release of excitatory amino acids [52] and paradoxically GABA release is reduced by adenosine receptor activation resulting from ischemia [53] or the administration of adenosine agonists [54]. Taken together these results suggest that GABA release is prevented and that the release of excitatory amino acids is blocked instead by adenosine. One advantage of such a strategy is that a

hypoxia- or ischemia-induced depolarisation is forestalled by the natural increase in adenosine accumulating as a result of slowed oxidative phosphorylation.

In this content, hypoxic preconditioning experiments demonstrated that increased receptor binding, coupled with the maintenance of high affinity binding and increased levels of GABA in the cerebellum could contribute to neuroprotective responses to diminished oxygen levels. Increased inhibition of neuronal excitability would partially ameliorate the mismatch between energy supply and demand and increase hypoxia-tolerance. Furthermore, if increased inhibition is provided by GABAergic stellate neurons it could be expected to have a pre-emptive role in reducing cerebellar activity to conserve brain energy charge in response to prolonged anoxic exposure (Wise, Renshaw and Dodd unpublished observations). The metabolic advantage of reduced neuronal activity has been demonstrated in turtles [55] and the benefit of reduced motor activity is indicated in the preference shown by many teleost fish for quiescence during hypoxia [4, 56]. We have previously shown that hypoxic preconditioning results in heterogenous neuronal hypometabolism in the brainstem of the epaulette shark [46] and that the pattern of neuronal hypometabolism corresponds to the pattern of increased GABA-IR (Mulvey and Renshaw, unpublished observations).

The interaction between the adrenergic and GABAergic system needs to be explored to determine the extent of protective and pre-emptive actions of these two molecular cascades. Further work needs to be done to identify the molecular trigger/triggers that regulate GABA and adenosine mediated inhibition and to examine the pre-emptive effect of GABA and adenosine on conserving cerebellar energy charge and forestalling neuronal death in tolerant species so that strategies can be developed to increase the window of protection for non tolerant species exposed to hypoxia or ischemia.

6. Anoxic preconditioning elevates the level of a neuroprotective molecular chaperone - heat shock protein 70

The induction of a protected phenotype in the epaulette shark also involved the up regulation of defense systems as part of a retaliatory molecular response. On a cellular level, molecular chaperones such as heat shock proteins assist in refolding damaged proteins [57, 58] and maintaining the tertiary structure of proteins during metabolic stress [59]. Hsps are up regulated from their constitutive level in response to a diverse array of physiological stressors. These stressors include exposure to: psychoactive drugs [60, 61]; neurodegenerative disease [62]; cellular injury [63]; acute temperature change [64], hypoxia, ischemia and environmental insult, [65, 66]. The theory of parsimony predicts that a broad range of triggers probably converge on a single molecular target,

which once activated serves to up regulate Hsp production and while this remains to be fully tested, there is evidence that induction of Hsp70 is linked to both an oxygen sensor and/or an energy sensor since Hsp70 promoter activation responds to low oxygen [67], as well as decreased cellular energy [68]. Furthermore, neuroprotection conferred in response to sub-lethal ischemic preconditioning was most effective if the level of inducible Hsp70 was elevated because the level of protection could be blocked by administering an antibody to Hsp70 to neutralize its action or Quercetin to prevent its synthesis [69], revealed that the elevation of this molecular chaperone during preconditioning provides defense against a subsequent ischemic challenge.

We used the hypoxia- and anoxia-tolerant epaulette shark to determine the conditions under which Hsp70 was induced [70]. Since Hsp70 induction reflects the vulnerability of regions of the brain to insult [59], these experiments were carried out on the most hypoxia-sensitive brain region, the cerebellum. In order to assess the role of putative oxygen sensors on Hsp70 levels, the effect of varying stressor intensity was examined by using either the hypoxic or anoxic preconditioning regimens (protocols presented above). To determine whether putative energy sensors are involved in the up regulation of Hsp70, we reduced brain energy charge by administering aminophylline intra-peritoneally, 20 minutes prior to anoxic challenge. Then Hsp70 protein levels were measured semi-quantitatively, in brain homogenates, using western blotting techniques.

