Properties and Expression of Na\(^{+}/K^{+}\)-ATPase \(\alpha\)-Subunit Isoforms in the Brain of the Swamp Eel, *Monopterus albus*, Which Has Unusually High Brain Ammonia Tolerance

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Abstract

The swamp eel, *Monopterus albus*, can survive in high concentrations of ammonia (>75 mmol l\(^{-1}\)) and accumulate ammonia to high concentrations in its brain (~4.5 \(\mu\)mol g\(^{-1}\)). Na\(^{+}/K^{+}\)-ATPase (Nka) is an essential transporter in brain cells, and since NH\(_4\)\(^+\) can substitute for K\(^+\) to activate Nka, we hypothesized that the brain of *M. albus* expressed multiple forms of Nka \(\alpha\)-subunits, some of which might have high K\(^+\) specificity. Thus, this study aimed to clone and sequence the nka \(\alpha\)-subunits from the brain of *M. albus*, and to determine the effects of ammonia exposure on their mRNA expression and overall protein abundance. The effectiveness of NH\(_4\)\(^+\) to activate brain Nka from *M. albus* and *Mus musculus* was also examined by comparing their Na\(^{+}/K^{+}\)-ATPase and Na\(^+\)/NH\(_4\)\(^+\)-ATPase activities over a range of K\(^+\)/NH\(_4\)\(^+\) concentrations. The full length cDNA coding sequences of three nka\(_{1}\) (nka\(_{1a}\), nka\(_{1b}\) and nka\(_{1c}\)) were identified in the brain of *M. albus*, but nka\(_{2}\) expression was undetectable. Exposure to 50 mmol l\(^{-1}\) NH\(_4\)Cl for 1 day or 6 days resulted in significant decreases in the mRNA expression of nka\(_{1a}\), nka\(_{1b}\) and nka\(_{1c}\). The overall Nka protein abundance also decreased significantly after 6 days of ammonia exposure. For *M. albus*, brain Na\(^+\)/NH\(_4\)\(^+\)-ATPase activities were significantly lower than the Na\(^+\)/K\(^+\)-ATPase activities assayed at various NH\(_4\)\(^+\)/K\(^+\) concentrations. Furthermore, the effectiveness of NH\(_4\)\(^+\) to activate Nka from the brain of *M. albus* was significantly lower than that from the brain of *M. musculus*, which is ammonia-sensitive. Hence, the (1) lack of nka\(_{2}\) expression, (2) high K\(^+\) specificity of K\(^+\)-binding sites of Nka1, Nka2a and Nka2b, and (3) down-regulation of mRNA expression of all three nka1 isoforms and the overall Nka protein abundance in response to ammonia exposure might be some of the contributing factors to the high brain ammonia tolerance in *M. albus*.

Introduction

Ammonia plays a crucial role in the maintenance of nitrogen homeostasis in almost all living organisms; but it is toxic if allowed to accumulate in the body. High concentration of ammonia affects the central nervous system, resulting in several neurological abnormalities [1] characterized by hyperactivity, convulsions, coma and eventually death [2]. Mammals, including humans, develop encephalopathy when brain ammonia content reaches 1–3 \(\mu\)mol g\(^{-1}\) [1], and ammonia remains as the leading candidate in the pathogenesis of hepatic encephalopathy in acute liver failure. Hepatic encephalopathy, if left untreated, can lead to hepatic coma and death. Several classical theories have been proposed to address the pathological consequences of increased ammonia concentration and the consequential changes in nitrogen metabolism in mammalian brains. These include glutamatergic dysfunction, glutamine accumulation leading to astrocyte swelling, and/or activation of N-methyl-D-aspartate (NMDA)-type glutamate receptors leading to ammonia-induced membrane depolarization [3,4]. Excessive activation of NMDA-type glutamate receptors is neurotoxic, resulting in oxidative stress and subsequent degeneration and death of neurons [5–7]. Recent findings have pointed to an important role of glutamine-mediated oxidative/nitrosative stress [8,9] and/or mitochondrial permeability transition [10] in the pathogenesis of cerebral ammonia toxicity.

Fishes are generally more tolerant to high internal ammonia concentrations than terrestrial vertebrates [11], but they are not exempted from the deleterious effects of high concentrations of ammonia on various cellular processes [12–14]. Fully aquatic fishes keep body ammonia levels low by excreting excess ammonia, mainly as NH\(_3\), through their gills [15]. However, some fishes dwelling in habitats with low oxygen tension have acquired the ability to breathe air, and air-breathing sometimes leads to degenerate gills with reduced functions [16]. Air-breathing
fishes can be exposed to environmental ammonia when stranded in puddles of water during dry season, during a stay inside a burrow, or after agricultural fertilization. Under these conditions, accumulation of endogenous and exogenous ammonia would occur, resulting in high concentrations of ammonia in the blood and various organs. Therefore, some air-breathing fishes have developed mechanisms to defend against ammonia toxicity at the branchial/epithelial surfaces through active ammonia excretion, lowering of the external pH, reducing ammonia permeability or ammonia volatilization [12-14]. In others, defence against ammonia toxicity can also take place at the cellular level by detoxifying ammonia to other nitrogenous compounds (e.g. glutamine or urea) or developing high cell/tissue ammonia tolerance [12-14].

The swamp eel, Monopterus albus (Zuiew, 1793), is an anguilliform bony fish, belonging to the family Synbranchidae, order Synbranchiformes, and class Actinopterygii. It is an obligate air-breather with degenerate gills which have been reduced to an opercular skin-fold [16]. Its natural habitat includes muddy ponds, swamps, canals, and rice fields [17], where it burrows in moist earth for long periods during drought [18]. It may also encounter high concentrations of environmental ammonia (90 mmol l⁻¹) [19] in rice fields during agricultural fertilization. Notably, the 48-h, 72-h and 96-h median lethal concentrations (LC50) of total NH₄Cl in rice fields during agricultural fertilization. Notably, the 48-h, 72-h and 96-h LC50 of total ammonia for M. albus are 209.9 mmol l⁻¹, 198.7 mmol l⁻¹ and 193.2 mmol l⁻¹, respectively [20], which are much higher than those for other fishes, many of which would succumb to <5 mmol l⁻¹ NH₄Cl. The LC50 for M. albus are even higher than those for some other tropical fishes known to have high environmental ammonia tolerance [21]. For instance, the 96-h LC50 of total ammonia for the giant mudskipper Periophthalmodon schlosseri, and the Boddart’s goggle-eyed mudskipper, Boleophthalmus boddaerti, are 115 mmol l⁻¹ and 13.8 mmol l⁻¹, respectively [22]. Furthermore, M. albus can tolerate extremely high levels of ammonia in its organs, especially the brain, during emersion or exposure to environmental ammonia [20,23,24]. After 144 h of exposure to 75 mmol l⁻¹ NH₄Cl at pH 7.0, ammonia concentration in the muscle, liver, brain and gut of M. albus reach 11.5, 15.2, 6.5 and 7.5 mmol g⁻¹, respectively [20]. More intriguingly, after an intraperitoneal injection of a sublethal dose of ammonium acetate, M. albus can tolerate extremely high levels of ammonia in its brain when confronted with high brain ammonia concentrations. Finally, an attempt was made to determine whether the abundance of Nka in the brain of M. albus was also examined through immunoblotting using commercially available anti-NKA antibodies. In addition, efforts were made to evaluate the differences between the effectiveness of NH₄⁺ and K⁺ to activate Nka from the brain of M. albus kept in freshwater or exposed to ammonia. Finally, an attempt was made to determine whether there was any difference between the effectiveness of NH₄⁺, in substitution of K⁺, to activate Nka from the brain of M. albus and that from the brain of the mouse, Mus musculus.

