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ADAPTATIONS OF VERTEBRATE NEURONS TO HYPOXIA AND ANOXIA: MAINTAINING CRITICAL Ca^{2+} CONCENTRATIONS

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Summary

Down-regulation of ion channel activity ('channel arrest'), which aids in preserving critical ion gradients in concert with greatly diminished energy production, is one important strategy by which anoxia-tolerant neurons adapt to O_2 shortage. Channel arrest results in the elimination of action potentials and neurotransmission and also decreases the need for ion transport, which normally requires a large energy expenditure. Important targets of this down-regulation may be channels in which activity would otherwise result in the toxic increases in intracellular [Ca^{2+}] characteristic of anoxia-sensitive mammalian neurons. In turtles, Na^+ channels and the Ca^{2+}-permeable ion channel of the *N*-methyl-D-aspartate (NMDA)-type glutamate receptor undergo down-regulation during anoxia. Inactivation of NMDA receptors during hypoxia occurs by a variety of mechanisms, including alterations in the phosphorylation state of ion channel subunits, Ca^{2+}-dependent second messenger activation, changes in Ca^{2+}-dependent polymerization/depolymerization of actin to postsynaptic receptors and activation of other G-protein-coupled receptors. Release of inhibitory neurotransmitters (e.g. γ-aminobutyrate) and neuromodulators (e.g. adenosine) into the brain extracellular fluids may play an important role in the down-regulation of these and other types of ion channels.

Key words: anoxia, hypoxia, neuron, Ca^{2+}, vertebrate, turtle, *Chrysemys picta belli*.

Introduction

Oxygen deprivation is a stress occasionally encountered to some degree by members of all vertebrate taxa, but true vertebrate anaerobes, which can tolerate anoxia for prolonged periods without damage to their organ systems, are unusual. The champion anaerobes, members of several groups of fishes and turtles, can withstand anoxia for periods more than 1000 times as long as hypoxia-sensitive species such as small mammals (Adolph, 1948; Nilsson, 1993; Ultsch and Jackson, 1982). In some vertebrates, hypoxic stress is largely confined to one particular developmental stage or period of life history (e.g. birth trauma in primates), and in other groups it is a daily experience associated with diving, burrowing or flying at extreme altitude.

In principle, neurons in the central nervous system could survive prolonged transitions from aerobic to anaerobic metabolism by using the following strategies.

Keeping the brain running. Anaerobic metabolism could increase *via* stimulation of glycolysis to provide ATP at almost the rate achieved under aerobic conditions. Critical organs (brain and heart) might adopt this strategy at the expense of others (e.g. muscle and liver), thus receiving substrates to meet their needs for heightened glycolysis, while other organs go wanting. Examples of this strategy are some anoxia-tolerant fishes (Lutz and Nilsson, 1997) which remain active in anoxic water.

Turning the brain off. Anaerobic metabolism would continue only at 'pilot light' levels, while normal neuronal functions would assume a dormant posture. An example is the brain of *Chrysemys picta belli*, the western painted turtle, which survives 5 months of anoxia during winter dormancy.

Of course, a combination of these strategies might also be adopted to serve neuronal function early and later into a bout of anoxia or dormancy. We hypothesize, however, that only the second strategy, with its attendant energy savings, would be a viable strategy for really long bouts of anoxia or dormancy. Just as the metabolic strategies for coping with oxygen deprivation are varied, hypoxia-tolerant nervous systems no doubt employ a number of different strategies to avoid injury from forced limitation of aerobic metabolism.

In contrast to our knowledge of metabolism, the study of how neurons adapt to anaerobic conditions remains in an embryonic stage. Several recent reviews concerning the adaptations of the vertebrate brain to anoxia are available (Lutz, 1992; Lutz and Nilsson, 1997; Nilsson, 1993). In this review, we will emphasize pathways and processes that appear to contribute to the second of the strategies outlined above, that of metabolic arrest coupled to ion channel arrest.

Down-regulation of energy utilization, decreased transmembrane ion fluxes through membrane ion channels and

decreased neuronal excitability are hallmarks of the responses of anoxia-tolerant neurons that apparently follow the second strategy. These processes allow the maintenance of critical ion gradients, particularly that of Ca^{2+}, despite drastically reduced rates of substrate utilization and ATP production. The processes that control ion channel activity in these neurons are almost completely unexplored and offer fertile ground for new explorations. The processes uncovered will be of value in understanding dormancy in a wide variety of organisms and will even lead eventually to improvements in the clinical care of patients with conditions such as strokes, degenerative nervous system diseases and chronic pain syndromes; they will also point to strategies for the suspended animation of organs for transplantation. Ultimately, such research will lead to the ability to create dormancy-like states for space travel and human adventures not yet imagined.

Problems for anaerobic neurons: energetics and ion gradients

For neurons exhibiting metabolic arrest strategies for surviving long-term anoxia, a critical problem is how to couple a decreased metabolic rate with membrane ion channel and ion pump activities. The maintenance of strongly hyperpolarized membrane potentials through energetically intensive membrane ion pumping is characteristic of hypoxia-sensitive vertebrate neurons, such as those found in 'typical' laboratory mammals and humans. The absence of aerobic ATP production in anoxia-sensitive neurons causes membrane depolarization and ion gradient collapse, excitatory neurotransmitter release and lethal increases in $[Ca^{2+}]_i$. Glutamate, the chief excitatory neurotransmitter in the vertebrate central nervous system (CNS), is a primary cause of Ca^{2+} influx and cell death *via* the 'glutamate cascade' (Choi, 1995). Hypoxia-tolerant neurons avoid this chain reaction (Hochachka *et al.* 1996; Lutz and Nilsson, 1997). We believe that decreases in the activity of key ion channels in hypoxia-tolerant neurons play a key role both in energy conservation and in decreasing the potential for injurious translocations of Ca^{2+} into the intracellular compartment.

Targets for energy savings: ion transport down-regulation

Hochachka (1986) proposed that a decreased permeability of membranes to ions would be a hallmark of anoxia- or cold-tolerant cells. The available evidence shows that energy production in hypoxia-tolerant animals during long-term anoxia decreases substantially and that increased anaerobic metabolism does not occur (Hochachka, 1986). At the same time, transmembrane ion gradients, which normally require large inputs of energy, remain almost unchanged. In some systems (for example, the anoxic turtle brain), the extracellular concentrations of key ions, such as Ca^{2+} and Mg^{2+}, increase by a factor of five or more during prolonged anoxia (Cserr *et al.* 1988), compounding the problems of ionic regulation. A substantial amount of energy (up to 70–80 % of total ATP turnover) is expended by ion transport pumps, with just one such pump, the Na^+/K^+-ATPase, accounting for at least 50 % of ATP turnover in mammalian cells (Else, 1991; Hochachka *et al.* 1996). The only known mechanism which can account for these facts is a large decrease in ion translocation – a tightening of the conduits through which large numbers of ions such as Ca^{2+} regularly flow. These conduits are undoubtedly protein ion channels; non-specific leaks through the lipid bilayer are unlikely to account for more than a small percentage of net ion translocation and, furthermore, would probably not be subject to the rapid down-regulation characteristic of ion transport in the anoxia-tolerant cells studied thus far. Such a functional constriction of ion flow through ion channels could be initiated by a number of processes, all leading to effectively decreased neurotransmission, reduced number of action potentials ('spiking'), decreased size of slow postsynaptic potentials, as well as suppression of miniature endplate potentials, etc. Some effects of decreased ion translocation through several important classes of ion channels and receptors are summarized in Table 1. While cessation of action potentials ('spike arrest') may alone account for a significant fraction of the decrease in net ion translocation, one would predict suppression of many types of ion translocation processes in order to achieve truly substantial energy savings during prolonged periods of anoxic dormancy. Furthermore, stabilization of some ionic gradients (e.g. that for Ca^{2+}) may be needed to prevent disordered intracellular signaling and induction of programmed cell death (apoptosis) during dormancy.

