

Neuronal injury in NCX3 knockout mice following permanent focal cerebral ischemia and in NCX3 knockout cortical neuronal cultures following oxygen-glucose deprivation and glutamate exposure

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Abstract

In this study we subjected NCX3 knockout and wildtype mice to permanent focal cerebral ischemia by intraluminal middle cerebral artery occlusion. Analysis of brain sections by 2,3,5-Triphenyl-2H-tetrazolium chloride staining, 12 hours after middle cerebral artery occlusion revealed no difference in infarct volume between NCX3 knockout and wildtype mice. In addition, we evaluated the effect of NCX3 protein knockdown on neuronal viability in primary cortical neuronal cultures following *in vitro* ischemia (oxygen-glucose deprivation) and L-glutamate (glutamate) exposure. *In vitro* experiments revealed that neuronal viability was higher in NCX3 knockout neuronal cultures than in the wildtype cultures following ischemic and glutamate insults. The reduced sensitivity of neurons from NCX3 knockout mice to *in vitro* ischemia and excitotoxicity indicates that NCX3 calcium entry mode activity contributes to calcium overload and neuronal death. However our *in vivo* finding of a lack of differential sensitivity on infarct volume in NCX3 knockout and wildtype mice suggests that any benefit of reduced NCX3 activity is overridden following permanent focal cerebral ischemia. Taken together, these findings suggest that reduced NCX3 activity limits calcium neurotoxicity during severe transient, but not during severe sustained ischemic insults. These results have important implications for the development of the NCX3 protein as a therapeutic target to reduce ischemic brain injury.

Keywords: NCX; sodium calcium exchanger 3; cerebral ischemia; permanent middle cerebral artery occlusion; glutamate; *in vitro* ischemia; neuroprotection

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1. Introduction

The sodium calcium exchanger (NCX) is an important trans-membrane transport protein involved in cellular calcium homeostasis. The exchanger's main function is to expel cytoplasmic calcium (calcium exit or forward mode), however under pathological

conditions such as ischemia, it can operate in calcium entry mode (reverse mode), causing calcium influx and neurotoxicity (Blaustein and Lederer, 1999; Annunziato et al., 2004). Three highly homologous genes (NCX1, NCX2, NCX3) are expressed in the brain (Lee et al., 1994; Quednau et al., 1997), and presently it is uncertain whether their functions exacerbate or limit neuronal calcium toxicity during and following cerebral ischemia (Jefferis et al., 2007). This uncertainty is due in part to the great number of *in vivo* and *in vitro* studies performed to elucidate the role of NCX following cerebral ischemia showing conflicting results (see Jefferis et al., 2007). Furthermore, depending on the intracellular status of sodium and calcium levels and energy (ATP) supply, NCX can operate in either calcium exit or entry modes and thereby increase or decrease excitotoxic

and ischemia-like induced calcium dysregulation (Annunziato et al., 2007; Jeffs et al., 2007).

With respect to NCX3, knockout mice have recently been shown to display increased brain injury following transient global and transient focal cerebral ischemia (Jeffs et al., 2008; Molinaro et al., 2008). These findings suggest that NCX3 has a positive role in maintaining neuronal intracellular calcium homeostasis following ischemia/reperfusion, and that when exchanger function is reduced neurons are less capable of managing calcium and are more susceptible to ischemic cell death. *In vitro* studies using organotypic slices and cortical neuronal cultures subjected to transient oxygen-glucose deprivation also revealed increased neuronal injury in NCX3 knockout mouse derived cultures (Molinaro et al., 2008).

Interestingly, under hypoxic (and normoxic) conditions NCX3 knockout derived neurons display a reduced capacity to operate in calcium entry mode (Molinaro et al., 2008), suggesting that NCX3 suppression reduces calcium influx, which would normally be expected to reduce ischemic insult severity. To further understand the role of the NCX3 exchanger in ischemic neuronal injury we subjected NCX3 knockout mice and primary cortical neuronal cultures from these mice to permanent focal cerebral ischemia and *in vitro* ischemia-like insults respectively. In line with our hypothesis of NCX3 suppression reducing ischemic severity our findings indicate that NCX3 knockdown has limited benefit in the ischemic brain, but appears beneficial to cultured neurons following ischemia and excitotoxicity.

