Paper II

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K_{ATP}-CHANNELS PLAY A MINOR ROLE IN THE PROTECTIVE HYPOXIC SHUT-DOWN OF CEREBELLAR ACTIVITY IN EIDER DUCKS (*SOMATERIA MOLLISSIMA*)

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Abstract—Eider duck (*Somateria mollissima*) cerebellar neurons are highly tolerant toward hypoxia *in vitro*, which in part is due to a hypoxia-induced depression of their spontaneous activity. We have studied whether this response involves ATP-sensitive potassium (K_{ATP}) channels, which are known to be involved in the hypoxic/ischemic defense of mammalian neural and muscular tissues, by causing hyperpolarization and reduced ATP demand. Extracellular recordings in the Purkinje layer of isolated normoxic eider duck cerebellar slices showed that their spontaneous neuronal activity decreased significantly compared to in control slices when the K_{ATP} channel opener diazoxide (600 μM) was added ($F_{1,70} = 92.781$, $p < 0.001$). Adding the K_{ATP} channel blocker tolbutamide (400 μM) 5 min prior to diazoxide completely abolished its effect ($F_{1,55} = 39.639$, $p < 0.001$), strongly suggesting that these drugs have a similar mode of action in this avian species as in mammals. The spontaneous activity of slices treated with tolbutamide in combined hypoxia/chemical anoxia (95% N_2–5% CO_2 and 2 mM NaCN) was not significantly different from that of control slices ($F_{1,203} = 0.071$, $p = 0.791$). Recovery from hypoxia/anoxia was, however, slightly but significantly weaker in tolbutamide-treated slices than in control slices ($F_{1,137} = 15.539$, $p < 0.001$). We conclude that K_{ATP} channels are present in eider duck cerebellar neurons and are activated in hypoxia/anoxia, but that they do not play a key role in the protective shut-down response to hypoxia/anoxia.

Key words: diving, hypoxia, cerebellum, neurons, spontaneous activity, K_{ATP}-channels.

INTRODUCTION

Due to its benthic feeding behavior (Cantin et al., 1974; Bustnes, 1998) the common eider duck (*Somateria mollissima*) is likely to be regularly exposed to hypoxia during breath-hold diving. As with diving mammals, ducks display an array of defense mechanisms that enable them to endure diving asphyxia better than non-diving birds. These include an enhanced capacity to store oxygen in blood and skeletal muscles, as well as cardiovascular adjustments involving bradycardia and profound peripheral vasoconstriction that cause partial ischemia in most tissues, thereby reducing their rate of oxygen use (Andersen, 1966; Bryan and Jones, 1980; Butler and Jones, 1997; Hawkins et al., 2000; Guillemette, 2001; Guillemette et al., 2004; Ramirez et al., 2007).

Additional protection may be provided via rapid, diving-associated brain cooling (Caputa et al., 1998). Apparently, their neural tissue is also inherently more hypoxia tolerant, since we in a previous study found that isolated cerebellar slices from eider ducks displayed significantly higher survival rates than did those of chicken, when subjected to identical hypoxic/anoxic insults (Ludvigsen and Folkow, 2009). That study also revealed two fundamentally different hypoxic responses of eider duck cerebellar neurons, in that the spontaneous activity of some actually persisted for durations of up to 1 h in hypoxia/chemical anoxia, while a majority of neurons displayed rapid cessation of activity, but subsequently resumed spontaneous activity upon return to normoxia (Ludvigsen and Folkow, 2009). This suggests that there exist two hypoxia defense mechanisms in duck cerebellar neurons, of which shut-down of spontaneous activity is one.

Hypoxia-sensitive neurons die from exposure to hypoxia due to a cascade of events that are triggered by energy deficit, eventually leading to loss of ion balance and membrane potential (Choi, 1988; Drew et al., 2004; Lutz and Nilsson, 2004; Szydlowska and Tymianski, 2010). An early cessation of activity could prevent such failure since much of the energy (ATP) is used to restore ion balance after generation of action potentials, and also because prevention of depolarization dampens the fatal glutamate excitotoxicity (Drew et al., 2004; Piña-Crespo et al., 2014) that eventually leads to cell death. ATP-sensitive potassium (K_{ATP}) channels are potassium-specific ion channels that open when ATP concentration drops below a certain level, which leads to membrane hyperpolarization and reduced neuronal activity (Hansen, 1985; Ballanyi and Kulik, 1998; Mironov and Richter, 2000; Ballanyi, 2004; Soundarapandian et al., 2007b). In this way, these channels may offer short-term protection against the fatal effects of energy deficiency (Pek-Scott and Lutz, 1998; Garcia de Arriba et al., 1999; Ballanyi, 2004; Yamada and Inagaki, 2005).