The net effects of the decreases in ion channel conductance (summarized in Table 1) on global neuronal function include a stable membrane potential, decreased excitability, decreased

Table 1. *Effects of ion channel inactivation on critical elements of neuronal signaling*

Na^+ channel inactivation
Elevated action potential threshold
Decreased nerve conduction and velocity
Decreased Na^+ leak, decreased likelihood of Na^+ gradient collapse
Stabilized Na^+-gradient-dependent transporters (glucose, Ca^{2+}, neurotransmitters, H^+)
K^+ channel inactivation
Altered action potential shape
Altered excitability
Glutamate receptor inactivation
Decreased excitatory neurotransmission, saving energy
Stabilized $[Ca^{2+}]_i$
Voltage-gated Ca^{2+} channel inactivation
Decreased excitability
Decreased neurotransmitter release
Decreased $[Ca^{2+}]_i$ accumulation
Acetylcholine receptor inactivation
Decreased excitatory neurotransmission
Decreased Ca^{2+} influx

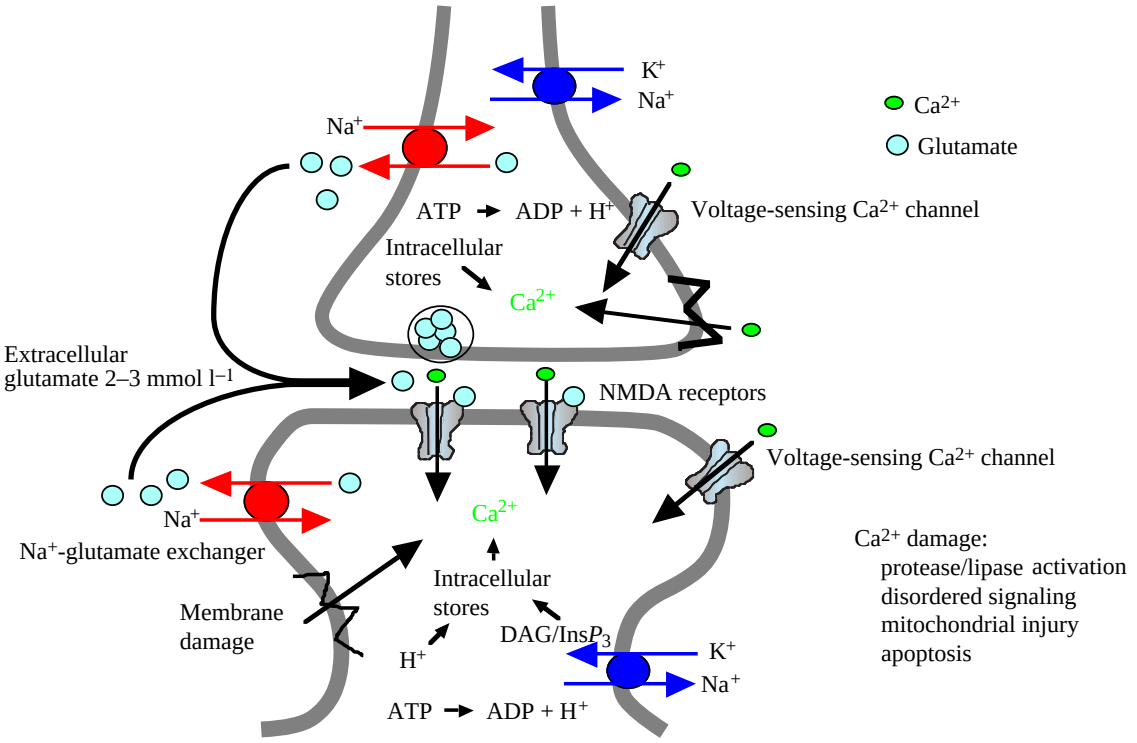

Fig. 1. The role of Ca^{2+} influx and the accumulation of intracellular Ca^{2+} in hypoxic/ischemic neuronal death. Processes are shown both for a presynaptic nerve ending (upper) and for a postsynaptic dendritic spine (lower). During energetic stress, cell membranes depolarize, triggering Ca^{2+} influx through voltage-sensitive Ca^{2+} channels. Depolarization results in Na^+ influx and the reversal of Na^+-gradient-dependent neurotransmitter re-uptake transporters, flooding the extracellular space with excitatory neurotransmitters such as glutamate. Vesicular release of glutamate does not make an important contribution to postsynaptic Ca^{2+} influx since vesicular release fails early on in hypoxia as a result of the loss of ATP. Glutamate triggers Ca^{2+} influx *via* *N*-methyl-D-aspartate (NMDA) receptors. Other contributions to the increase in intracellular $[Ca^{2+}]$ include membrane damage due to free-radical generation, acidification resulting from anaerobic metabolism and Ca^{2+}-triggered Ca^{2+} release from intracellular stores in organelles. DAG, diacyl glycerol; InsP_3, inositol trisphosphate.

neurotransmission and a decreased propensity for ion gradients or fluxes to become disordered during energetic compromise.

One of the key targets for protective inactivation of ion channel function during anoxia or dormancy is likely to be the stabilization of the various avenues responsible for potentially uncontrollable Ca^{2+} influx and accumulation. As will be mentioned in the following sections, Ca^{2+} is recognized as playing a critical role in inducing cell injury and death during hypoxia or ischemia and as a target for ion channel inactivation in hypoxia-tolerant neurons.

Ca^{2+} as the cause of hypoxia-induced cell death and as a target for protective regulation in hypoxia-tolerant neurons

Ca^{2+} is a double-edged sword. In its role as an intracellular second messenger, it regulates the excitability of neurons and controls the formation and modification of synapses. When energy is scarce, it can also set in motion a cascade of events triggering cell death (Choi, 1992; Siesjö, 1992; Szatkowski and Attwell, 1994). Some of the ways in which Ca^{2+} enters and injures neurons during hypoxia or ischemia are shown in Fig. 1. Key points shown include Ca^{2+} influx *via* voltage-gated Ca^{2+} channels, the collapse of Na^+ gradients and subsequent reversal of Na^+/H^+-gradient-dependent neurotransmitter re-uptake transporters, and flooding of the extracellular space with neurotransmitters such as glutamate, acetylcholine and dopamine. Hypoxia-sensitive neurons depolarize rapidly because they cannot generate sufficient ATP to make up for the loss of aerobic metabolism (Hansen, 1985), i.e. there is a mismatch between ATP production and demand. Depolarization directly increases intracellular $[Ca^{2+}]$ *via* the voltage-dependent activation of a number of different types of voltage-gated Ca^{2+} channels. Ca^{2+} can also enter as a result of events initially unrelated to Ca^{2+}-permeable channels *per se* (e.g. as a result of the collapse of the Na^+ gradient and the reversal of the Na^+/Ca^{2+} exchanger). Finally, the $[Ca^{2+}]$ increase in the cytosol can derive from the liberation of Ca^{2+} from intracellular stores (e.g. Ca^{2+}-stimulated release from the endoplasmic reticulum). Elevated intracellular free $[Ca^{2+}]$ causes disordered activation of protein kinases, phosphatases, lipases and proteases, all of which damage cellular constituents and lead to necrosis or apoptosis (Choi, 1995).

A priori, we might expect that hypoxia-tolerant neurons would be able to avoid some of the processes just mentioned, including the loss of neurotransmitter homeostasis (stabilizing

Na^+ gradients), depolarization and Ca^{2+} channel activation, and inactivation of other receptors and ion channels contributing to Ca^{2+} influx (e.g. the Ca^{2+}-permeable glutamate receptors and acetylcholine receptors). We will next review some of the specifics known about these possibilities.

Neuronal ion channel regulation during anoxia

Na^+ channels

Current flowing through Na^+ channels constitutes a major component of action potentials ('spikes'). Spike arrest has been proposed as an important mechanism in the adaptation of neurons to hypoxia by Sick, Rosenthal, Lutz and others (Sick *et al.* 1993) because it would decrease the energetic costs of neurotransmission and nerve conduction. The arrest of Na^+ channels would favor the maintenance of transcellular Na^+ gradients, which are crucial for preventing cellular swelling, the failure of neurotransmitter re-uptake and Ca^{2+} influx (Table 1).