Materials and Methods

Ethics Statement

Approval to undertake this study was obtained from the Institutional Animal Care and Use Committee of the National University of Singapore (IACUC 021/10, protocol for M. albus; C11/09, protocol for M. musculus).

Animals

Specimens of M. albus (150-250 g) were purchased from a local fish distributor in Singapore. Fish were maintained in plastic tanks in freshwater at 25°C under a 12 h: 12 h dark: light regime. No aeration was provided because M. albus is an obligate air-breather. No attempt was made to separate the sexes. Fish were acclimated to laboratory conditions for at least 1 week before experimentation. Food was withheld during the experimental period. Specimens of M. musculus were obtained and maintained by the Animal Holding Unit, National University of Singapore.
Experimental Conditions and Collection of Samples

Control fish (total \( N = 18 \); \( N = 5 \) each for ammonia assay, Nka assay and molecular work, and \( N = 3 \) for Western blot) were immersed in 25 volumes (v/v) of freshwater in plastic tanks with free access to air. Fish subjected to ammonia exposure were immersed in freshwater containing 50 mmol l\(^{-1}\) NH\(_4\)Cl (pH 7), for either 1 day or 6 days (total \( N = 18 \) for each group). Control fish and fish exposed to ammonia were killed with an overdose of neutralized MS-222 (0.2%) followed with a strong blow to the head. The whole brain from an individual fish was quickly excised within 2 min, frozen in liquid nitrogen and stored at \(-80^\circ C\) until further analysis. For the measurement of Nka activity, the entire brain was suspended in 1 ml of solution containing 100 mmol l\(^{-1}\) imidazole-HCl (pH 7.2), 300 mmol l\(^{-1}\) sucrose, 20 mmol l\(^{-1}\) ethylenediamine tetraacetic acid (EDTA) following the method of Zaugg [33], frozen in liquid nitrogen and stored at \(-80^\circ C\) until further analysis.

Mice (\( N = 5 \)) were euthanized using carbon dioxide gas and regarded as dead when there was no observable respiratory activity and no reaction to mechanical stimulation. The whole brain was quickly excised within 2 min and suspended in 1 ml of Zaugg’s solution [39], frozen in liquid nitrogen and stored at \(-80^\circ C\) until the assay of NKA activity.

Determination of Ammonia Concentrations in the Brain

The frozen brain samples of \( M. \) albus were weighed, ground to a powder in liquid nitrogen, and homogenized three times in 5 volumes (v/w) of ice-cold 6% perchloric acid at 24,000 rpm for 20 s each using an Ultra-Turrax homogenizer (Ika-werk, Staufen, Germany) with intervals of 10 s between each homogenization. The homogenate was centrifuged at 10,000 \( g \) for 30 min to obtain the supernatant. The pH of the supernatant obtained was adjusted to between 6.0 and 6.5 with 2 mol l\(^{-1}\) NaOH solutions. The homogenate was then centrifuged at 12,000 \( g \) for 2 min at \( 4^\circ C \) to obtain the pellet. The pellet was re-suspended in 1 ml of homogenizing buffer containing 100 mmol l\(^{-1}\) imidazole-HCl (pH 7.2), 300 mmol l\(^{-1}\) sucrose, and 1 g l\(^{-1}\) of sodium deoxycholate (without EDTA), which interfered with the subsequent phosphate analysis, and homogenized twice at 15,500 rpm for 10 s each with an interval of 10 s. The supernatant obtained was centrifuged for 6 min at 2,000 \( g \) and 4 \( ^\circ C \). The supernatant obtained was assayed for Nka activity.

Determination of Nka/NKA Activity

Frozen brain samples of both \( M. \) musculus and \( M. \) albus were thawed on ice and homogenized for 2 s at 7,000 rpm using an Ultra-Turrax homogenizer. The homogenate was then centrifuged at 2000 \( g \) for 7 min at 4 \( ^\circ C \) to obtain the pellet. The pellet was re-suspended in 1 ml of homogenizing buffer containing 100 mmol l\(^{-1}\) imidazole-HCl (pH 7.2), 300 mmol l\(^{-1}\) sucrose, and 1 g l\(^{-1}\) of sodium deoxycholate (without EDTA), which interfered with the subsequent phosphate analysis, and homogenized twice at 15,500 rpm for 10 s each with an interval of 10 s. The supernatant obtained was centrifuged for 6 min at 2,000 \( g \) and 4 \( ^\circ C \). The supernatant obtained was assayed for Nka activity on the same day. Brain samples were pre-incubated at 25 \( ^\circ C \) for 10 min in the presence of 30 mmol l\(^{-1}\) imidazole-HCl buffer (pH 7.2) and 100 mmol l\(^{-1}\) NaCl, 20 mmol l\(^{-1}\) KCl and 5 mmol l\(^{-1}\) MgCl\(_2\), in the presence or absence of 3 mmol l\(^{-1}\) ouabain. The reaction was subsequently initiated by the addition of 0.05 ml of 3.5 mmol l\(^{-1}\) ATP (pH 7.0), incubated at 25 \( ^\circ C \) and the reaction terminated by the addition of 0.05 ml of ice-cold 100% trichloroacetic acid. The Nka/NKA activity was calculated as a difference of activities assayed in the presence and absence of ouabain.

The reaction mixture was centrifuged at 12,000 \( g \) for 2 min at 4 \( ^\circ C \). The amount of inorganic phosphate (Pi) released from ATP during the incubation period represented the activity of Nka/NKA. An aliquot (0.4 ml) of the supernatant was diluted with 4 volumes of 100 mmol l\(^{-1}\) sodium acetate for Pi assay. To this diluted aliquot, 0.2 ml of 1% ascorbic acid and 0.2 ml of 1% ammonium molybdate in 0.025 mol l\(^{-1}\) H\(_2\)SO\(_4\) were added. Absorbance was determined at 700 nm using a UV160 UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan), and the Pi concentration calculated with reference to a standard made from K\(_2\)HPO\(_4\) and assayed in the presence of trichloroacetic acid and sodium acetate. The protein content of the sample was determined by the method of Bradford [35]. Bovine gamma globulin dissolved in 25% glycerol was used as a standard. The activity of Nka is expressed as \( \mu \)mol Pi released min\(^{-1}\) mg\(^{-1}\) protein. To evaluate if there were changes in the affinity of Nka/NKA to its substrates (i.e. K\(^{+}\) or NH\(_4\)\(^{+}\)), enzyme activities were also determined at various sub-saturating substrate concentrations (1, 2.5, 5, 10 or 20 mmol l\(^{-1}\) of KCl or NH\(_4\)Cl. The effectiveness of substitution by NH\(_4\)\(^{+}\) is expressed as the ratio of Na\(^{+}\)/NH\(_4\)\(^{+}\)-ATPase activity to Na\(^{+}\)/K\(^{+}\)-ATPase activity at various K\(^{+}\) or NH\(_4\)\(^{+}\) concentrations.

Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from brain samples of \( M. \) albus using Tri Reagent\textsuperscript{TM} (Sigma-Aldrich Co., St. Louis, MO, USA) and further purified using the RNeasy Plus Mini Kit (Qiagen GmbH, Hilden, Germany). After extraction, RNA was quantified spectrophotometrically using a Helma TrayCell (Helma GmbH & Co. KG, Mullheim, Germany) and checked electrophoretically to verify the RNA integrity. Total RNA (1 \( \mu \)g) isolated from the brain of \( M. \) albus was reverse transcribed into first strand cDNA using oligo (dT)\(_{18}\) primers and the RevertAid\textsuperscript{TM} First Strand cDNA synthesis kit (Fermentas International Inc., Burlington, ON, Canada) following the manufacturer’s protocol.

Polymerase Chain Reaction (PCR) and Cloning

Partial nka sequences were obtained using the primers (Forward: 5’-CAC TTC ATC CAC ATC ATC AC-3’; Reverse: 5’-ATG GCA GGG AAC CAT GTC-3’) designed from the highly conserved regions based on multiple alignments of the nka1, nka2 and nka3 sequences from various animal and fish species available in Genbank (http://www.ncbi.nlm.nih.gov/Genbank). PCR was carried out in a Bio-Rad Peltier thermal cycler (Bio-Rad Laboratories, Hercules, CA) using DreamTaq\textsuperscript{TM} DNA polymerase (Fermentas International Inc.) under the following cycling conditions: 95 \( ^\circ C \) for 3 min, followed by 35 cycles of 95 \( ^\circ C \) for 30 s, 55 \( ^\circ C \) for 30 s, 72 \( ^\circ C \) for 2 min and a final cycle of extension at 72 \( ^\circ C \) for 10 min. PCR products were separated by electrophoresis in 1% agarose gel. Bands of the expected sizes (\( ~3000 \) bp) were excised and purified from the gel using FavorPrep\textsuperscript{TM} Gel Purification Mini Kit (Favorgen Biotech Corp., Ping-Tung, Taiwan) according to the manufacturer’s protocol. Purified PCR products were ligated into pGEM-T easy vector (Promega Corporation, Madison, WI, USA), transformed into JM109 Escherichia coli competent cells and plated onto Luria-Bertani (LB) agar with ampicillin, IPTG and X-gal. Colony-PCR was performed on selected white colonies. Colonies with insert of estimated size were grown overnight in LB/ampicillin broth in a shaking incubator (37 \( ^\circ C \), 250 rpm). Plasmid extraction was performed using AxyPrep\textsuperscript{TM} Plasmid Miniprep Kit (Axygen Biosciences, Union City, CA, USA). Multiple clones of each fragment were sequenced bidirectionally by cycle sequencing using BigDye\textsuperscript{®} Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA, USA), and subsequently purified by ethanol/sodium acetate precipitation. Purified products were automatically sequenced using the 3130XL Genetic Analyzer (Applied Biosystems Inc.). The fragments were verified to be nka z-subunit isoforms from GenBank database. Cloning results.
obtained from the sequencing of the extracted plasmid inserts indicated the presence of three isoforms of nka α-subunits (nka1 and nka2α and nka3α).

**Rapid Amplification of cDNA Ends (RACE)**

Total RNA (1 µg) isolated from the brain of *M. albus* was reverse transcribed into 3′-RACE-Ready cDNA and 3′-RACE-ready cDNA using the SMARTer™ RACE cDNA Amplification kit (Clontech Laboratories, Mountain View, CA, USA). RACE-PCR was performed using Advantage® 2 PCR kit (Clontech Laboratories) to generate the 5′ and 3′ cDNA fragments. RACE primers (Table 1) were designed based on the partial cDNA sequences obtained for all three isoforms of nka α-subunits. RACE-PCR cycling conditions were: 25 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 4 min. RACE-PCR products were separated using gel electrophoresis, purified and sequenced.

**Deduced Amino Acid Sequences and Phylogenetic Analysis**

The partial fragments of nka1, nka2α, and nka3β obtained were aligned using BioEdit version 7.0.9 [36] to obtain their full-length nucleotide coding sequences, which were subsequently translated into deduced amino acid sequences using ExPASy Proteomic server (http://web.expasy.org/translate/). The deduced amino acid sequences were aligned and compared with selected Nka/NKA (http://genome.jp/tools/clustalw/). The deduced amino acid sequences using ExPASy Proteomic server into deduced amino acid sequences using ExPASy Proteomic server nucleotide coding sequences, which were subsequently translated aligned using BioEdit version 7.0.9 [36] to obtain their full-length a

### Table 1. Primer sequences for RACE and quantitative (q) RT-PCR.

| Gene   | Primer type | Primer sequence (5′-3′) |
|--------|-------------|------------------------|
| nka1   | RACE-PCR    | 5′-RACE GTC TCT CTT CAG GAT GGG AAT GTT GC |
|        | 3′-RACE CTT CCT GGC GCA GGA GAG CAA CA |
|        | q RT-PCR    | Forward GTT GCT TCT CCT ACT ACC AAG AG |
|        | Reverse ATC ACC AAC CAC TAC ATC CT |
| nka2α  | RACE-PCR    | 5′-RACE GCG CTT AAG GAT GGG CAA AGA TTC |
|        | 3′-RACE TCA CGA AAC TGA GGA TGA AAA TGA CAA T |
|        | q RT-PCR    | Forward AGT GGG TCA AGT TGC TGT CT |
|        | Reverse GTC GGC TGG TGC TGC TCT GTG |
| nka3β  | RACE-PCR    | 5′-RACE GAT CTT GGT CTT CAT GGC CGT CT |
|        | 3′-RACE GTG ATG TGG TGA GTG ATG AGT |
|        | q RT-PCR    | Forward GTG CTT GGT CTT GAT TGA TGG |
|        | Reverse GGC GTG GTC ATT AGG ATC TTC TG |

Primer used for RACE and q RT-PCR of Na+/K+-ATPase (nka) α-subunit isoforms from the brain of *Monopterus albus*. doi:10.1371/journal.pone.0084296.t001

**Tissue Expression of nka1, nka2α and nka3β**

Total RNA (1 µg) isolated from the brain, operculum membrane, liver, anterior gut, posterior gut, kidney and skin of *M. albus* kept in freshwater were reverse transcribed into cDNA using oligo(dT)12 primer and the RevertAid™ first strand cDNA synthesis kit (Fermentas International Inc.). PCR was performed on the cDNAs of these tissues using the specific qPCR primers (Table 1) to detect the mRNA expression of each gene in various tissues. Each PCR was carried out in 10 µl reaction volumes using DreamTaq polymerase (Fermentas International Inc.) with thermal cycling conditions: 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final extension of 72°C for 10 min. PCR products were then separated by electrophoresis in 2% agarose gel.

**Quantitative Real-time PCR (qPCR)**

Total RNA (1 µg) from the brain samples of *M. albus* was reverse transcribed using random hexamer primers with RevertAid™ first strand cDNA synthesis kit (Fermentas International Inc.). qPCR was performed in triplicates using a StepOnePlus™ Real-Time PCR System (Life Technologies Corporation, Carlsbad, California). The mRNA expression of nka1, nka2α and nka3β in the brain of *M. albus* were determined using specific qPCR primers (Table 1). For nka1, the specificity of each pair of qPCR primers was verified by PCR using three different plasmid clones containing fragments of nka1, nka2α and nka3β as templates. The identities of these plasmid clones had been verified through cloning and sequencing (see above). The specificity of each pair of primers was demonstrated by the presence of a single band using the plasmid clones of the targeted nka isoform as the template and the absence of detectable band using the plasmid clones of the other two isoforms.