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Abbreviations: aCSF, artificial cerebrospinal fluid; DMSO, dimethyl sulfoxide; I/R, ischemia–reperfusion; K_{ATP}, ATP-sensitive potassium; MUA, multi-unit activity.

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In this study we investigated the possible presence and influence of K_{ATP} channels on neuronal activity in the Purkinje layer of isolated cerebellar slices from eider ducks, by manipulating these channels pharmacologically. We hypothesized that if K_{ATP} channels are present, opening the channels in normoxia should decrease spontaneous neuronal activity through hyperpolarization. Moreover, we anticipated that if they are, indeed, involved in the shut-down of activity in hypoxia (Ludvigsen and Folkow, 2009), blocking them before or after cessation of activity in hypoxia/anoxia should result in a blunted shut-down response, and thereby possibly poorer survival, as the neurons would then no longer be hyperpolarized.

EXPERIMENTAL PROCEDURES

Animals and handling

Eider duck (*S. mollissima*) eggs were collected in a colony on the island Grindøya outside Tromsø, under permits issued by the relevant Norwegian authorities (Directorate for Nature Management/County Governor in Troms), or from birds breeding in captivity at the research animal facility of Department of Arctic and Marine Biology, University of Tromsø - the Arctic University of Norway. The eggs were incubated and hatched at this facility, and birds were raised and maintained in indoor and outdoor enclosures with access to a fresh water pond at both locations, and to concentrate feed (FKnr 12516, Felleskjøpet, NO) *ad libitum*. Altogether 18 birds were used. All birds (of both sexes) were more than 1 year of age, with body mass ranging from 1.1 to 2.1 kg.

Cerebellar slice preparation and extracellular recordings

Cerebellar slices were prepared and recordings conducted as described by Ludvigsen and Folkow (2009). In brief, birds were decapitated in deep anesthesia (combination of ketamin HCl (Ketalar, Pfizer; 20 mg kg^{-1}) and xylazine (Rompun, Bayer; 4 mg kg^{-1})) in accordance with Norwegian regulations (the Norwegian Animal Welfare Act). Heads were immediately transferred to a container with ice-cold oxygenated artificial cerebrospinal fluid (aCSF; composition: NaCl (128.0 mM), KCl (2.95 mM), $CaCl*2H_2O$ (1.5 mM), $MgSO_4*7H_2O$ (1.0 mM), $NaHCO_3$ (24.0 mM), NaH_2PO_4 (0.5 mM) and D-Glucose (30.0 mM), pH 7.4), and the brain was removed within 3–5 min. Four hundred-micrometer-thick longitudinal slices were cut through the vermis region of the cerebellum using a vibrating blade microtome (Leica VT1000 S, Leica Microsystems Nussloch Gmbh, Germany), with the tissue still submerged in ice-cold oxygenated aCSF. The slices were kept for $\geqslant$30 min in oxygenated aCSF at room temperature ($\sim$23 °C), and then transferred to recording chambers (volume 50 ml), in which temperature was maintained at 34 °C. Here, the slices were placed on a stainless steel mesh, stabilized under a platinum frame with nylon threads, and left for a minimum of 30 min before any recordings were started. The system was superfused with oxygenated aCSF (total volume 200 ml), at a flow rate of $\sim$30 ml min^{-1}. In normoxia, the aCSF was gassed with 95% O_2–5% CO_2, while hypoxia/chemical anoxia was induced by gassing with 95% N_2–5% CO_2 and adding NaCN to 2 mM. The aCSF oxygen level in the recording chambers was routinely monitored using Clark-type oxygen tension (P_{O2})-sensitive electrodes (OX25 microsensors, Unisense A/S, Aarhus, Denmark) connected to a polarographic amplifier (1900 Polarographic Amplifier, A-M Systems, Inc., Carlsborg, WA, USA) or an oxymeter (Oxymeter-1, Unisense A/S, Aarhus, Denmark). Before measurements, the electrodes were polarized for at least 2 h and calibrated in aCSF at 34 °C, saturated with either 95% O_2–5% CO_2 or 95% N_2–5% CO_2. Recording electrodes (tip diameter 50–80 μm) were produced from borosilicate glass capillaries (G150F-4, Warner Instruments Inc., Hamden, CT) using a micropipette puller (P-97 Flaming/Brown, Sutter Instrument Co., Novato, CA, USA). They were carefully positioned on the surface of the Purkinje cell layer of the isolated slices to record spontaneous extracellular multiunit activity (MUA) as previously described (Ludvigsen and Folkow, 2009).