Several differences in Na^+ channel abundance between anoxia-sensitive and anoxia-insensitive animals (rats *versus* turtles) have been found and could be related to the phenomenon of spike arrest. The density of Na^+ channels in turtles is lower than in mammals (Edwards *et al.* 1989), in synaptosomes and in several fractions of brain homogenate (Xia and Haddad, 1991), and is perhaps related to the tenfold lower metabolic rate of reptiles compared with mammals (Else, 1991). Na^+ channels further decrease in density during anoxia in turtles. The most dramatic change with anoxia is a 42 % decrease in density of Na^+ channels in cerebellum (Perez-Pinzon *et al*. 1992). It is not clear whether this difference (based solely on the numbers of binding sites in brain homogenates) is alone enough to account for the 100-fold reduction in metabolic rate in turtle brain, even if it leads to complete 'spike arrest'. Whether or not the open probability of individual Na^+ channels is regulated during anoxia needs study, because channel numbers cannot be used to infer channel activity or channel currents, and Na^+ channels, like all channels studied thus far, are subject to a large number of allosteric controls involving phosphorylation, etc. Unfortunately, to our knowledge, no direct measurements of Na^+ channel activity during anoxia in anoxia-tolerant neurons have been made. This is required, since determining the numbers of channels through binding studies gives no useful information on channel activity *per se*.

Na^+ channel cycling from an active membrane-bound form into one in the cytosol is a form of regulation that could be involved in anoxia-induced channel inactivation. The movement of Na^+ channels from the cell membrane to the cytoplasm and back again has been demonstrated in some systems. Both protein kinase A and protein kinase C phosphorylation modulate the activity of neuronal Na^+ channels (Frohnweiser *et al*. 1995; Murphy *et al.* 1993), and these phosphorylation/dephosphorylation events could either directly influence receptor activity (open probability) or label receptors for internalization or for persistence in an active role in the membrane. As a variety of other channels are anchored to the cytoskeleton, it is conceivable that, during anoxia, Ca^{2+}-dependent events (e.g. depolymerization of channels to actin in the cytoskeleton) remove the Na^+ channels from the membrane or inactivate them *in situ*. Much exciting work on these possibilities seems inevitable in the near future.

K^+ channels

K^+ channels are diverse in function and structure, and many types are still being defined genetically and electrophysiologically. Increased activity of K^+ channels brings cell membrane potential closer to the K^+ equilibrium potential, which is hyperpolarized from the normal resting potential. Hyperpolarization makes neurons less excitable in the sense that they are farther from the action potential threshold, but the hyperpolarization comes at the cost of increased gradients for Na^+ and Ca^{2+}, requiring greater tonic energy expenditure to expel these ions. It is difficult to see, therefore, that K^+ channel activation would form a reasonable strategy for saving energy in an energy-scarce situation. K^+ channels also have other functions, of course, such as shaping the upstroke and duration of action potential spikes, because they are active at depolarized potentials and will tend to pull the membrane potential back towards its resting level.

Measurements of K^+ channel activity during anoxia have also only been made indirectly. The efflux of K^+ from neurons has been used as an index of K^+ channel activity in intact turtle brain or isolated cerebellum (Chih *et al*. 1989). Measurements of K^+ accumulation in extracellular fluid (ECF) have been made when metabolic production of ATP is blocked by anoxia and an inhibitor of glycolysis (usually iodoacetate). Measurements of K^+ accumulation in the extracellular fluid made using this technique depend upon a number of factors, including the activation of multiple types of ion channels and, of course, on rates of ATP depletion. These problems aside, reduced efflux of K^+ has been seen in anoxia-adapted turtle cerebellum and cortex and has been interpreted as evidence of channel arrest (Fig. 2). The efflux of K^+ is depressed by extracellular adenosine, a 'retaliatory' adenylate metabolite accumulated in turtle ECF during anoxia (Pek and Lutz, 1997; Perez-Pinzon *et al.* 1993).

One specific group of K^+ channels, those sensitive to ATP (K_{ATP} channels), is involved in the *initial* hyperpolarization (i.e. before the cells depolarize and die) seen in anoxic mammalian neurons. These channels could play a role in stabilizing membrane potential in anoxia-tolerant cells. However, glibenclamide, an antagonist of mammalian K_{ATP} channels, fails to alter K^+ release from turtle brain (Jiang *et al*. 1992) or carp brain during anoxia or during depolarization with ouabain (Johansson and Nilsson, 1995). Much work remains to be done to define the roles of K^+ channels in the adaptations of neurons to hypoxic conditions, including the use of more specific tools for measurement (e.g. patch-clamp studies or whole-cell K^+ current measurements).

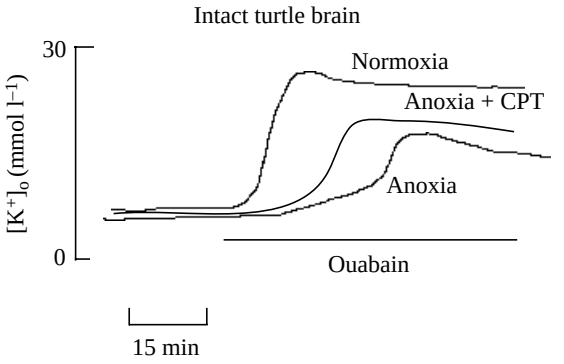

Fig. 2. Evidence for ion channel arrest in intact turtle brain. Extracellular accumulation of K^+ was measured (using an ion-sensitive microelectrode) before and after superfusion of the brain with 20 mmol l^{-1} ouabain in normoxic animals and those treated with prior anoxia or with an adenosine receptor antagonist (cyclopentyl theophylline, CPT). Delayed release of K^+ and partial reversal of the delay by CPT is evidence for ion channel arrest. Modified from Pek and Lutz (1997).

Regulation of intracellular [Ca^{2+}] and glutamate receptor ion channels

Glutamate receptors are responsible both for the bulk of excitatory neurotransmission in the vertebrate brain and for transducing lethal Ca^{2+} fluxes during anoxia. Their regulation in anoxia-tolerant neurons is therefore a relevant target for study.

In hypoxia-sensitive brains (e.g. rats), intracellular free [Ca^{2+}] increases to lethal levels after only minutes of anoxia, and this increase is mediated in significant part by glutamate receptors (Bickler and Hansen, 1994; Lipton and Loebner, 1990). In contrast, [Ca^{2+}] remains relatively stable in turtle cerebrocortical neurons (Bickler, 1992) (Fig. 3). In turtle

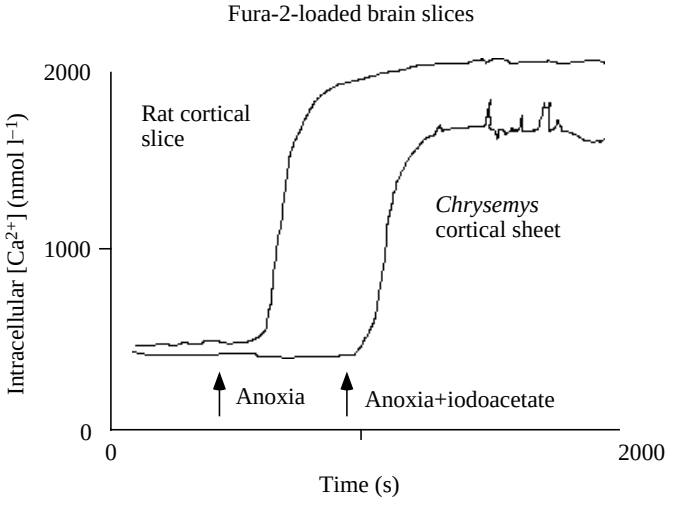

Fig. 3. Comparison of changes in intracellular [Ca^{2+}] in rat and turtle cortical slices (sheets) during anoxia and during anoxia in the presence of 3 mmol l^{-1} iodoacetate to block glycolytic energy production. Modified from Bickler (1992).