Since it is essential to compare the mRNA expression of the three nka isoforms in the brain of *M. albus*, the method of absolute quantification with reference to a standard curve was adopted in this study. Relative quantitation methods produce only fold-change data, which do not allow the interpretation of which isoform being the predominant one being expressed in a certain condition. Although absolute quantification provides more information, it is considered to be more labor-intensive than relative quantification. Absolute quantification is not commonly adopted because of the necessity to create reliable standards for quantification and include these standards in every PCR. Therefore, to determine the absolute quantity of transcripts of each of the 3 nka in a qPCR reaction, efforts were made to produce a pure amplicon (standard) of a defined region of each of the 3 cDNA from the brain of *M. albus* following the method of Gerwick et al. [39]. The
amplicon sizes were 137 bp, 113 bp and 122 bp for Nkaα1, Nkaα3a and Nkaα3b, respectively. PCR was performed with a specific set of qPCR primers (Table 1) and cDNA as a template in a final volume of 25 μl with the following cycling conditions: initial denaturation 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 1 min 30 s and 1 cycle of final extension of 72°C for 10 min. The PCR product was separated in a 2% agarose gel then excised and purified using FavorPrepTM Gel Purification Mini Kit (Favorgen Biotech Corp., Ping-Tung, Taiwan). The nucleotide fragment in the purified product was cloned using pGEM®-T Easy vector (Promega Corporation). The presence of the insert in the recombinant clones was confirmed by sequencing. The cloned circular plasmid was quantified using a spectrophotometer with a Hellma TrayCell.

The standard cDNA (template) was serially diluted (from 10⁶ to 10² specific copies per 2 μl). The PCR reactions contained 5 μl of 2 x Fast SYBR® Green Master Mix (Life Technologies Corporation), 0.2 pmol μl⁻¹ of forward and reverse primers each (Table 1) and 1 ng of sample cDNA or various quantities of standard in a total volume of 10 μl. Cycling conditions were 95°C for 20 s (1 cycle), followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. Data (Ct values) were collected at each elongation step. A melt curve analysis was performed after each run by increasing the temperature from 60°C to 95°C in 0.3°C increments to confirm the presence of only a single product. The PCR products obtained were also separated in a 2% agarose gel to verify the presence of a single band. A standard curve was obtained from plotting threshold cycle (Ct) on the Y-axis and the natural log of concentration (copies μl⁻¹) on the X-axis. The Ct slope, PCR efficiency, Y-intercept and correlation coefficient (R²) were calculated using the default setting of StepOneTM Software v2.1 (Life Technologies Corporation). Diluted standards were stored at −20°C. The amplification efficiencies for Nkaα1, Nkaα3a and Nkaα3b were 91.2%, 97.1% and 83.8%, respectively. The quantity of the target sequence in a sample was determined from the linear regression line derived from the standard curve and expressed as copy number per ng cDNA. Copy numbers were calculated from the Ct values of the standards.

Western Blot

Brain samples of M. albus were homogenized three times in five volumes (v/w) of ice cold buffer containing 50 mmol l⁻¹ Tris HCl, (pH 7.4), 1 mmol l⁻¹ EDTA, 150 mmol l⁻¹ NaCl, 1 mmol l⁻¹ NaF, 1 mmol l⁻¹ Na₂VO₄, 1% NP-40, 1% sodium deoxycholate, 1 mmol l⁻¹ PMSF, and 1xHALT protease inhibitor cocktail (Pierce, Rockford, USA) at 24,000 rpm for 20 s each with 10 s intervals using the Polytron PT 1300D homogenizer (Kinematica, Luzern, Switzerland). The homogenate was centrifuged at 10,000 x g for 20 min at 4°C. The protein concentration in the supernatant obtained was determined according to the method of Bradford [35] and adjusted to 2 μg μl⁻¹ with Laemmli buffer [40]. Samples were heated at 70°C for 15 min, and then kept at −80°C until analysis.

Proteins were separated by SDS-PAGE (8% acrylamide for resolving gel, 4% acrylamide for stacking gel) under conditions as described by Laemmli [41] using a vertical mini- slab apparatus (Bio-Rad Laboratories). Proteins were then electrophoretically transferred onto PVDF membranes using a transfer apparatus (Bio-Rad Laboratories). After transfer, membranes were blocked with 10% skim milk in TTBS (0.05% Tween 20 in Tris-buffered saline: 20 mmol l⁻¹ Tris-HCl; 500 mmol l⁻¹ NaCl, pH 7.6) for 1 h before being incubated overnight at 4°C with NKAα3 specific anti-NKA antibody (1:800 dilution; Y-13, Santa Cruz Biotechnology Inc., Texas, USA) or NKAα5 antibody (1:600 dilution; Developmental Studies Hybridoma Bank/DSHB, Iowa City, IA, USA) or pan-actin antibody (1:5000 dilution; Thermo Fisher Scientific, United Kingdom). The anti-NKAα5 antibody was developed by Douglas M. Farmbrough (Johns Hopkins University, MD, USA) and is known to react pan-specifically with Nka α-subunit isoforms in fish and other animals. All primary antibodies were diluted in 1% bovine serum albumin in TTBS. The membranes were then incubated in horseradish peroxidase-conjugated secondary antibodies (anti-goat for NKAα3; 1:40,000 dilution; goat anti-mouse for NKAα5 and pan-actin; 1:10,000; Santa Cruz Biotechnology Inc.) for 1 h at room temperature. Bands were visualized by chemiluminescence (Western Lightning™, PerkinElmer Life Sciences, Boston, MA, USA) using X-ray film (Thermo Scientific) and were processed by a Kodak X-OMat 3000 RA processor (Kodak, Rochester, NY, USA). The films were scanned using CanonScan 4400F flat bed scanner in TIFF format at 300 dpi resolution. Densitometric quantification of band intensities were performed using ImageJ (version 1.40, NIH), calibrated with a 37 step reflection scanner scale (#R3703-1C; Stouffer Graphic Arts, South Bend, IN, USA). Results were presented as relative protein abundance of Nka normalized with actin.

Statistical Analysis

Results were presented as means ± standard errors of the mean (S.E.M.). Differences between means were evaluated using one-way analysis of variance (ANOVA), followed by multiple comparisons of means by Tukey’s post-hoc test. Ratios were processed with arcsine transformation before statistical analysis. Differences were regarded as statistically significant at P<0.05.

Results

Ammonia Concentration in the Brain of M. albus exposed to Ammonia

After 1 day or 6 days of exposure to 50 mmol l⁻¹ NH₄Cl, the concentration of ammonia in the brain of M. albus (N = 5 for each group) increased significantly to 2.85±0.43 and 4.38±0.82 μmol g⁻¹, respectively, as compared with that of the freshwater control (N = 5; 0.83±0.21 μmol g⁻¹). No mortality of experimental fish was recorded.

Nucleotide Sequences of nkaz and Phylogenetic Analysis of the Deduced Nkaz Amino Acid Sequences

Three different nkaz α-subunit isoforms were cloned and sequenced from the brain of M. albus. The complete cDNA coding sequence of nkaz1 [GenBank: KC620448] consisted of 3078 bp, coding for 1025 amino acids, with an estimated molecular mass of 113 kDa. Similarly, the full length of nkaz3a [GenBank: KC620449] cDNA sequence was comparable to that of nkaz1 with 3069 bp and coded for 1022 amino acids, with an estimated molecular mass of 113 kDa. However, the full cDNA coding sequence of nkaz3b [GenBank: KC620450] was longer at the 3' end, containing 3282 bp which translated into 1093 amino acids, with a calculated molecular mass of 120 kDa.