The signals were amplified 1000 times and filtered (bandwidth: 0.1–3 kHz) using a headstage and a Grass P55 amplifier (Grass Telefactor, West Warwick, RI, USA). The raw signal was recorded online using PowerLab (mod. ML796, ADInstruments Inc., Colorado Springs, CO, USA), and Chart 7 for Windows software (ADInstruments Inc., Colorado Springs, CO, USA). MUA was always monitored for at least 20 min before any experiments were made.

In one series of experiments, diazoxide (K_{ATP} channel opener (Ballanyi and Kulik, 1998; Domoki et al., 1999; Ballanyi, 2004; Soundarapandian et al., 2007b), 1.2 ml of 100 mM in dimethyl sulfoxide (DMSO) yielding 600 μM in the bath) was added under normoxic conditions (95% O_2–5% CO_2), and responses were recorded for at least 20 min. The drug vehicle, DMSO (1.2 ml), was added 10 min prior to this, to ensure that it had no effect. Activity levels after adding DMSO or diazoxide in DMSO were compared to control experiments consisting of recordings in normoxia without any manipulation. To test if diazoxide actually targeted K_{ATP} channels, we also added tolbutamide (K_{ATP} channel blocker (Ballanyi and Kulik, 1998; Yamada and Inagaki, 2005; Soundarapandian et al., 2007b), yielding 400 μM in bath) 5 min prior to adding diazoxide (to 600 μM) under normoxic conditions, and recorded the response for 20 min. In a third series of experiments, hypoxia/chemical anoxia was introduced after an initial 10 min recording period in normoxia, by switching to 95% N_2–5% CO_2 and adding NaCN (to 2 mM). We then awaited the hypoxic/anoxic response to secure that this was of the shut-down type (Ludvigsen and Folkow, 2009), before either tolbutamide (0.8 ml of 100 mM in DMSO, yielding 400 μM in bath) or only DMSO (0.8 ml – control), was added. Drug and hypoxia responses were recorded for 60 min before reoxygenation and rinse of the aCSF, by switching back to 95% O_2–5% CO_2 and rinsing the system three times with fresh aCSF (34 °C). Recordings were continued for another 40 min to monitor