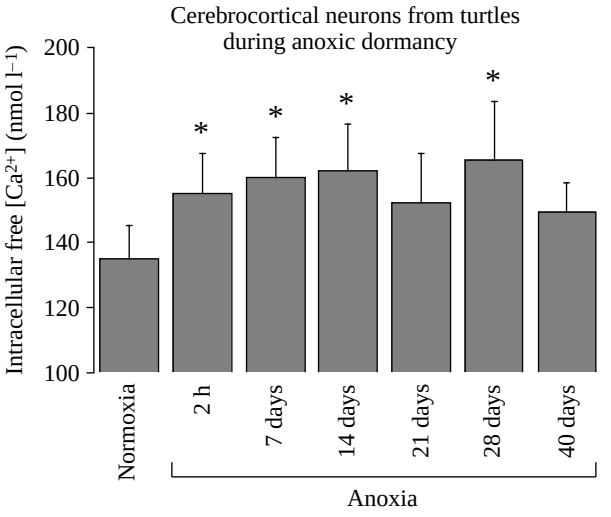

Fig. 4. Intracellular free Ca^{2+} concentration in neurons in cortical sheets from *Chrysemys picta* during normoxia and after 2 h to 40 days of anoxia at 3 °C. $[Ca^{2+}]_i$ was measured using the Fura-2 technique. An asterisk indicates a significant difference from the control (normoxia) group (Dunnett's test, $P<0.05$). Values are means + S.E.M., N=4–6.

neurons, several hours to several weeks of anoxia result in slight (but significant) increases in intracellular free [Ca^{2+}]. Ca^{2+} levels decrease towards control values during weeks 3–5 of anoxia (Fig. 4). This pattern of relatively stable intracellular Ca^{2+} concentrations during even prolonged anoxia suggested to us that glutamate receptors may be undergoing some form of suppression during anoxia. In addition, the slight elevation in free [Ca^{2+}] observed in turtle neurons during both brief and prolonged anoxia may indicate a role for Ca^{2+} as an important intracellular second messenger in view of the multiple known effects of Ca^{2+} in controlling electrical excitability, gene expression, etc.

By far the most complete functional picture of ion channel regulation during hypoxia and anoxia comes from studies of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors conducted on turtle cerebrocortical neurons and in hippocampal pyramidal neurons from neonatal rats. The NMDA receptor is a highly Ca^{2+}-permeable glutamate receptor subtype, and its hyperactivation during ischemia makes an important contribution to cell death (Ascher and Nowak, 1987; Choi, 1995). Studies with turtles and rat neurons have shown that glutamate receptor activity decreases during hypoxia and that the stabilized intracellular [Ca^{2+}] contributes to cell survival, at least in the hypoxia-sensitive mammalian neurons (Bickler and Hansen, 1997; Buck and Bickler, 1998). The most profound case of glutamate receptor inactivation occurs in the anoxia-tolerant turtle neurons, but in moderately sensitive neurons (e.g. neurons from newborn rat hippocampus) a similar pattern of receptor inactivation occurs. In mature rats, the inactivation occurs but it is poorly expressed and correlates with high rates of hypoxia-induced cell death. These cases make a

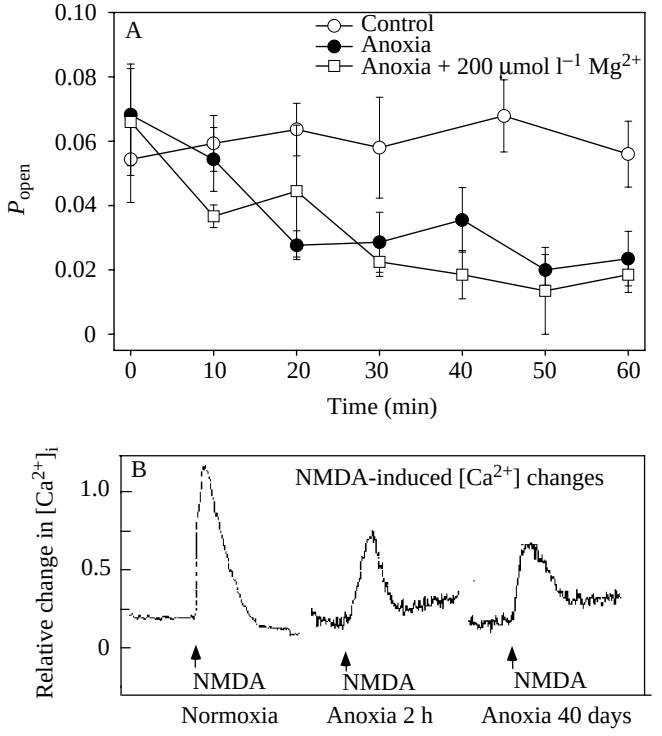

Fig. 5. Reduction in *N*-methyl-D-aspartate (NMDA) receptor open probability (P_{open}) during anoxia in *Chrysemys* cortical neurons (A). To exclude a membrane-potential-related reduction in open probability, the Mg^{2+} concentrations in the recording patch pipette were lowered to 200 µmol l^{-1}. This low Mg^{2+} concentration removes the voltage-sensitive Mg^{2+} channel block present at physiological Mg^{2+} concentrations. Values are means ± S.E.M., *N*=8. (B) The reduction in NMDA-receptor-mediated changes in $[Ca^{2+}]$ in cortical sheets measured using the Fura-2 technique.

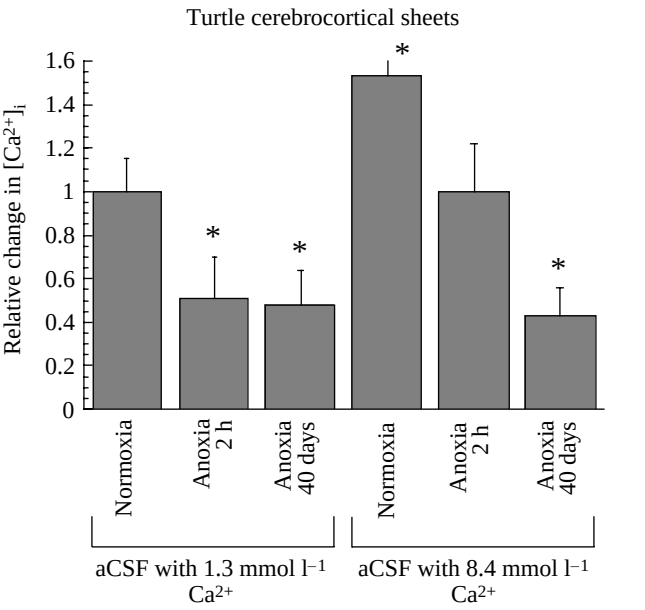

Fig. 6. *N*-methyl-D-aspartate (NMDA) receptor activity, measured as NMDA-mediated changes in $[Ca^{2+}]$, during long-term anoxia. Measurements were made with anoxic artificial cerebrospinal fluid (aCSF) containing the same ion concentrations as in normoxic turtles (in mmol l^{-1}: Na^+, 129; Cl^-, 91; K^+, 1.9; Ca^{2+}, 1.3, Mg^{2+}, 1.2; lactate, 2.9; glucose, 10; HCO_3^-, 41; pH 7.63 at 25 °C) or with aCSF containing the levels of ions found in CSF after prolonged dormancy (in mmol l^{-1}: Na^+, 131; Cl^-, 62; K^+, 4; Ca^{2+}, 8.4; Mg^{2+}, 5.1; lactate, 69; glucose, 10; HCO_3^-, 31, pH 7.25 (Cserr *et al.* 1988). An asterisk indicates a significant difference from the control normoxic group with aCSF containing 1.3 mmol l^{-1} Ca^{2+}. Values are means + S.E.M., *N*=6–8.

convincing argument for the importance of ion channel inactivation in protecting neurons during hypoxic stress.