A phylogenetic analysis confirmed that Nka3b of M. albus was grouped together with teleost Nka3l and is distinct from various Nka2 and Nka3 isoforms (Fig. 1). On the other hand, both Nka3a and Nka3b of M. albus were closely related to teleost Nka3 instead of Nka3l and Nka32 isoforms (Fig. 1).

The deduced amino acid sequences of Nka3l, Nka3a and Nka3b from the brain of M. albus had ten predicted transmembrane domains and an alignment of these three deduced amino acid sequences, together with those of O. mossambicus, X. laevis, R. norvegicus and H. sapiens, revealed large areas of conserved regions (Fig. 2; Fig. S1). Based on the homology modeling of human NKA
Figure 1. Phylogenetic analysis of Na\(^+\)/K\(^+\)-ATPase (Nka) \(\alpha1\), Nka\(\alpha3a\) and Nka\(\alpha3b\). A phylogenetic tree to illustrate the relationship between Nka1, Nka3a and Nka3b from the brain of Monopterus albus and Nka of selected teleost species. Numbers presented at each branch point represent bootstrap values from 100 replicates. Ciona intestinalis Nka is used as an outgroup.

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α-subunit [41], three Na\(^+\) and two K\(^+\) binding sites are known to be present in the NKA α-subunit. Indeed, the coordinating residues responsible for Na\(^+\) or K\(^+\) binding were found to be highly conserved across all three Nka α-subunit isoforms present in the brain of M. albus (Fig. 2). A region containing a lysine-rich sequence that plays a critical role in cation binding and occlusion [42] was also present in all three isoforms of the Nka α-subunits with Nkaα1 containing the greatest number of lysine residues. In addition, potential phosphorylation sites that could serve as targets for protein kinase A [43] were present in all three Nka α-subunit isoforms. While potential targets for regulatory phosphorylation by protein kinase C [43] were found to be present in Nkaα2a and Nkaα2b (Fig. 2).

Comparison of Nka α-subunits from the Brain of M. albus with those from the Gills of Anabas testudineus

An alignment of the amino acid sequences of Nkaα1, Nkaα3a and Nkaα3b from the brain of M. albus, with Nkaα1a, Nkaα1b and Nkaα1c from the gills of A. testudineus [44] revealed that all of them shared the highest percentage similarity with Nkaα1c (Table 2). More importantly, a detailed analysis of the amino acid residues which constitute one of the K\(^+\)-binding sites revealed that those of Nkaα1, Nkaα3a and Nkaα3b from the brain of M. albus were identical to Nkaα1c, but distinct from those of Nkaα1a and Nkaα1b from the gills of A. testudineus (Fig. 5).

Tissue Expression of the Three nka α-subunit Isoforms

nka3 was expressed in the brain, operculum membrane, liver, anterior gut, posterior gut and kidney, but not the skin, of M. albus kept in freshwater (Fig. 4). By contrast, nka3a was expressed only in the brain. On the other hand, nka3b was detected readily in the brain, operculum membrane, kidney and anterior gut, but weakly in the operculum membrane, posterior gut and skin (Fig. 4).

Effects of Exposure to Environmental Ammonia on the mRNA Expression of the Three nka α-subunit Isoforms in the Brain of M. albus

In the brain of M. albus kept in freshwater, the quantity (copies per ng cDNA) of nka3a was the highest (~20,000 copies), followed by nka1 (~13,000 copies) and nka3b (~5,000 copies). Ammonia exposure led to significant decreases in the mRNA expression of all three nka α-subunit isoforms in the brain of M. albus. After 1 and 6 days of exposure to 50 mmol l\(^{-1}\) NH\(_4\)Cl, the mRNA expression of nka1 decreased by 77.7% and 50.4%, respectively (Fig. 3A). The corresponding decreases in mRNA expression were 68.7% and 48.4% for nka3a (Fig. 3B) and 79.4% and 69.3% for nka3b (Fig. 3C).

Overall Protein Abundance of Nka α-subunit during Environmental Ammonia Exposure

The protein abundance of Nka α-subunit (Fig. 6) from the brain of M. albus, based on the α3 anti-NKA antibody which is pan-specific for Nka α-subunit isoforms, and that of Nkaα3 (Fig. 7), based on α3-specific antibody, decreased significantly after exposure to 50 mmol l\(^{-1}\) of NH\(_4\)Cl for 6 days as compared with the freshwater control.

Effectiveness of NH\(_4\)\(^+\), Substituting for K\(^+\), to Activate Nka/NKA from the Brains of M. albus and M. musculus

The Na\(^+\)/NH\(_4\)\(^+\)-ATPase activities from the brain of M. musculus assayed at low NH\(_4\)Cl concentrations (1, 2.5 or 5 mmol l\(^{-1}\)) were significantly lower than the NKA activities assayed at corresponding KCl concentrations (Fig. 8A). However, at high concentrations of KCl or NH\(_4\)Cl (10 or 20 mmol l\(^{-1}\)), there was no significant difference between the NKA and the Na\(^+\)/NH\(_4\)\(^+\)-ATPase activities. These results confirm that NH\(_4\)\(^+\) could effectively substitute for K\(^+\) as a substrate for NKA from the mouse at high substrate concentrations. In contrast, the brain Na\(^+\)/NH\(_4\)\(^+\)-ATPase activities of M. albus (Fig. 8B) assayed at 1, 2.5, 5, 10 or 20 mmol l\(^{-1}\) NH\(_4\)Cl were significantly lower than the Nka activities assayed at similar KCl concentrations (Fig. 8B).

At substrate concentrations of 1, 2.5, 5, 10 and 20 mmol l\(^{-1}\), the effectiveness of NH\(_4\)\(^+\) substituting for K\(^+\) to activate Nka (expressed as ratios of Na\(^+\)/NH\(_4\)\(^+\)-ATPase to Na\(^+\)/K\(^+\)-ATPase activities) from the brain of M. albus were 0.16, 0.33, 0.43, 0.60 and 0.71, respectively (Table 3). In comparison, the effectiveness of NH\(_4\)\(^+\) substituting for K\(^+\) to activate NKA from the brain of M. musculus was significantly higher (0.55, 0.74, 0.78, 0.94 and 0.99, respectively) than those of M. albus (Table 3). Exposure to 50 mmol l\(^{-1}\) NH\(_4\)Cl for 6 days had no significant effect on the effectiveness of NH\(_4\)\(^+\) to activate Nka activity from the brain of M. albus (Table 3).

Discussion

Air-breathing fishes, particularly amphibious ones, are equipped with various strategies to ameliorate ammonia toxicity during emersion or ammonia exposure [12–14]. Active ammonia excretion, exhibited by P. schlosseri [45,46] and A. testudineus [47], is theoretically the most effective strategy to maintain low internal (plasma and tissue) ammonia concentrations. Recent reports on A. testudineus have revealed that both active salt excretion during seawater acclimation and active NH\(_4\)\(^+\) excretion during ammonia exposure (in freshwater) involve similar transport mechanisms (cystic fibrosis transmembrane conductance regulator, Nkcc1 and Nka) but two different types of Nka-immunoreactive cells in its gills [44,48,49]. In comparison, M. albus has degenerate gills and is incapable of active ammonia excretion. Therefore, it is imperative for M. albus to develop high tolerance of ammonia at the cellular level, especially in the brain. Our results suggested for the first time a possible relationship between the high brain ammonia tolerance of M. albus and (1) the high effectiveness of its brain Nka to differentiate K\(^+\) from NH\(_4\)\(^+\), and (2) the ability of its brain to down-regulate the mRNA and protein expression of nka3/Nkaα when confronted with ammonia toxicity.