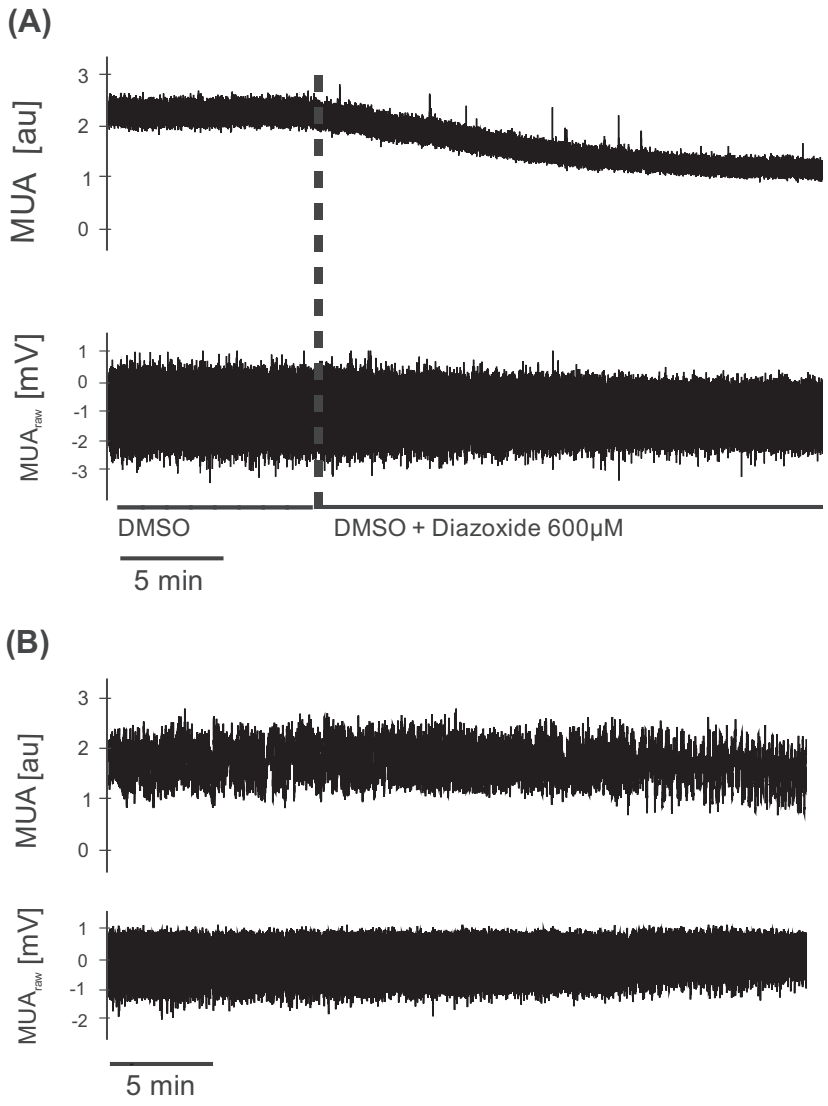

Fig. 1. Typical recording of spontaneous neuronal multi-unit activity (MUA) in normoxic isolated slices of eider duck cerebellum; (A) after adding, first, the drug vehicle (DMSO, 1.2 ml), and second, diazoxide in DMSO (1.2 ml, to yield 600 µM in bath – at dashed vertical line), and (B) during control conditions. Adding tolbutamide before diazoxide produced a similar recording to (B).

the extent of recovery of spontaneous activity. In a fourth set of experiments, tolbutamide was added as described above, but prior to, instead of after, introduction of hypoxia/chemical anoxia, to investigate whether potential K_{ATP}-channel-linked hypoxic responses might have confounded the effect of tolbutamide in the third series of experiments. Prior to tolbutamide administration, slices were subjected to a brief hypoxia/chemical anoxia episode to confirm that the neurons under investigation actually displayed the shut-down type of response. When observed (usually within ~3–5 min), the slice was reoxygenated, rinsed with fresh aCSF and then, after full recovery of spontaneous activity for ~5 min, tolbutamide (400 µM) was added 5 min prior to a 60-min hypoxia/anoxia exposure. Control experiments were performed according to the same protocol but without tolbutamide exposure, to check for a potential preconditioning effect of the brief pre-exposure to hypoxia/anoxia, before the full exposure and recovery was conducted (Schurr et al., 1986; Gidday, 2006).

Data analyses

Data analyses were made offline, using Chart 7 for Windows software. The raw signal (MUA_{raw}) was rectified, integrated and multiplied with spike-frequency and the product (MUA) was used for statistical analyses. Timed cursor values for mean spontaneous neuronal MUA were calculated over ±0.5 min around the time intervals that are given in the results. Resulting MUA levels are expressed relative to the mean control MUA in normoxia during the last 5 min prior to manipulations, which was set to 100%. Statistical analyses were performed using SPSS (version 21.0 for Windows; SPSS Inc., Chicago, IL, USA). A linear mixed model approach was used to analyze the data. A