Regulation of NMDA receptors in anoxic turtle neurons

The activity of NMDA receptors decreases by 30–50 % within a few hours of the onset of anoxia, when measured either using Ca^{2+}-sensitive dyes (NMDA-mediated Ca^{2+} influx measured with Fura-2) or using single-channel patch-clamp recordings (Fig. 5). NMDA receptor activity remains stably depressed during 5 weeks of anoxia at 3–4 °C (Fig. 6). During prolonged anoxia, $[Ca^{2+}]$ in cerebrospinal fluid increases four- to eightfold as a result of acidosis and demineralization of the skeleton. This increase in $[Ca^{2+}]$ causes NMDA-receptor-mediated Ca^{2+} fluxes to double in aerobic-adapted neurons, but in neurons adapted to 5 weeks of *in vivo* anoxia, NMDA receptor activity in this high-$[Ca^{2+}]$ environment decreases progressively to a level below that in neurons tested with normal Ca^{2+} concentrations. This strongly suggests that NMDA receptor sensitivity to acidity, Mg^{2+} or other compounds is altered during long-term anoxia. This fascinating situation is under study at the moment.

An interesting candidate for a long-term regulatory mechanism is a 95 kDa postsynaptic density protein (PSD 95) that binds to NMDA receptors and affixes the receptor to the cytoskeleton (Kornau *et al*. 1995). In the presence of high intracellular $[Ca^{2+}]$, PSD 95 unbinds and NMDA receptor activity decreases. In anoxic turtle brain, the sustained increase in $[Ca^{2+}]_i$ may be a signal for PSD 95 to dissociate from the NMDA receptor, thereby decreasing its sensitivity to glutamate and increasing $[Ca^{2+}]_e$.

Adenosine, which accumulates in turtle ECF during anoxia as a result of ADP hydrolysis (Nilsson and Lutz, 1992), decreases $[K^+]$ leakage (Pek and Lutz, 1997) and also acts to decrease Ca^{2+} fluxes and NMDA receptor open probability (Buck and Bickler, 1995) (Fig. 7). Although adenosine receptor antagonists prevent inactivation, other factors are no doubt also involved. Neuronal phosphatases, which are involved in the modulation of NMDA receptor activity during memory formation, apparently play a role because antagonism of neuronal phosphatase 2A or 1 with calyculin prevents anoxia-induced receptor inactivation (Fig. 8). Inhibition of phosphatase 2b with cypermethrin or activation of adenylate cyclase with forskolin fails to alter anoxia-induced NMDA receptor inactivation. Thus, we believe that at least the following events occur as signaling processes to control NMDA receptor activity during anoxia: an increase in

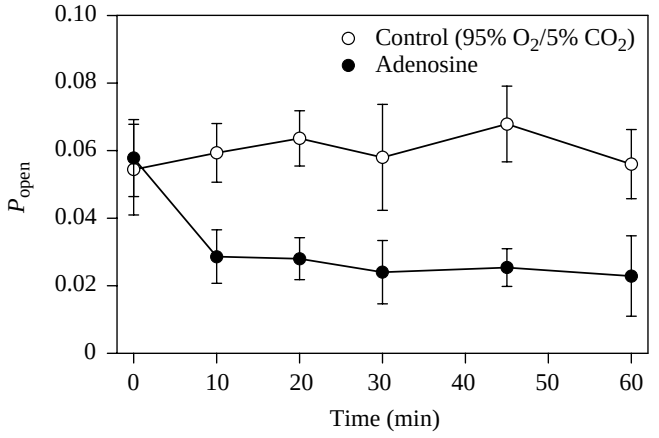

Fig. 7. Reduction in open probability (P_{open}) of *N*-methyl-D-aspartate (NMDA) receptors on *Chrysemys* cortical neurons during exposure to 200 μmol l^{-1} adenosine. Values are means ± S.E.M., *N*=7.

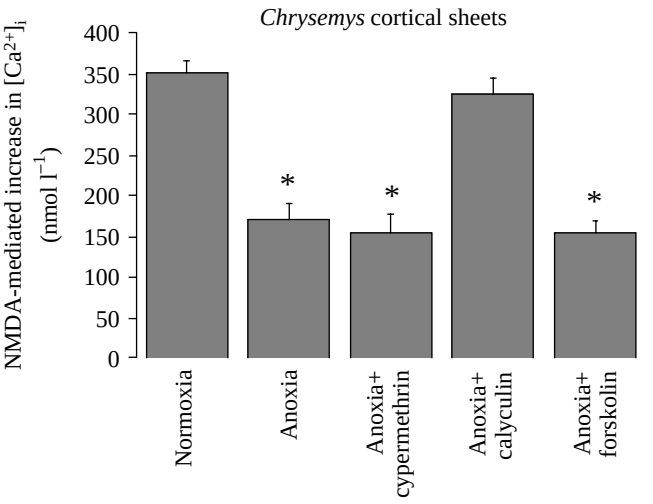

Fig. 8. Reduction of *N*-methyl-D-aspartate (NMDA) receptor activity (NMDA-mediated changes in $[Ca^{2+}]$ measured using Fura-2) during anoxia in the presence and absence of inhibitors of second messenger signaling. The results with calyculin (1 μmol l^{-1}) (an inhibitor of protein phosphatase 1 and 2A) suggest a role for dephosphorylation of NMDA receptor subunits, while the lack of effect of cypermethrin (an inhibitor of protein phosphatase 2b) (100 nmol l^{-1}) suggests that protein phosphatase 2b is not involved. The effect of forskolin (1 μmol l^{-1}), which stimulates the activity of adenylate cyclase, suggests that increased cyclic AMP levels may not be needed for anoxia-induced NMDA receptor inactivation. Values are means + S.E.M., *N*=6–12. An asterisk indicates a significant difference from the control (normoxia) value.

extracellular [adenosine], an increase in intracellular $[Ca^{2+}]$ and activation of Ca^{2+}-dependent protein kinases and phosphatases which allosterically modulate the NMDA receptor (Fig. 9). In addition, longer-term changes in the behavior of the receptor, possibly involving changes in subunit composition, adapt the receptor for stable function at acidic pH and at extremely high extracellular $[Ca^{2+}]$ and $[Mg^{2+}]$. Indeed, the NMDA receptor is known to undergo phosphorylation by protein kinase A (Cerne *et al.* 1993) and protein kinase C (Tingley *et al.* 1993). Conversely, the receptor is dephosphorylated by the corresponding phosphatases: tyrosine phosphatase (Wang and Salter, 1994), protein phosphatases 1 and 2A (Wang and Salter, 1994) and Ca^{2+}-dependent phosphatase (Lieberman and Mody, 1994). In general, phosphorylation increases receptor activity and dephosphorylation decreases receptor activity. Each of these second messenger pathways is potentially under the regulation of adenosine A1 receptors. Adenosine is well established as a potent pre- and postsynaptic neuromodulator. Mechanisms underlying these effects have recently been characterized (Fig. 10). Presynaptically, adenosine reduces neurotransmitter release (Burke and Nadler, 1988), probably as a result of reduced Ca^{2+} entry through N-type channels (rat hippocampal pyramidal neurons, Scholz and Miller, 1991; rat hippocampal CA3 region, Mogul *et al.* 1993; chick ciliary ganglia, Yawo and Chuhma, 1993; mouse motor neurons, Mynlieff and Beam, 1994; rat hippocampal CA1 region, Wu and Saggau, 1994). Postsynaptically, adenosine activates K^+ and Cl^- currents in hippocampal neurons, causing membrane hyperpolarization (Dunwiddie, 1985; Gerber *et al.* 1989; Rudolphi *et al.* 1992; Wu and Saggau, 1994). Hyperpolarization decreases the likelihood of NMDA receptor activation *via* a voltage-dependent Mg^{2+} block (Ascher and Nowak, 1987). An adenosine-evoked decrease in electrical activity in the CA1 region of rat hippocampus was shown to be Mg^{2+}-dependent and to be modulated by both A1 receptor and NMDA receptor ligands (Bartrup and Stone, 1990; de Mendonca and Ribiero, 1993). These data suggest that membrane hyperpolarization is one link between A1 receptors and reduced NMDA receptor activity, although de Mendonca *et al.* (1995), using whole-cell patch-clamp methods, recently demonstrated that the adenosine analog 2-chloroadenosine reversibly inhibited NMDA-mediated currents independently of the presence Mg^{2+}. Furthermore, our results, employing cell-attached patch-clamp methods and low extracellular $[Mg^{2+}]$, are consistent with this finding (Fig. 5).