After hypoxic preconditioning, there was no significant difference in the level of Hsp70 in the cerebella from control and hypoxia preconditioned animals, suggesting that the intensity of the stressor was not sufficient to increase Hsp70 [70]. When exposure to anoxia was used to provide a greater level of physiological stress there was a significant increase in the level of Hsp70 in the cerebella of saline treated animals exposed to anoxia compared to

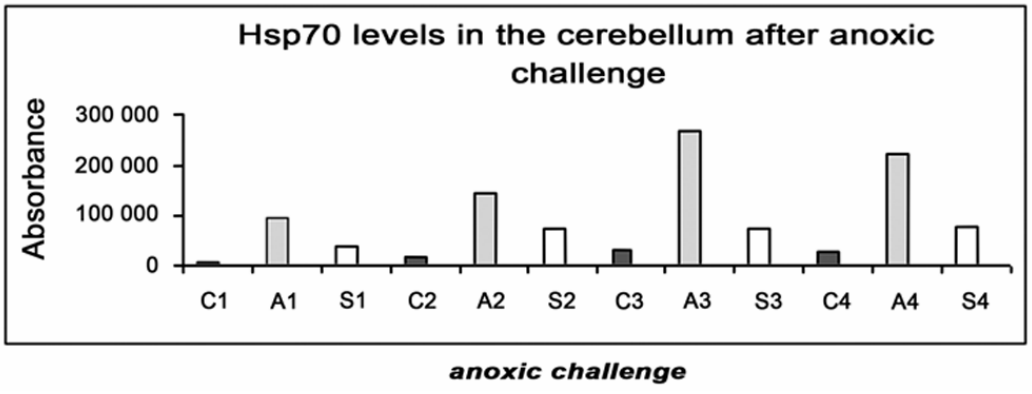

Figure 4. Changes in the level of Hsp70 in control and aminophylline treated animals after an anoxic challenge. These changes refer to the absorbance of cerebellar homogenates from control animals (C) kept in normoxia, saline treated (S) and aminophylline (A) treated animals exposed to a 50 min. anoxic challenge (Adapted from [70]).

their normoxic controls (Fig. 4). Since brain energy charge was unchanged by anoxic exposure, it was suggested that the increase in Hsp70 levels was linked to the activity of oxygen sensors alone [70]. Activation of HIF-1 alpha is known to up regulate Hsp70 expression but it is not known at present whether elasmobranchs express HIF-1 alpha.

Comparison of the cerebellar Hsp70 levels in animals pre-treated with aminophylline prior to anoxic challenge with their saline treated controls showed that a significant increase ($p<0.005$) in Hsp70 occurred. Comparison of total brain energy charge in saline and aminophylline treated animals confirmed that aminophylline treated animals had a significantly lower brain energy charge than saline treated controls ($p<0.001$). Furthermore, a 7.4 fold increase in the level of Hsp70 was significantly higher ($p<0.01$) than that elicited by anoxic preconditioning alone. Since brain energy charge was significantly lower in the aminophylline treated animals, it was suggested that a metabolic sensor may be involved in further up regulating the level of Hsp70 above the level that could be induced by anoxia alone [70]. Taken together, these experiments revealed the role of energy sensors and oxygen sensors acting synergistically to regulate Hsp70 levels.

In this case, while the precise cascade of signals involved in neuroprotective plasticity remains obscure, there is considerable support for the role of adenosine in sensing not only oxygen stress but also metabolic stress and initiating a retaliatory series of signals, which culminate in hypoxic tolerance. In hypoxia- and anoxia-tolerant teleosts that over-winter at 0°C in hypoxic or anoxic conditions, brain adenosine levels rise as ATP utilisation exceeds generation. This rise in adenosine triggers the onset of metabolic depression, which in turn conserves ATP [31, 55]. In the epaulette shark, exposure to anoxia with or without aminophylline resulted in a 3.5-fold increase in brain adenosine levels [15]. It is likely that in this species, the significant increase in adenosine acts not only as a trigger to initiate metabolic depression but also may act as a signal of metabolic stress to trigger increased Hsp70 [15]. There is evidence that a negative cellular energy balance activates the Hsp70 promoter [68] and activation of the adenosine receptor A_1 also increases Hsp70 via a Protein Kinase C dependent pathway [71]. While hypoxic preconditioning results in increased levels of Hsp70 mRNA and adenosine mediated neuroprotection via K_{ATP} channels there is also evidence that when adenosine A1 receptors are stimulated the level of Hsp mRNA is decreased [72]. These seemingly disparate actions of adenosine may reflect that there are damage and/or energy thresholds that need to be reached in order to trigger increased Hsp70 expression. Since Hsp70 expression can be up regulated by the blockade of adenosine receptors by the non-specific antagonist aminophylline, there is a need for detailed adenosine receptor studies targeted at the downstream effects of adenosine-mediated preconditioning in order to identify the receptor subtypes involved in eliciting the protected phenotype.

During metabolic stress, Hsp70 is a potent protective agent because it is able to actively prevent apoptosis [73] as well as acting as a chaperone to refold proteins that have been damaged during the metabolic stress caused by ischemia [59]. The increase in the level of Hsp70 in response to diminished oxygen and decreased brain energy charge [70] support the proposal by Lutz and Prentice that metabolic and molecular sensors, such as adenosine receptors and oxygen sensors respectively, trigger compensatory gene regulation in response to a physiological crisis [74]. It is not clear whether cell damage *per se* causes a retaliatory increase in protective molecular chaperones, which then exert a pre-emptive/inhibitory effect on the apoptotic pathway. The ability to respond to a physiological crisis with an appropriately protected phenotype could provide a driving force for successful evolutionary strategies that have enabled animals to successfully exploit extreme environments.