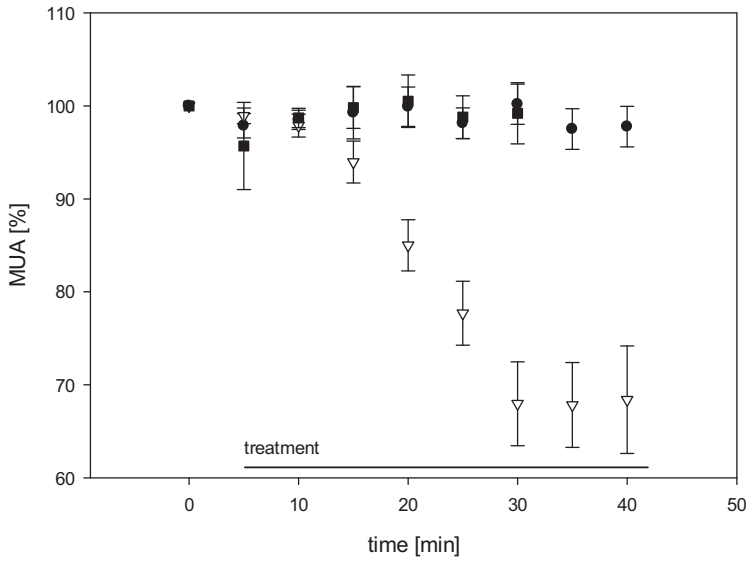

Fig. 2. Mean ± SD spontaneous multi-unit neuronal activity (MUA), in percent of mean MUA during 5 min prior to manipulations, in isolated cerebellar slices from eider ducks subjected to normoxia, under control conditions (black circles, 0–30 min: $n = 6$, 35–40 min: $n = 5$), in the presence of diazoxide (600 μM) (white triangles, 0–30 min: $n = 11$, 35 min: $n = 9$, 40 min: $n = 6$) and after adding tolbutamide (400 μM) 5 min prior to diazoxide (600 μM) (black squares, 0–30 min: $n = 4$).

significance level of < 0.05 was taken to indicate statistically significant differences. *N* indicates number of animals, if multiple slices from one animal were investigated, the mean value was used.

RESULTS

Responses of eider duck cerebellar neurons to agonists and antagonists in normoxia

Adding the K_{ATP} channel opener diazoxide (600 μM) had an inhibiting effect on the normoxic spontaneous activity of eider duck cerebellar neurons (Fig. 1A), as reflected in a significant decrease in activity level over time relative to control slices ($F_{4,70} = 3.506$, $p = 0.011$) (Fig. 2). Relative activity levels of control slices displayed little change (Fig. 1B), and were found to be significantly higher than in diazoxide experiments (Fig. 2) ($F_{1,70} = 92.781$, $p < 0.001$). Administration of tolbutamide (5 min) prior to diazoxide caused no change in activity, just like in control slices ($F_{1,39} = 0.471$, $p = 0.497$), showing that the inhibiting effect of diazoxide was abolished, being significantly different from that caused by diazoxide alone ($F_{1,55} = 39.639$, $p < 0.001$) (Fig 2).

Responses of eider duck cerebellar neurons to antagonists in hypoxia/chemical anoxia

Switch to hypoxia/anoxia (95% N_2–5% CO_2 + 2 mM NaCN) caused aCSF O_2 content to drop to < 5% within the first 2 min, eventually reaching 1.2% at the end of the hypoxic period (Fig. 3C). Adding the K_{ATP} channel blocker tolbutamide (400 μM) after hypoxia/anoxia had induced silencing of spontaneous activity, caused weak activity to reappear (Fig. 3B), typically within a period of 30 s to 6 min, in 75% of the studied slices ($n = 10$), while none of the control slices displayed any activity whatsoever in hypoxia/anoxia (Fig. 3A) The weak activity in response to tolbutamide was, however, not significantly different from control slice activity during the hypoxia/anoxia challenge ($F_{1,203} = 0.071$, $p = 0.791$), but tolbutamide-exposed slices displayed a significantly reduced recovery of spontaneous activity compared to controls ($F_{1,137} = 15.539$, $p < 0.001$) in the recovery period (Fig. 4). Adding tolbutamide prior to the hypoxia/chemical anoxia exposure yielded similar results, as the treated slices ($n = 4$) showed no significant difference in activity compared to control slices ($n = 3$) during hypoxia ($F_{1,171} = 0.1$, $p = 0.753$), and a slightly but significantly reduced recovery compared to control slices ($F_{1,102} = 5.729$, $p = 0.019$). The brief pre-exposure to hypoxia/anoxia did not appear to produce a preconditioning effect (Schurr et al., 1986; Gidday, 2006), as the pre-exposed control slices ($n = 3$) did not show a different level of activity during either hypoxia ($F_{1,123} = 2.765$, $p = 0.099$) or recovery ($F_{1,80} = 0.096$, $p = 0.758$) compared to non-pre-exposed control slices.