It is becoming clear that a complex interplay of second messenger phosphorylation/dephosphorylation cycles regulates neuronal excitability and that these cycles could be involved in decreasing excitability in hypoxia-tolerant neurons such as those from western painted turtle (*Chrysemys picta*).

NMDA receptor regulation in neonatal rats during hypoxia

Neonatal rats survive anoxia for more than 10 times as long as mature rats without sustaining central nervous system damage (Adolph, 1948; Fazekas *et al.* 1941). Some explanations for this developmental change have focused on metabolic causes (e.g. pathways or processes that enhance anaerobic energy production or delay energy store depletion (Duffy *et al.* 1975), but none has incorporated the important role of glutamate in the cascade of injury which ensues from hypoxia or ischemia. The enhanced survival of the neonatal brain during anoxia occurs despite the increased Ca^{2+}

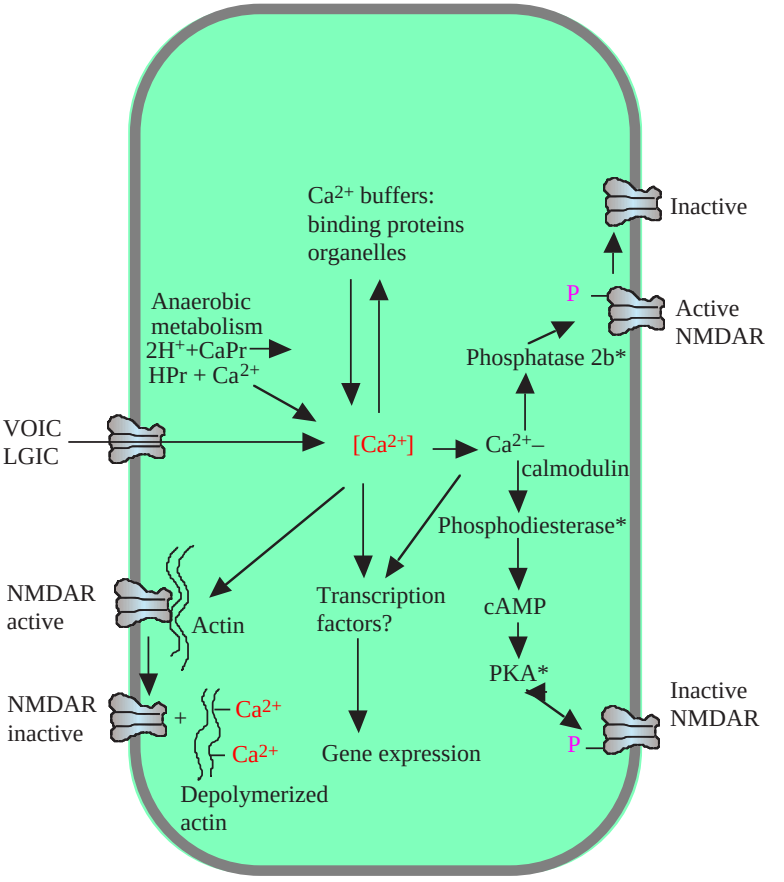

Fig. 9. Possible Ca^{2+} signaling pathways during anoxia in turtle cortical neurons. Elevations in intracellular free $[Ca^{2+}]$ (see Fig. 4) could trigger the activation of Ca^{2+}-dependent phosphatases and kinases *via* the intermediate calmodulin. An increase in $[Ca^{2+}]$ also results in depolymerization of ion channels from cytoskeletal elements, modifying their activities. Gene expression, modified by Ca^{2+}-sensitive transcription factors, may also occur. PKA, protein kinase A; NMDAR, NMDA receptors; Pr, protein; VOIC, voltage-operated ion channels; LGIC, ligand-gated ion channels. Asterisks indicate activated enzymes.

permeability of NMDA receptors (probably correlating with an enhanced propensity for synapse formation in the developing brain) and the increased sensitivity of the newborn brain to neurotoxicity from drugs that hyperexcite NMDA receptors. Of note, the newborn brain contains somewhat more glutamate per unit mass than does the mature brain (Reynolds and Brein, 1992). We hypothesized that this state of affairs could co-exist with hypoxia resistance only if one of the following scenarios occurred in the neonatal neurons: (1) the expression of the glutamate cascade is avoided by mechanisms that delay ATP depletion (e.g. hypometabolism – metabolic arrest), resulting in reduced glutamate release; (2) NMDA receptors are functionally inactivated during hypoxia to prevent glutamate from triggering Ca^{2+} influx through NMDA receptors.

We have shown that both the above mechanisms occur and result in enhanced survival of hippocampal neurons in the neonate (Bickler and Hansen, 1977). These investigations were carried out on hippocampal neurons of the stratum pyramidalis

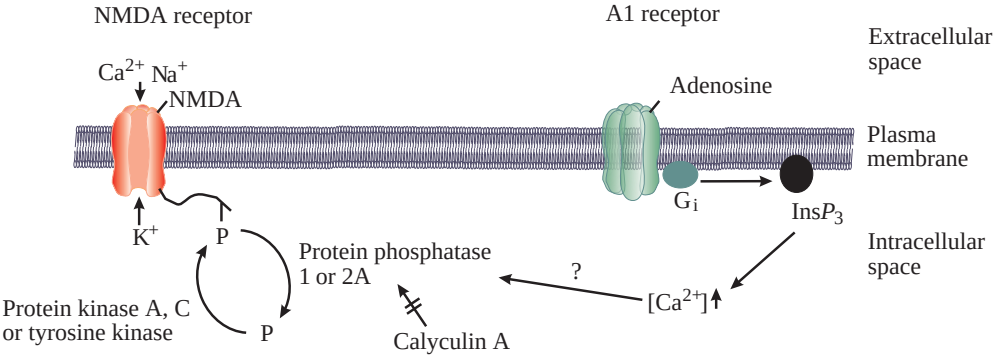

Fig. 10. Diagram showing adenosine signaling during anoxia. The *N*-methyl-D-aspartate (NMDA) receptor consists of five subunits with an intracellular peptide tail containing several potential phosphorylation sites. The mechanism leading to decreased receptor open probability during anoxia is unknown, but some evidence points to a role for adenosine. With the onset of anoxia, adenosine concentrations in the interstitial space increase. Adenosine, acting *via* its A1 receptor, stimulates inositol trisphosphate ($InsP_3$) production and leads to an increase in intracellular $[Ca^{2+}]$. It is as yet unclear how intracellular traffic is regulated in a way that activates phosphatases and kinases selectively; however, $[Ca^{2+}]_i$ increases modestly in anoxic turtle brain and NMDA receptor activity is sensitive to calyculin A. Taken together, it is therefore reasonable to speculate that increased Ca^{2+} levels stimulate the calyculin-A-inhibitable protein phosphatase, thereby dephosphorylating NMDA receptors. The result is decreased glutamate sensitivity and protection from neuroexcitatory cell death. G_i, inhibitory G-protein.

(CA1), one of the groups of neurons in the brain which is amongst the most sensitive to hypoxic or ischemic insults. These neurons are studded with glutamate receptors and play a key role in memory formation. They are extensively studied for this purpose, and the phenomenon of long-term potentiation has been defined largely in synapses involving these neurons. For the purposes of comparison, we have studied slices prepared from very young rats (3–7 days old; an age at which animals show a very profound degree of resistance to anoxia) and in older juvenile rats (18–22 days old), when whole-animal hypoxia tolerance approximates that of the young adult (Adolph, 1948).