2. Materials and Methods

2.1. Animals used to establish experiments models

Generation of the NCX3 knockout (NCX3 $-/-$) mice bred on a C57BL/NHSD background strain has been previously described (Sokolow et al., 2004). Control C57BL/NHSD wildtype mice were obtained from Harlan, France and both strains were maintained at the University of Western Australia Animal Care Unit. Male homozygous NCX3 knockout mice and male wildtype mice (weighing 25–30g) were used for the permanent focal cerebral ischemia experiments. The animals were housed under diurnal light conditions (12 hours light, 12 hours darkness) and had free access to food and water before and following the surgery. NCX3 knockout and NHSD wildtype female mice were time-mated with their respective strain male in order to obtain embryonic day 17 (E17), embryos for preparation of primary neuronal cultures. All animal experiments were approved by the University of Western Australia Animal Ethics Committee.

2.2. Permanent middle cerebral artery occlusion model

Anaesthesia was induced with isoflurane (3% initial, 1.5 - 2% maintenance), 30% O₂: 67% N₂O using a facemask. A single dose of atropine was given subcutaneously (0.04 mg/kg) at the onset of ventilation to reduce secretions. Rectal temperature was measured throughout the procedure and maintained between 36 - 37°C using a heatpad and/or fan heater. A laser Doppler flowmeter probe (Bloodflow meter, ADInstruments, Australia) attached to the right hemisphere of the skull was used to monitor the regional cerebral blood flow. Permanent middle cerebral artery occlusion (MCAO) was induced by the intraluminal insertion of a nylon thread as described previously with slight modification (Minematsu et al., 1992). Briefly, a 6–0 silk suture (Ethicon, Johnson and Johnson, Australia) was permanently tied at the origin of the external common carotid artery and at the distal end of the common carotid artery (CCA). A coated nylon suture (6.0; Doccol, Redlands, CA, USA) was introduced through an incision in the right CCA into the internal carotid artery (ICA), then advanced approximately 8-10 mm intracranially from the CCA bifurcation into the ICA. Successful MCAO was confirmed by a sudden drop in the laser Doppler reading. The occluding thread was tied in place and incisions sutured. Anaesthetic was discontinued and animals maintained on a face mask breathing 100% oxygen until mobile. Thereafter the animals were transferred to a recovery room maintained at 28°C, and body temperature and recovery were closely monitored for the remainder of the experiment. In order to avoid an additional invasive procedure and the risk of mortality, arterial blood gas analysis was not performed in this study. Animal experiments were performed randomly with the operator blinded to the mouse strain.

2.3. Assessment of infarct volume

Immediately prior to the animals being sacrificed, a basic neurological test was performed. The following grading system was used: 0- no obvious deficit, 1- asymmetrical paw extension; torsion to ipsilateral side when held by tail, 2- circling to affected side, 3- no spontaneous movement. Twelve hours post occlusion, the animals were sacrificed by lethal injection of pentobarbitone, brains carefully removed and sliced into 2 mm coronal sections using a mouse brain matrix. Brain sections were stained using a 2% solution of 2,3,5-Triphenyl-2H-tetrazolium chloride, (TCC; Sigma, USA) and scanned and infarct volume quantified by ImageJ analysis software (NIH, USA). Total brain infarct volume was calculated and corrected for edema as previously described by (Campbell et al., 2008). Statistical analysis was

performed *t*-test; $P < 0.05$ was considered statistically significant. A total of 8 wildtype mice and 10 NCX3 knockout mice were successfully operated on and analyzed.