DISCUSSION

K_{ATP}-channels contribute to protect neurons from hypoxia in several species

Since the first description of K_{ATP} channels in cardiac muscle (Noma, 1983), these channels have been found to be widely distributed in mammalian excitable tissues, including the brain, in which they may convey temporary protection against energy deprivation, by reducing cellular excitability through hyperpolarization (Wind et al., 1997; Erdemli et al., 1998; Zawar et al., 1999; Ballanyi, 2004; Yamada and Inagaki, 2005; Soundarapandian et al., 2007b; Hibino et al., 2010; Kawano, 2012), and by thereby retarding excitatory neurotransmitter release

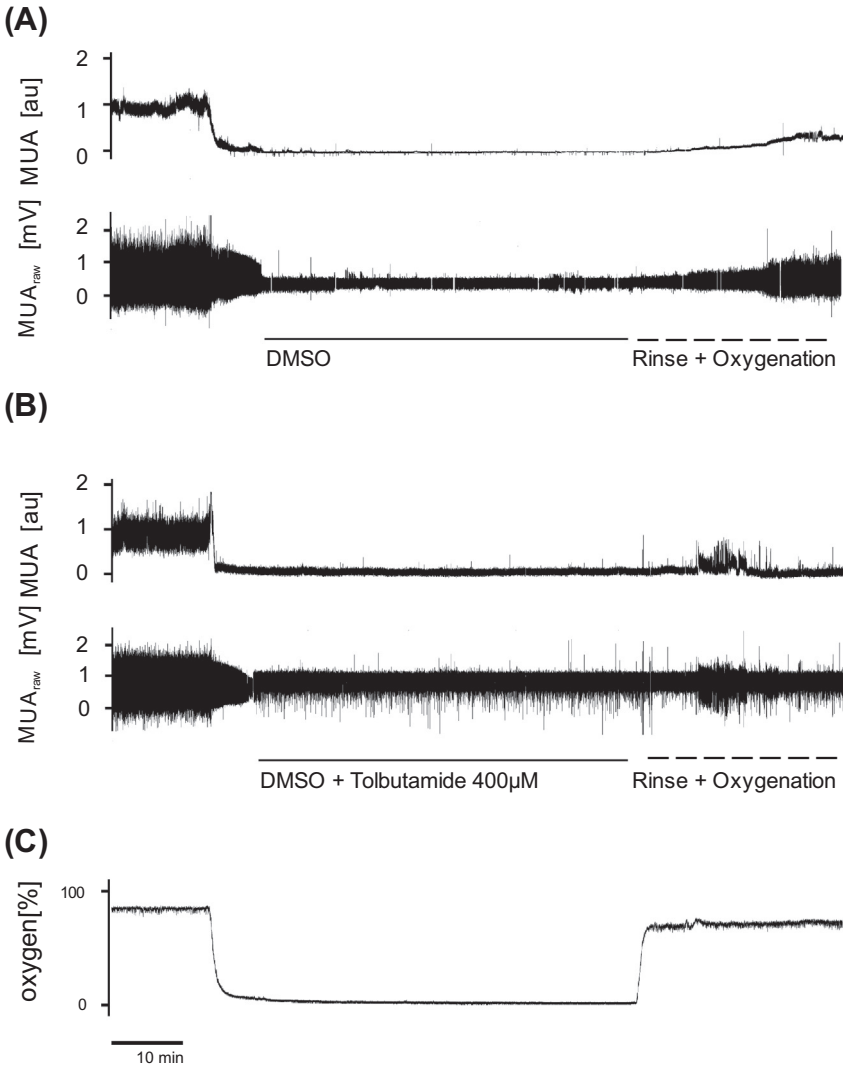

Fig. 3. Typical recording of spontaneous multi-unit neuronal activity before, during and after a 60-min anoxia challenge (95% N_2–5%CO_2 + 2 mM NaCN), (A) during control conditions (0.8 ml of DMSO added after silencing), and (B) after adding tolbutamide in DMSO (0.8 ml, to yield 400 µM in bath), (C) the aCSF oxygen level during the experiment.