The rate of ATP depletion in neonatal brain slices following blockade of energy production pathways is slower than in mature slices and is probably related to acutely reduced metabolism. This is true whether metabolism is blocked with cyanide or with both cyanide and iodoacetate to prevent both aerobic and anaerobic metabolism (Bickler *et al.* 1993). The neonatal slice preparation also shows limited release of glutamate, whether release is due to stimulation with anoxia or to depolarization with potassium chloride. Another feature common to neonatal hippocampal neurons in brain slices is a reduced contribution of glutamate receptors to anoxia-induced Ca^{2+} influx. Following a 5 or 10 min period of anoxia, intracellular [Ca^{2+}] in neonatal CA1 neurons returns quickly to baseline levels, whereas Ca^{2+} levels in neurons in slices prepared from animals more than 14 days old remain elevated (Bickler and Hansen, 1997). A most interesting finding has been that, as in the turtle, NMDA receptors in the brain of neonatal rats are partially inactivated during hypoxia. Taken together, these events interrupt the glutamate cascade and enhance the survival of neurons. The relationship between the anoxia-induced increase in intracellular [Ca^{2+}], the amount of glutamate released and cell survival as a function of rat age is illustrated in Fig. 11 and suggests a possible causal relationship between these phenomena.

The phenomenon of NMDA receptor inactivation during hypoxia in neonatal hippocampal neurons is of interest since similar reductions in NMDA receptor activity have been seen in turtle neurons. Does this protective mechanism represent a phylogenetically ancient response to hypoxia, one that is lost during postnatal development in mammals? In neonatal neurons, among the mechanisms responsible for receptor inactivation is Ca^{2+}-dependent actin–NMDA receptor depolymerization (Fig. 12), probably triggered by NMDA-receptor-mediated Ca^{2+} influx. Phosphatases involved in NMDA receptor regulation in the turtle brain could also be involved in receptor inactivation.

Gene expression and adaptation to hypoxia

Since hypoxia-tolerant neurons survive anoxia for very long periods and may increase their tolerance of anoxia as part of their normal preparation for dormancy, a strong possibility exists that differential gene expression may play a role in protection. This possibility has recently been reviewed by Hochachka *et al.*

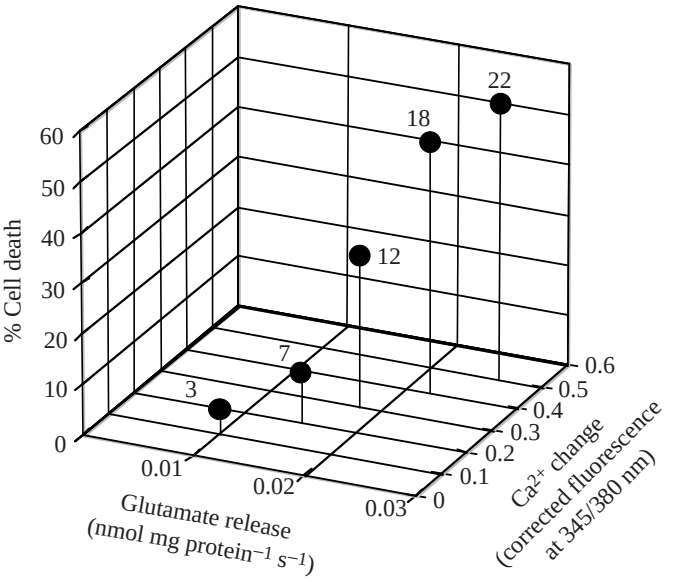

Fig. 11. Relationship between the postnatal age of hippocampal slices and hypoxia-induced glutamate release, hypoxia-induced changes in cytosolic [Ca^{2+}] and hypoxia-induced changes in cell viability index in hippocampal slice CA1 neuron cell bodies. Rates of glutamate release and total changes in [Ca^{2+}] are mean maximal rates during immersion of the slices in artificial cerebrospinal fluid with a P_{O_2} of less than 15 mmHg for 10 min. The cell viability index was determined using the calcein/ethidium homodimer method; an index of one represents a viability equivalent to that of freshly prepared slices; an index of zero represents a viability equal to that of neurons exposed to cyanide and iodoacetate for 1 h (from Bickler and Hansen, 1997).

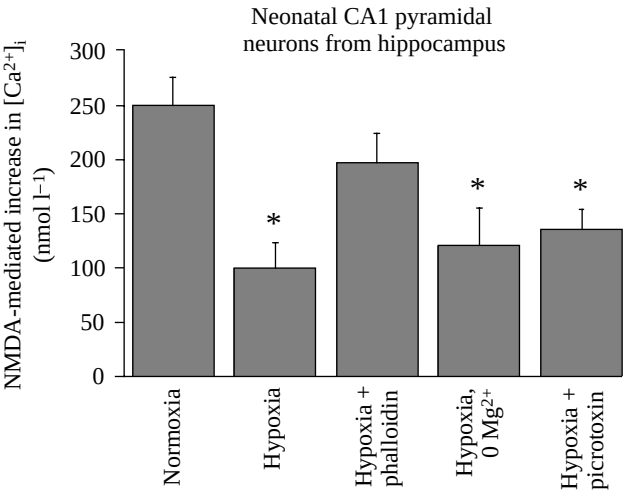

Fig. 12. *N*-methyl-D-aspartate (NMDA) receptor inactivation in immature CA1 neurons during moderate hypoxia (P_{O_2} 25 mmHg). Phalloidin (1 μmol l^{-1}), which stabilizes actin–NMDA receptor polymerization, partially prevented the inactivation, but altered the membrane potential (the effect was reversed with low [Mg^{2+}] in artificial cerebrospinal fluid) and inhibition of γ-aminobutyric acid (GABA) receptors by picrotoxin (10 μmol l^{-1}) did not. Values are means + S.E.M., *N*=8–12. An asterisk indicates a significant difference from the control (normoxia) value.