The Brain of M. Albus Expressed nka1, nka3a and nka3b, but not nka2

Three nka α-subunit isoforms were expressed in the brain of M. albus. Based on phylogenetic analysis, they were identified as nka1, nka3a and nka3b. The PCR primers used in this study were designed against the highly conserved regions of nka1, nka2 and nka3. However, cloning and sequencing results confirmed the expression of nka1 and nka3, but not nka2, in the brain of M. albus. To further verify the lack of expression of nka2 in the brain of M. albus in response to ammonia exposure, we performed suppression subtractive hybridization PCR using control brain as driver and brain of fish exposed to 50 mmol l\(^{-1}\) NH\(_4\)Cl as tester, and confirmed the absence of nka2 in the forward and reverse libraries (Y. K. Ip, unpublished results). In contrast, Semple et al. [50] reported the expression of nka2 in the brain and muscle of F. heterochirus, while Guyn et al. [51] reported the expression of Nka1, Nka2 and Nka3 in the brain of the Antarctic nototheniid, Trematomus bernacchii, and the temperate nototheniid, Nototobius angustatus. Thus, the lack of expression of nka2 in the brain of M. albus is uncommon, and could have a physiological reason.
In mammalian brain, three isofoms, NKA1, NKA2 and NKA3, have been identified [27,32]. In adult mouse brain and in cultured mouse brain cells, the NKA1 isoform is expressed in both neurons and astrocytes, while NKA2 is an astrocyte specific isoform and NKA3 is expressed in neurons [52]. These three NKA α-subunit isoforms differ with regard to their Na⁺, K⁺ and ATP sensitivity [53,54], but they all have a specific binding site for ouabain and its analogues, the cardiotonic steroids [55,56]. Rodent NKA1 has a much lower affinity to ouabain (KD of 9.3 μM and 1.5 μM in cultured mouse astrocytes and neurons, respectively) than NKA2 (KD of ~80 nM in cultured mouse astrocytes) and NKA3 (KD of ~110 nM in cultured mouse neurons) and NKA1 from other species [57].

Similar to the brain of M. albus, the spiral ganglion and organ of Corti of rat cochlea were found to express NKA1 and NKA3 but not NKA2 [58]. It is probable that NKA1 and NKA3 may play more prominent roles in handling the physiological demands of myelinated axons. In comparison with NKA1, NKA3 has relatively low affinity for intracellular Na⁺ [52,53,55,59], high affinity for ATP [52], and a lack of inhibition at hyperpolarized potentials [59,60]. Thus, NKA3 appears to be especially suited for myelinated axons which can sustain high rates of activity leading to an increase in intracellular Na⁺ concentrations, a depletion of ATP, and prolonged hyperpolarization of membrane potentials [58]. Expression of various rat NKA α-subunit isoforms in Xenopus oocytes reveals that NKA2 isoform has a three-fold higher sensitivity to extracellular Na⁺ when compared with NKA1 and NKA3, causing the NKA2 isoform to be more sensitive to the membrane potential [61]. Hence, it is logical to deduce that the lack of expression of nka2 in the brain of M. albus could have a physiological relevance related to its high brain ammonia tolerance.

Recently, Xue et al. [62] investigated (i) effects of ammonia on mRNA and protein expression of Nkaα1/NKA1 and Nkaα2/NKA2 in primary cultures of mouse astrocytes; (ii) effects of hyperammonia obtained by urease injection on mRNA and protein expression of Nkaα1/NKA1 and Nkaα2/NKA2 in the mouse brain in vivo; and (iii) effect on observed upregulation of gene expression of Nkaα2/NKA2 induced by tyrphostin AG1478, an inhibitor of the EGF receptor-tyrosine kinase, PP1, an inhibitor of Src, and GM6001, an inhibitor of Zn²⁺-dependent metalloproteinasises in cultured mouse astrocytes. They [62] established that mRNA and protein expression of Nkaα2/NKA2, but not Nkaα1/NKA1, were upregulated in cultured astrocytes after 1–4 days of exposure to 3 or 5 mmol l⁻¹ ammonia, and that similar upregulation, contrasted by a down-regulation of the neuronal Nkaα3/NKA3, occurred in the hyperammonemic brain. Based on the effects of the inhibitors (AG1478, PP1 and GM6001) and how they affect the mRNA and protein expression of Nkaα2/NKA2, Xue et al. [62] concluded that ammonia activated the formation of an endogenous ouabain-like compound, which binds to NKA and activates Src, which in turn stimulates the receptor-tyrosine kinase of the EGF receptor. This led to the activation of the Ras, Raf, MEK pathway and phosphorylation of ERK1/2, which eventually causes an upregulation of Nkaα2 mRNA expression. Assuming that a similar molecular pathway exists in fish brain in general, the lack of expression of nkaα2 in the brain of M. albus could be an important adaptation to its mode of living (air-breathing and emersion) and conditions of its habitat (high environmental ammonia concentration), as it would contribute in part to its high brain ammonia tolerance.

Molecular Characterization of Nkaα1, Nkaα3a and Nkaα3b from Brain of M. albus

Three Na⁺ and two K⁺ binding sites are known to be present in the NKA α-subunit [41,63]. The coordinating residues present in the binding sites are arranged within the transmembrane domains such that the release of one type of cation coordinates with the binding of the other. Based on the homology modeling of human NKA α-subunit [41], these coordinating residues are found to be highly conserved in all three isoforms of the Nka α-subunit obtained from the brain of M. albus. Furthermore, it has been established that Na⁺ and K⁺ are occluded within NKA during each turnover of the pump and this occlusion requires conformational changes in the enzyme [64]. Proteolytic cleavage at a lysine-rich region near the N-terminal alters the equilibrium between the El and E2 conformations [65]. Hence, this conformational shift could involve the movement of the lysine-rich sequence, which could serve as a movable, ion-selective gate, controlling the passage of Na⁺ and K⁺ during certain stages of the transport process [42]. Indeed, the highly conserved lysine-rich sequence is present in Nkaα1, Nkaα3a and Nkaα3b from the brain of M. albus. Thus, this indirectly implies that the mechanisms of ion transport in Nka from M. albus could be similar to those of other species and they might share close structural-functional relationships.

Our results indicate that Nka activity could be regulated by phosphorylation in the brain of M. albus. Both cAMP-dependent protein kinase A and protein kinase C are known to be involved in

### Table 2. Percentage similarity between Na⁺/K⁺-ATPase (Nka) α-subunits from the brain of Monopterus albus and those from gills of Anabas testudineus.

| M. albus | A. testudineus | Similarity |
|----------|----------------|------------|
| Nkaα1 [KC620448] | Nkaα1c [JN180942] | 90.1% |
|         | Nkaα1b [JN180941] | 85.2% |
|         | Nkaα1a [JN180940] | 80.2% |
| Nkaα3a [KC620449] | Nkaα1c [JN180942] | 82% |
|         | Nkaα1b [JN180941] | 79.3% |
|         | Nkaα1a [JN180940] | 76.4% |
| Nkaα3b [KC620450] | Nkaα1c [JN180942] | 74.6% |
|         | Nkaα1b [JN180941] | 70.6% |
|         | Nkaα1a [JN180940] | 68.5% |

The percentage similarity between the deduced amino acid sequence of Nkaα1, Nkaα3a and Nkaα3b from the brain of M. albus with Nkaα1a, Nkaα1b and Nkaα1c from the gills of A. testudineus obtained from GenBank (accession numbers in brackets; Ip et al. [44]). Sequences are arranged in a descending order of similarity.