(Murphy and Greenfield, 1992; Soundarapandian et al., 2007a). Use of K_{ATP} channel activators (e.g., diazoxide, nicorandil) consequently causes hyperpolarization and neuronal ischemic/hypoxic protection (Ballanyi, 2004; Bancila et al., 2004; Soundarapandian et al., 2007a), while use of K_{ATP} channel inhibitors (e.g., tolbutamide, glibenclamide) counteracts the hyperpolarizing effect and thereby may increase neuronal vulnerability, at least in some tissues (Jiang et al., 1992; Ballanyi and Kulik, 1998; Pek-Scott and Lutz, 1998; Garcia de Arriba et al., 1999; Centonze et al., 2001; Ballanyi, 2004).

K_{ATP}-channels appear to be present and cause hyperpolarization also of cerebellar neurons in eider ducks

We have here shown that diazoxide caused diminished spontaneous neuronal activity in the Purkinje layer of normoxic isolated cerebellar slices from eider ducks, while tolbutamide prevented this effect (Fig. 2). In addition, although statistically non-significant, tolbutamide tended to cause anoxia-silenced slices to resume some activity, and was responsible for a significantly poorer recovery upon reoxygenation (Fig. 4). This suggests that K_{ATP} channels also contribute to regulate neuronal excitability during hypoxia in the CNS (cerebellum) of birds, as they have also previously been shown to do in other non-mammalian species (Jiang et al., 1992; Pek-Scott and Lutz, 1998).

Although our data suggest that K_{ATP} channels may provide some temporary protection against energy deprivation in the anoxic eider duck cerebellum, the effect of tolbutamide was quite weak, which implies that additional factors are probably involved in the observed shut-down response to hypoxia (Ludvigsen and Folkow, 2009). Preliminary results from experiments with hooded seals (*Cystophora cristata*), which also display enhanced inherent neuronal hypoxia tolerance (Folkow et al., 2008), reveal a similar, quite weak cerebellar response to tolbutamide (Ludvigsen and Folkow, unpublished observations). This is in contrast to rodents, in which

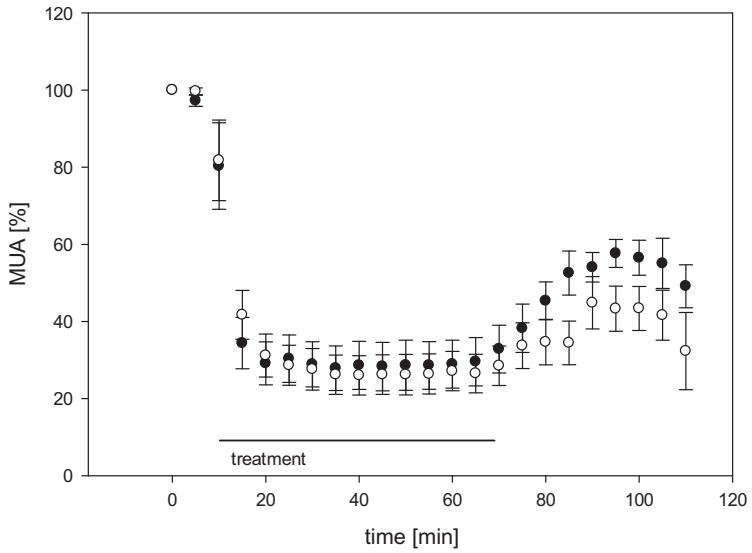

Fig. 4. Mean ± SD spontaneous multi-unit neuronal activity (MUA), in percent of mean MUA in normoxia, in isolated cerebellar slices from eider ducks before, during and after a 60-min anoxia challenge (95% N_2–5%CO_2 + 2 mM NaCN), under control conditions (0.8 ml DMSO added) (black; 0–100 min: n = 8, 105 min: n = 5, 110 min: n = 3), and in the presence of tolbutamide (400 μM) (white; 0–100 min: n = 10, 105 min: n = 8, 110 min: n = 5). Tolbutamide was added after introduction of anoxia. Adding tolbutamide prior to anoxia did not change the result (see text).