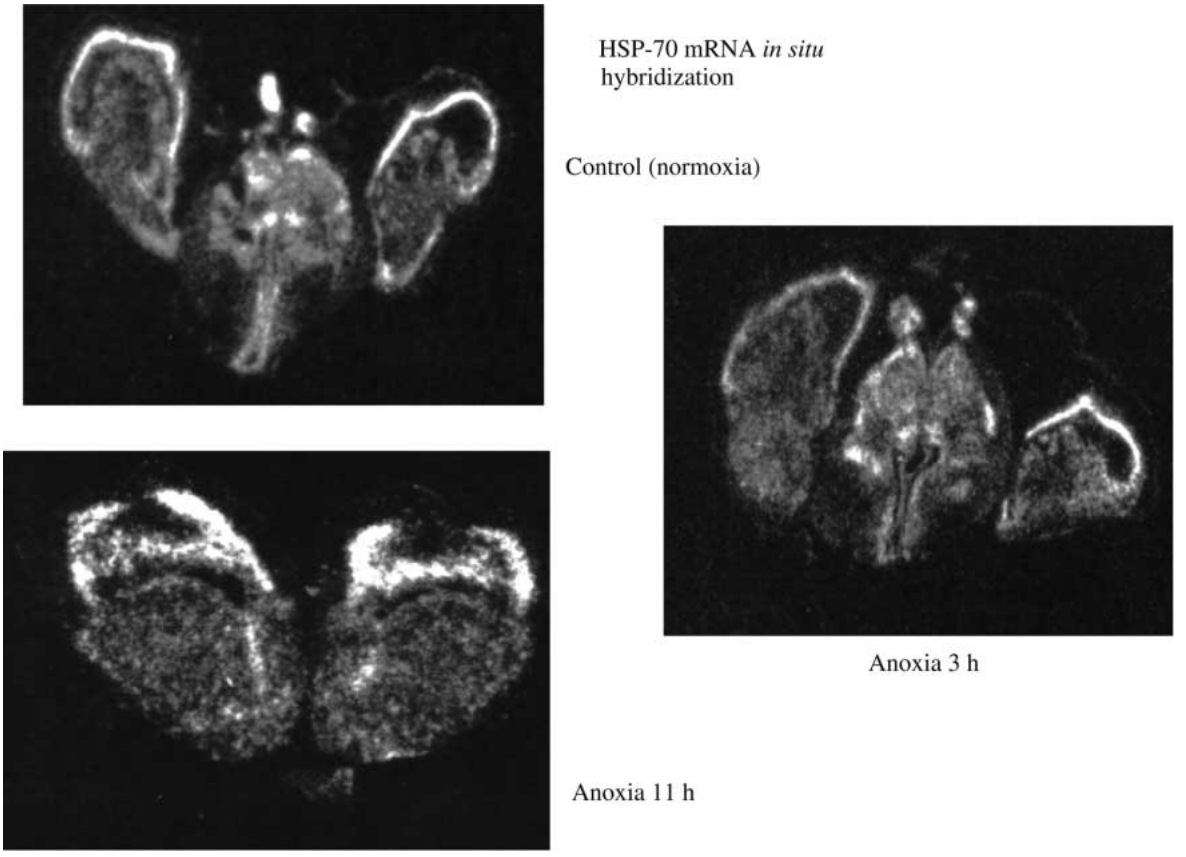

Fig. 13. Expression of HSP-70 in turtle cortex during anoxia. HSP-70 was constitutively expressed in cortex during normoxia and anoxia, but expression was increased in the cortex and appeared for the first time in deeper brain structures after 11 h of anoxia.

(1996). Although protein synthesis is globally depressed during anoxia, selective gene translation and protein synthesis may be important (Hochachka *et al.* 1996). Specific information on gene expression in neurons is scarce – almost all the data have been obtained from mammals, in which hypoxia-induced injury makes interpretation difficult. An opportunity exists, therefore, to use hypoxia-tolerant animals to help identify gene products that are adaptive and protective (since hypoxia is not causing injury, there is no injury cascade to confuse things). Towards that goal, we have measured the expression of several gene products during anoxia in the intact turtle brain. These studies were carried out on turtles kept in anoxic atmospheres for 11 h at room temperature prior to study. We have thus far found that the expression of a heat-shock protein (HSP-70) is increased during anoxia throughout the cortex, midbrain and cerebellum of all turtles studied (Fig. 13). The protein is also constitutively expressed in these areas. Pyramidal neurons in the cerebrocortex stained the most intensely. In contrast, levels of cyclo-oxgenase (COX) II and the proto-oncogene *c-fos*, which are extensively expressed in mammalian brain following hypoxia/ischemia, are not increased in turtles. These results suggest that HSP-70 is associated with surviving hypoxia while COX and *c-fos* are perhaps associated only with injury. Present studies are defining whether the induction of anti-apoptosis genes (e.g. *Bcl-2* and *Bcl-x*) occurs in turtle neurons during anoxia.

Conclusions

Some key differences in the responses of hypoxia-tolerant and intolerant neurons to anoxia are shown in Fig. 14. Whereas hypoxia-sensitive neurons lose ATP, depolarize and are flooded with Ca^{2+} before life-sustaining protective mechanisms can come into play, those from hypoxia-tolerant animals are able successfully to initiate processes that decrease energy use and ion channel function in a coordinated manner.

Hypoxia-tolerant neurons no doubt use numerous strategies for dealing with oxygen lack. We propose that among the most significant are those that contribute to stabilizing $[Ca^{2+}]_i$, so that Ca^{2+} remains useful as a second messenger in controlling the metabolic and ion channel arrest characteristic of neurons from animals able to tolerate long-term anoxia. Decreases in the activity of key ion channels, directly or indirectly, contribute to regulating $[Ca^{2+}]_i$ to a new setpoint during anoxia. The processes that regulate ion channels during hypoxia no doubt include phosphorylation by protein kinases, dephosphorylation by protein phosphatases, Ca^{2+}-dependent depolymerization of cytoskeletal elements controlling channel function, receptor removal/insertion into the membrane and expression of new receptor subtypes. The effects of the extracellular ionic milieu (changes in $[Ca^{2+}]$, $[Mg^{2+}]$ and pH) must also be important.

Regulated decreases in the activity of key ion channels are

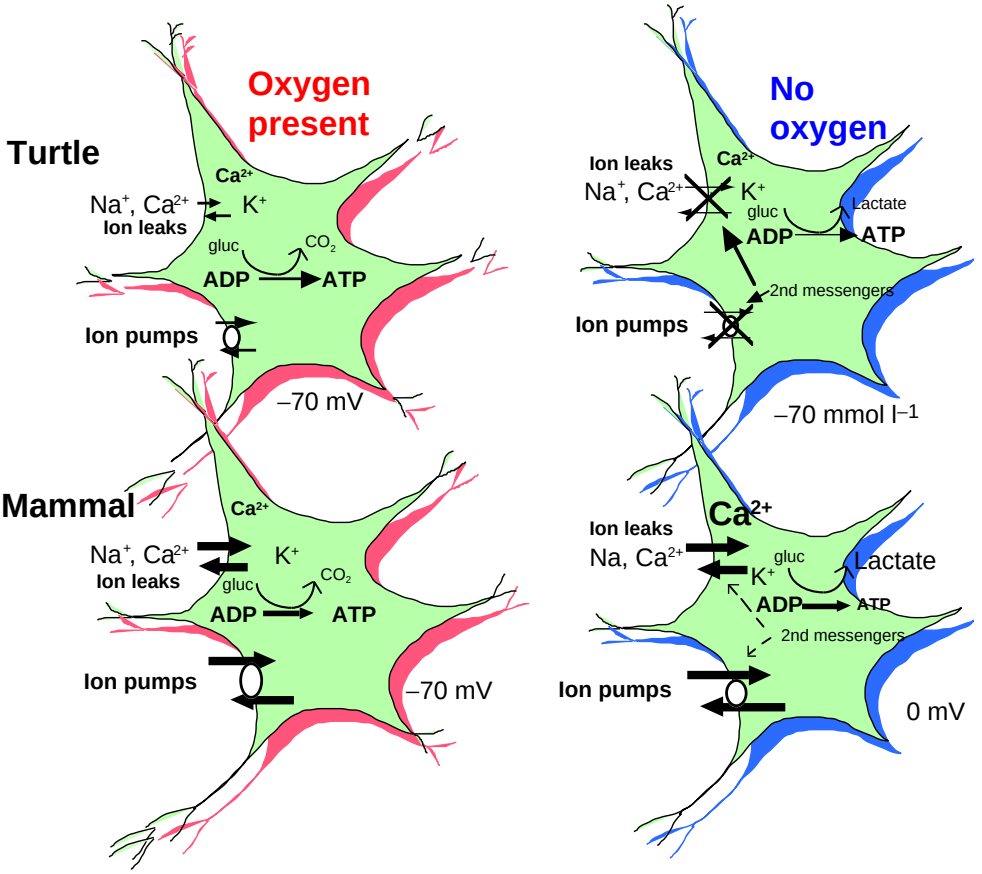

Fig. 14. Summary of the differences between hypoxia-tolerant (turtle) and hypoxia-sensitive (mammalian) neurons. Relative concentrations of ions, substrates and rates of reactions are indicated by the size of the arrows and lettering. In mammals, ion pumping and ATP turnover rates during normoxia are greater than in turtles and ion pumping rates are not decreased during anoxia, depleting ATP, depolarizing the membrane and increasing intracellular [Ca^{2+}]. Second messengers are not effective in preventing catastrophic ATP loss, [Ca^{2+}] elevation and cell swelling/membrane damage. In contrast, lower rates of ATP depletion allow turtle neurons to activate a cascade of second messengers to inactivate ion pumps, reduce energy turnover and maintain relatively stable ATP and Ca^{2+} levels. gluc, glucose. Membrane potentials are also given.

common to hypoxia-tolerant neurons from both mammals and reptiles, and thus these processes may represent fundamental cellular adaptations to stresses that may decrease energy availability. The key differences between hypoxia-sensitive mammalian and hypoxia-tolerant reptilian neurons may not be the mechanism, but the time required: the lower metabolic rate of hypoxia-tolerant neurons allows for the expression of a coordinated suite of processes before catastrophic energy failure occurs.

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