2.4. Primary cortical neuronal cultures

Primary cortical neuronal cultures were prepared from NCX3 knockout and wildtype embryos using methods as previously described for rat embryos (Meloni et al., 2001). Briefly, cortical brain tissue from E17 embryos was dissociated in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Australia) supplemented with 1.3 mM L-cysteine, 0.9 mM NaHCO₃, 10 units/ml papain (Sigma) and 50 units/ml DNase (Sigma) and washed in cold DMEM/10% horse serum. Cells were re-suspended in Neurobasal medium (NB; Invitrogen) containing 2% B27 (Invitrogen) and seeded in either plastic (Costar, USA) or glass (All-tech, Australia) 96-well sized wells with 55,000 (75 μ l) and 70,000 (90 μ l) cells per well respectively. Before seeding, plastic wells were pre-coated with poly-D-lysine (50 μ g/mL; 70 - 150K; Sigma) and glass wells were pre-coated sequentially with poly-D-lysine and laminin (50 μ g/ml; Invitrogen). After coating and before seeding, wells were filled with (plastic: 50 μ l and glass: 60 μ l) NB containing 2% B27; 4% fetal bovine serum; 1% horse serum; 62.5 μ M L-glutamate (glutamate); 25 μ M 2-mercaptoethanol; and 30 μ g/mL streptomycin and 30 μ g/mL penicillin. Culture wells were maintained at 37°C in 95% air, 5% CO₂ incubator. On day *in vitro* (DIV) 4, one third of the culture media was replaced with fresh NB/2%B27 containing the mitotic inhibitor cytosine arabinofuranoside (1 μ M final concentration; Sigma) to inhibit glia cell proliferation, and on DIV 8, one half of the media was replaced with NB/2% B27. Cultures were used on DIV 10 or 11.

2.5. *In vitro* ischemia model

In vitro ischemia involved depriving neurons of oxygen and glucose by removing media from glass culture wells, washing in 315 μ l of balanced salt solution (BSS; mM: 116 NaCl, 5.4 KCl, 0.8 MgSO₄, 1 NaH₂PO₄; pH 7.0) and then adding 50 μ l of BSS. Cultures were placed into an anaerobic chamber (Don Whitely Scientific, England) with an atmosphere of 5% CO₂, 10% H₂ and 85% argon, 98% humidity at 37°C for periods ranging from 20 to 50 minutes. Following removal from the anaerobic incubator, cultures were supplied with an equal volume (50 μ l) of NB media containing 2% N2 supplement (Invitrogen) before incubation in a 5% CO₂ incubator for 24 hours. Culture controls received the same BSS wash steps and media additions as ischemic cultures but were maintained in a 5% CO₂ incubator throughout the experiment.

2.6. Glutamate model

For the glutamate excitotoxicity model, the media from plastic culture wells was removed and replaced with 100 μ l of a 50:50 mixture of conditioned media and fresh NB2/2% B27 containing glutamate at concentrations ranging from 25 to 100 μ M. Following a 5 minute incubation at 37°C the media was replaced with 100 μ l of NB/1% N2 media and cultures were placed into a CO₂ incubator at 37°C for 24 hours. Control cultures received media additions without glutamate.

2.7. Assessment of cell viability

Neuronal viability was assessed 24 h after *in vitro* ischemia and glutamate exposure using the (MTS) assay (Promega, Australia). The MTS assay measures the mitochondrial conversion of the tetrazolium salt to a water-soluble brown formazan salt, which is detected spectrophotometrically at 495nm (Segu et al., 1998). MTS absorbance readings from controls and treatments were converted to percentage viability. Light microscopy was used to assess the percentage of morphological cell death in the cultures. Viability in control cultures was taken as 100% while viability in cultures exposed to *in vitro* ischemia (40 minutes) and glutamate (100 μ M) was based on light microscopy and estimated to be 5% and 1%, respectively. Four to six wells were used for each treatment and control and experiments were repeated a minimum of three times using neuronal cultures established from embryos obtained from different pregnant mice. MTS absorbance data was analysed by ANOVA, followed by a post-hoc Fisher's PLSD test; $P < 0.05$ was considered statistically significant

3. Results

3.1. Permanent middle cerebral artery occlusion

Mean total infarct volumes for NCX3 knockout and wildtype mice determined after 12 hours of permanent MCAO are represented graphically in Fig 1A. A representative picture of the brain infarcts from NCX3 knockout and wildtype mice is shown in Fig 1B. Infarct volumes were similar in NCX3 knockout and wildtype mice and comprised most of the left cerebral hemisphere, indicative of maximal infarction. In addition, there was no significant difference in brain swelling between NCX3 knockout and wildtype mice (mean 25.30; SD \pm 11.49mm³ and 20.24; SD \pm 7.75mm³ respectively, $P = 0.2892$) or neurological deficit scores (data not shown). TTC staining at a 3 hour time point revealed slightly reduced staining in the affected cerebral hemisphere, indicative of decreased tissue metabolism, but not infarction. Hence, due to

the variable TTC staining, it was not possible to quantify brain infarct at this time point.