7. Concluding remarks

Organisms can be viewed as self-organizing systems and as such the evolution of a protected phenotype is the net effect of selecting agents on the capacity for plasticity. The protected phenotype depends on cross-talking events both between cells and within cells. One way in which to investigate the switch to a protected phenotype, developed as an evolutionary survival strategy, is to use hypoxic or anoxic preconditioning to provide the physiological stress threshold needed to elicit retaliatory and pre-emptive transitions in gene expression.

The switches responsible for the transition to a protected phenotype in a tropical hypoxia- and anoxia-tolerant animal presented here represent both pre-emptive and retaliatory strategies that involve compensatory neuronal plasticity. There is compelling evidence that elevated levels of adenosine acts on its receptor to result in the temporary loss of cerebellar responsiveness, manifested as the loss of righting reflex, which could be blocked by the administration of aminophylline. This cerebellar shut down appears to have a neuroprotective functional correlate because the administration of aminophylline not only prolonged the time to loss of righting reflex but also resulted in a significant decrease in brain energy charge [15]. Furthermore, the significantly higher levels of neuroprotective Hsp70 in aminophylline treated animals, which had significantly lower brain energy charge than controls, may indicate that increased cellular stress occurred when adenosine receptors were blocked. Taken together these results reveal that elevated adenosine associated with anoxic exposure provided a pre-emptive state of metabolic depression, which would serve to conserve ATP in this successfully hypoxia- and anoxia-tolerant tropical species.

The increase in $GABA_A$ receptors could be expected to make the cerebellum more sensitive to GABA. This in turn would enhance adenosine

induced metabolic depression and reduce neuronal energy expenditure. Collectively such strategies prolong the time to a catastrophic brain energy crisis. GABA accumulation occurred in neurons and axons of the cerebellum as part of the neuroprotected phenotype in response to hypoxic preconditioning. Elevated levels of GABA could serve to increase neuronal inhibition and/or minimize the effect of excitatory neurotransmitters in the event of an energy failure induced depolarization. Further work is needed to determine whether the accumulation of GABA is linked to its increased synthesis or its deceased release.

Due to the success that molecular biologists have identified and manipulated genes, the control of gene expression and the interactions of gene products leading to expression of unique phenotypes remain poorly understood. Bridging this genome/transcriptome-to-phenotype 'gap' is perhaps the most important challenge in molecular biology. Gene profiling of metabolic depression in the zebra fish in response to hypoxia has recently revealed the repression of genes involved in oxidative phosphyorylation [75]. Ischemia/reperfusion–induced “death signals” can be replaced by preconditioning induced “survival signals” elicited by genomic responses (see review [20]).

Hypoxic preconditioning turns on the oxygen sensitive transcription factor HIF-1 alpha that alters the expression of a suite of protective genes. Hypoxia also results in the build up of adenosine which acts as a retaliatory molecule to signal a second messenger cascade resulting in an altered cellular phenotype and this can certainly better withstand a second hypoxic insult. Recent studies, using human glioblastoma cells *in vitro* have implicated the A3 adenosine receptor subtype in increasing the level of HIF-1 alpha and one of its target genes, vascular endothelial growth factor [76]. *In vivo* preconditioning experiments are needed to clarify the relationship between A3 receptor stimulation and the increased expression of the HIF-1 alpha gene targets.

While the link between HIF-1 alpha gene targets and changes in GABA levels has not yet been investigated, it is known that HIF-1 alpha increases neuronal erthropoetin (EPO) levels and recent evidence demonstrates that EPO protected cells *in vitro* from glutamate excitotoxicity in a dose dependent manner [77]. Furthermore EPO reduced glutamate release from cultured cerebellar granule neurons [78]. It is possible that either HIF-1 alpha activation decreases the need for GABA release to counteract excitotoxicity because EPO acts to pre-empt excitotoxicity or that elevated EPO as a result of HIF-1 alpha activation augments the action of GABA. Experiments designed to turn on HIF-1 alpha using either hypoxia or chemically induced stress are needed to clarify the neuroprotective effects of HIF-1 alpha activation and the relationship of its gene targets to neuroprotective changes.

The effects of genomic, proteomic and metabolomic changes in phenotype in response to hypoxic preconditioning and the functional correlates of such changes could lead to the identification of novel regulatory genes that can be

targeted in a clinical setting. Identifying genes/paths involved in hypoxia- and anoxia-tolerance would assist in the development of improved strategies for tissue/organ preservation and storage, new intervention therapies for myocardial or cerebral ischemic disorders and improve the outcome from surgical ischemia.

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