suppression of anoxia-induced hyperpolarization by use of K_{ATP} channel blockers significantly alters the typical hypoxic response and increases neuronal vulnerability (Jiang et al., 1992; Garcia de Arriba et al., 1999; Ballanyi, 2004). Moreover, in other hypoxia-tolerant species (turtles), activation of K_{ATP} channels in anoxia has been shown to provide initial protection from anoxia-induced damages, until other hypometabolic mechanisms that allow maintenance of adequate intracellular ATP-levels are activated (Pek-Scott and Lutz, 1998).

Role of cell membrane-based K_{ATP} channels versus mitochondrial membrane-based K_{ATP} channels (mitoK_{ATP})

In the brain, K_{ATP} channels in the cellular membrane of neurons play key roles in regulating neuronal excitability and synaptic transmission (Cowan and Martin, 1992; Wind et al., 1997; Erdemli et al., 1998; Zawar et al., 1999; Ballanyi, 2004; Yamada and Inagaki, 2005), but in relation to protection toward (hypoxia-induced) lack of energy it has been suggested that mitoK_{ATP} may also play a central role, by reducing mitochondrial Ca^{2+} accumulation and the induction of reactive oxygen species (Domoki et al., 1999; Nicholls and Budd, 2000; Bajgar et al., 2001; Liu et al., 2002, 2003; Virgili et al., 2013; Ren et al., 2014). The same issue has been studied in cardiomyocytes in relation to the role of preconditioning in protection against damage during ischemia–reperfusion (I/R), and it seems widely accepted that mitoK_{ATP} channels are responsible for this defense to I/R damage (Xu et al., 2001; Zhuo et al., 2005), even though sarcolemmal K_{ATP} channels are essential for cardioprotection in ischemia (Quindry et al., 2012). Indications that brain mitochondria contain 6–7 times more mitoK_{ATP} per milligram of mitochondrial protein than liver or heart (Bajgar et al., 2001) may indeed be seen as support of the hypothesis that mitoK_{ATP} channels are just as important in the brain as in the heart in this respect. Another hypothesis suggests that K_{ATP} channels from different organelles within one cell collaborate to regulate cell energy metabolism, and that in case of energy crisis, all channels are ultimately involved in the response to the insult (Zhuo et al., 2005). The distribution density of K_{ATP} channels in different cell types in the brain vary (Dunn-Meynell et al., 1998; Karschin et al., 1998; Zawar et al., 1999; Yamada and Inagaki, 2005), and it has been speculated that there may be a direct relationship between hypoxia tolerance and K_{ATP} channel density in the individual cell types (Zawar et al., 1999). Such relationships remain to be elucidated in the eider duck, but could contribute to explain the weak effects observed in the present study.

CONCLUSION: K_{ATP} CHANNELS APPEAR TO MAKE A LIMITED CONTRIBUTION TO PROTECT CEREBELLAR NEURONS OF EIDER DUCKS FROM HYPOXIC INJURY

Our results with isolated cerebellar slices from eider ducks show that the spontaneously active cerebellar neurons of this avian diver possess K_{ATP} channels. However, these do not appear to be responsible for the hypoxia-induced cessation of spontaneous activity that may lend this tissue its enhanced hypoxia tolerance (Ludvigsen and Folkow, 2009), since adding the K_{ATP} channel blocker tolbutamide had no or only a weak effect on the neuronal hypoxic response, regardless of whether the drug was added before or during hypoxia exposure. Therefore, the mechanism underlying the presumed protective shutdown response must be sought elsewhere, possibly among candidates such as KCa channels (Yamada and Inagaki, 2005), enhanced release of inhibitory neurotransmitters and/or reduced release of excitatory neurotransmitters (Lutz and Nilsson, 2004).

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