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the phosphorylation of the NKA α-subunit [66] although the functional effects of protein kinases remain controversial [67]. One possible site of cAMP-dependent protein kinase A phosphorylation, serine-945 [43], was present in all three Nka α-subunit isoforms from the brain of M. albus. However, out of these three Nka α-subunit isoforms, only Nka1α contains cAMP-dependent protein kinase C phosphorylation sites, serine-16 and threonine-15 [43]. Hence based on previous reports [43,66,67], it is probable that NH₄⁺ enters mitochondrion-rich cells through basolateral Nkcc1 before being actively transported across the apical membrane. However, the operation of Nkcc1 during active ammonia excretion would lead to an increase in the intracellular Na⁺ concentration of the mitochondrion-rich cells. Therefore, an up-regulation of Nka activity would be necessary to remove the excess Na⁺. In order to maintain intracellular K⁺ homeostasis, the gills of A. testudineus must express more than one type of Nka α-isofom, with at least one isoform that can differentiate K⁺ from NH₄⁺, rendering NH₄⁺ ineffective to substitute for K⁺ to induce Nka activity. Indeed, Ip et al. [44] reported that three nka α-isofom (α1a, α1b and α1c) were expressed in the gills of A. testudineus, and their results suggested that nkaα1a was a freshwater isoform while nkaα1b was a seawater isoform. They also demonstrated that environmental ammonia exposure led to significant increases in the mRNA expression of nkaα1c, the overall Nka protein abundance, the Nka activity, and the up-regulation of nkaα3b expression served to remove excess Na⁺ from, and to transport K⁺ in preference to NH₄⁺ into, mitochondrion-rich cells in order to maintain intracellular Na⁺ and K⁺ homeostasis [44].

**The Implications of High Similarity in K⁺ Binding sites between Nka1α, Nka2α and Nka3α of M. albus and Nka1c of A. testudineus**

A detailed analysis of the amino acid residues constituting the K⁺ binding sites of Nka1α, Nka2α and Nka3α from the brain of M. albus revealed that they are identical to those of Nka1c, but different from those of Nka1α and Nka1b, from the gills of A. testudineus. Exposure of A. testudineus to 100 mmol l⁻¹ NH₄Cl in freshwater resulted in a significant increase in the mRNA and protein expression of Nkcc1 in the gills [48]. Hence, it is probable that NH₄⁺ enters mitochondrion-rich cells through basolateral Nkcc1 before being actively transported across the apical membrane. However, the operation of Nkcc1 during active ammonia excretion would lead to an increase in the intracellular Na⁺ concentration of the mitochondrion-rich cells. Therefore, an up-regulation of Nka activity would be necessary to remove the excess Na⁺. In order to maintain intracellular K⁺ homeostasis, the gills of A. testudineus must express more than one type of Nka α-isofom, with at least one isoform that can differentiate K⁺ from NH₄⁺, rendering NH₄⁺ ineffective to substitute for K⁺ to induce Nka activity. Indeed, Ip et al. [44] reported that three nka α-isofom (α1a, α1b and α1c) were expressed in the gills of A. testudineus, and their results suggested that nkaα1a was a freshwater isoform while nkaα1b was a seawater isoform. They also demonstrated that environmental ammonia exposure led to significant increases in the mRNA expression of nkaα1c, the overall Nka protein abundance, the Nka activity, and the up-regulation of nkaα3b expression served to remove excess Na⁺ from, and to transport K⁺ in preference to NH₄⁺ into, mitochondrion-rich cells in order to maintain intracellular Na⁺ and K⁺ homeostasis [44].
Figure 5. Effects of ammonia exposure on Na+/K+-ATPase (nka) α1, nkaα3a and nkaα3b mRNA expression. Absolute quantification (copies of transcript per ng cDNA) of mRNA expression of (A) nkaα1, (B) nkaα3a and (C) nkaα3b, in the brain of Monopterus albus kept in freshwater (FW; control), or after exposure to 50 mmol l⁻¹ NH₄Cl for 1 day or 6 days. Results represent mean±S.E.M. (N=5). Means not sharing the same letter are significantly different (P<0.05).

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Therefore, the similarity in the K\(^+\) binding sites between all three Nka\(a\)-subunit isoforms from the brain of \textit{M. albus} and Nka\(1\)c from the gills of \textit{A. testudineus} indicate that the overall Nka activity from the brain of \textit{M. albus} might exhibit high substrate specificity for K\(^+\).

The Nka from the Brain of \textit{M. albus} can Differentiate K\(^+\) from NH\(_4\)^+ Better than the NKA from the Brain of \textit{M. musculus}

Indeed, our results reveal for the first time that the Nka from the brain of \textit{M. albus} has a high specificity for K\(^+\), as compared to NH\(_4\)^+, at physiological concentrations (~1 mmol l\(^{-1}\)) of K\(^+\) or NH\(_4\)^+. For control fish, NH\(_4\)^+ was only 15\% effective in substituting for K\(^+\) to induce Nka activity. More importantly, our results confirm that Nka from the brain of \textit{M. albus} had a greater K\(^+\) specificity than NKA from the mouse brain. This would imply that, when confronted with high brain ammonia concentration, cells in the brain of \textit{M. albus} could maintain intracellular K\(^+\) homeostasis and low intracellular ammonia concentration through the normal functioning of Nka with only a low level of K\(^+\) being substituted by NH\(_4\)^+. Thus, the high K\(^+\) specificity of Nka from the brain of \textit{M. albus} could have a major contribution to its extraordinarily high brain ammonia tolerance. As for the mouse brain, the ineffectiveness of its NKA to differentiate K\(^+\) from NH\(_4\)^+ could be one of the contributing factors to its low tolerance of ammonia [1]. Exposure of \textit{M. albus} to ammonia had no significant effect on the effectiveness of NH\(_4\)^+ or K\(^+\) to activate Nka from its brain. Hence, a down-regulation of the mRNA and protein expression of these nka/Nka\(a\)-subunit isoforms would be essential to further ameliorate the deleterious effects of ammonia under hyperammonia conditions.

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\textbf{Figure 6. Effects of ammonia exposure on total Na\(^+\)/K\(^+\)-ATPase (Nka) protein abundance.} Protein abundance of Nka, based on the \(\alpha5\) anti-NKA antibody which is known to react with all Nka/NKA \(\alpha\)-isoforms, in the brain of \textit{Monopterus albus} kept in freshwater (FW; control) or exposed to 50 mmol l\(^{-1}\) NH\(_4\)Cl for 1 day or 6 days. (A) An example of the immunoblots of Nka and actin. (B) The intensity of the Nka band normalized with respect to that of actin. Results represent mean±S.E.M. (N = 3). Means not sharing the same letter are significantly different (P<0.05).

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Down-regulation of mRNA and Protein Expression of nka/Nka α-subunit Isoforms in the Brain of M. albus Exposed to Ammonia

Mammalian brains have low brain ammonia tolerance, and an increase in brain ammonia concentration is known to induce higher NKA gene expression and NKA activity. It has been established that an injection of large doses of ammonia into rats leads to the depletion of brain ATP [29]. Kosenko et al. [29] reported that an injection with ammonia into rat increased the brain NKA activity by 76%, which could be prevented by a previous injection of MK-801, an antagonist of the NMDA receptor. After normalizing NKA activity in samples from ammonia-injected rats by in vitro incubation with phorbol 12-myristate 13-acetate, an activator of protein kinase C, Kosenko et al. [29] obtained results indicating that ammonia-induced ATP depletion was mediated by the activation of NMDA receptor, which resulted in decreased protein kinase C-mediated phosphorylation of NKA and, therefore, increased NKA activity and increased consumption of ATP. It has also been reported that ammonia increases the production of ouabain-like substances and NKA activity in cultured mouse astrocytes [70]. Thus, increased activity of NKA could also be the result of enhanced production of ouabain-like compounds, as cultured rat astrocytes react to prolonged exposure to a high concentration of ouabain with an upregulation of NKA3 [71].

By contrast, we report for the first time that 1 day or 6 days of exposure to ammonia resulted in significant decreases in mRNA expression of nka1/Nka α1, nka3a, and nka3b in the brain of M. albus. In addition, there were significant decreases in the protein abundance of total Nka α-subunit isoforms (based on the commercially available pan-specific antibody α5), and Nka3 (based on the commercially available α3-specific antibody) in the brain of M. albus after 6 days of exposure to ammonia. Therefore, it is probable that reduction in the mRNA expression and protein abundance of nka1/Nka α-subunit...
isoforms in the brain of *M. albus* exposed to ammonia could directly ameliorate the severity of ammonia toxicity, leading to high brain ammonia tolerance.

In mammals, swelling of astrocytes represents the most prominent neuropathological abnormality in acute liver failure [72], and ammonia has been shown to induce swelling of astrocytes *in vivo* [73], and *in vitro* [74]. Aquaporin 4, which acts as a water channel, has been implicated in the swelling process [75]. Recently, Illarionova et al. [76] reported that aquaporin 4 could assemble with its regulator metabotropic glutamate receptor 5 and NKA, forming a macromolecular transporting microdomain in astrocytes. Therefore, it is probable that the reduction in the mRNA expression and protein abundance of *nkaa*-subunit isoforms in the brain of *M. albus* exposed to ammonia could suppress the function of aquaporin 4 and ameliorate the severity of ammonia-induced astrocyte swelling and brain edema, the confirmation of which awaits future studies.

Perspective

Our results suggest for the first time that the ability to down-regulate the mRNA expression of *nkaa1, nkaa3a* and *nkaa3b* and protein abundance of Nka α-subunit isoforms in the brain could be some of the contributing factors to the extraordinarily high brain ammonia tolerance in *M. albus*. Another contributing factor could be the ineffectiveness of NH₄⁺, as compared with K⁺, to activate Nka from the brain of *M. albus*. Efforts are being made in our laboratory to determine the localization of Nka in the brain of *M. albus*, and its functional relationship with other transporters, e.g. Nkcc1 and aquaporin. Since exposure to environmental ammonia also resulted in a reduction in *nkcc1b/Nkcc1b* expression [26], it is highly probable that these two transporters work in concert to control NH₄⁺ influx into brain cells to ameliorate the toxic effects of high environmental ammonia exposure.

Figure 8. Effects of varying K⁺ or NH₄⁺ concentrations on Na⁺/K⁺-ATPase or Na⁺/NH₄⁺-ATPase activities from brains of *Mus musculus* and *Monopterus albus*. Specific activity (μmol inorganic phosphate 20 min⁻¹ mg⁻¹ protein) of NKA were determined from the brain of (A) *M. musculus* and (B) *M. albus* kept in freshwater with varying concentrations of K⁺ or NH₄⁺. Results represent mean ± S.E.M. (N=5). Asterisks indicate significant difference from the corresponding potassium-induced specific activity (P<0.05).

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Brain NKA and Ammonia Tolerance in M. albus

Table 3. A comparison of Na+/K+-ATPase and Na+/NH₄⁺-ATPase activities, from the brains of Monopterus albus or Mus musculus, at various concentrations of K⁺/NH₄⁺.

| KCl or NH₄Cl concentration (mmol l⁻¹) | Ratio of Na+/NH₄⁺-ATPase activity to Na+/K⁺-ATPase activity |
|--------------------------------------|-------------------------------------------------------------|
|                                      | M. musculus | M. albus |
| Freshwater                           | 50 mmol l⁻¹ NH₄Cl for 6 days |
| 1                                   | 0.55±0.04b | 0.16±0.03* | 0.26±0.04* |
| 2.5                                  | 0.74±0.06b | 0.33±0.05* | 0.33±0.02* |
| 5                                    | 0.78±0.03b | 0.43±0.05* | 0.49±0.03* |
| 10                                   | 0.94±0.04b | 0.60±0.04* | 0.70±0.04* |
| 20                                   | 0.99±0.06b | 0.71±0.08* | 0.85±0.03b* |

Effectiveness of NH₄⁺, substituting for K⁺ (expressed as ratio of Na+/NH₄⁺-ATPase activity to Na+/K⁺-ATPase activity) to induce Nka activities from the brain of M. musculus, or from the brain of M. albus kept in freshwater or exposed to 50 mmol l⁻¹ NH₄Cl for 6 days.

Values are means ± S.E.M. (N=5).

Means not sharing the same letter are significantly different, P<0.05.

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Supporting Information

Figure S1 Multiple amino acid sequence alignment of Na+/K⁺-ATPase (Nka) α-subunits. A multiple amino acid sequence alignment of Nkaα1, Nkaα3 and Nkaα3b from the brain of Monopterus albus was performed with those of Oreochromis mossambicus Nkaα1 [GenBank: AAD11455.2], Xenopus laevis NKAα1 [GenBank: NP_001084064.1], Rattus norvegicus NKAα1 [GenBank: NP_036636.1], Homo sapiens NKAα1 [GenBank: NP_000692.2], O. mossambicus Nkaα3 [GenBank: AAF75108.1], X. laevis NKAα3 [GenBank: NP_00108440.1], R. norvegicus NKAα3 [GenBank: NP_036638.1], and H. sapiens NKAα3 [GenBank: NP_689509.1]. Identical amino acid residues are indicated by asterisks, strongly similar amino acids are indicated by colons and weakly similar amino acids are indicated by periods.

Effectiveness of NH₄⁺, substituting for K⁺ (expressed as ratio of Na+/NH₄⁺-ATPase activity to Na+/K⁺-ATPase activity) to induce Nka activities from the brain of M. musculus, or from the brain of M. albus kept in freshwater or exposed to 50 mmol l⁻¹ NH₄Cl for 6 days.

Values are means ± S.E.M. (N=5).

Means not sharing the same letter are significantly different, P<0.05.

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Author Contributions

Conceived and designed the experiments: YKI SFC. Performed the experiments: XLC NLJEW KCH SFC. Analyzed the data: XLC NLJEW. Contributed reagents/materials/analysis tools: WPW. Wrote the paper: XLC YKI. Edited the manuscript: XLC JLIYO YRC BC YKI.

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