3.2. *In vitro* ischemia and glutamate exposure

Representative graphs for *in vitro* ischemia and glutamate experiments are presented in Figs 2 and 3. NCX knockout neuronal cultures were consistently

more resistant to ischemic and glutamate exposure. Prolonged *in vitro* ischemic durations (>40 min) and high glutamate concentrations (>75µM) resulted in extensive neuronal death (<5% neuronal viability) that did not differ significantly between NCX3 knockout and wildtype cultures.

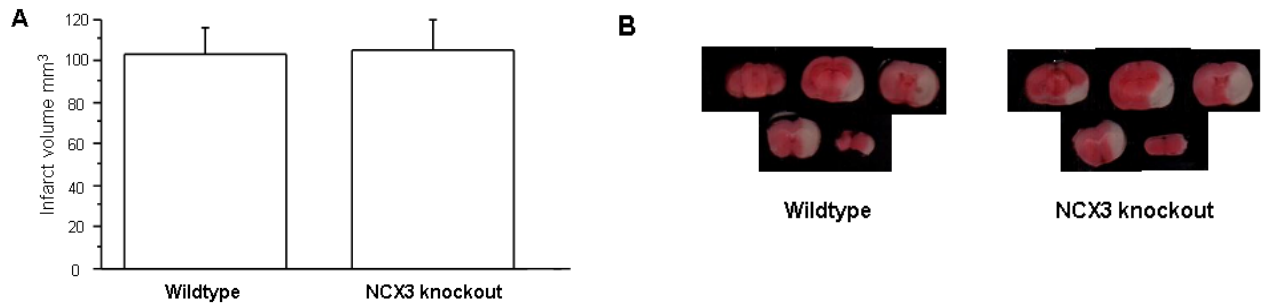


Fig. 1 - Permanent focal cerebral ischemia in wildtype and NCX3 knockout mice. A: Total infarct volumes for wildtype and NCX3 knockout mice when assessed 12 hours following permanent MCAO. B: Representative TTC stained brain slices from a wildtype and NCX3 knockout mouse. Values are means ± SE; N = 8 wildtype; N = 10 NCX3 knockout.

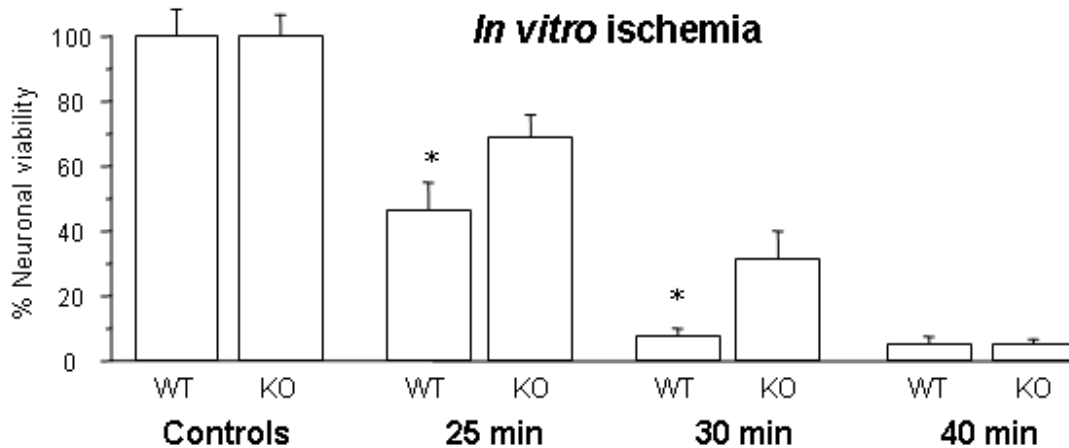


Fig. 2 - *In vitro* ischemia duration experiment using cortical neuronal cultures derived from wildtype and NCX3 knockout mice. MTS data were expressed as percentage neuronal viability with control (no insult) taken as 100% viability. Values are means ± SE; N = 6 wells; * *P* < 0.05 compared to corresponding treatment in NCX3 knockout neuronal cultures.

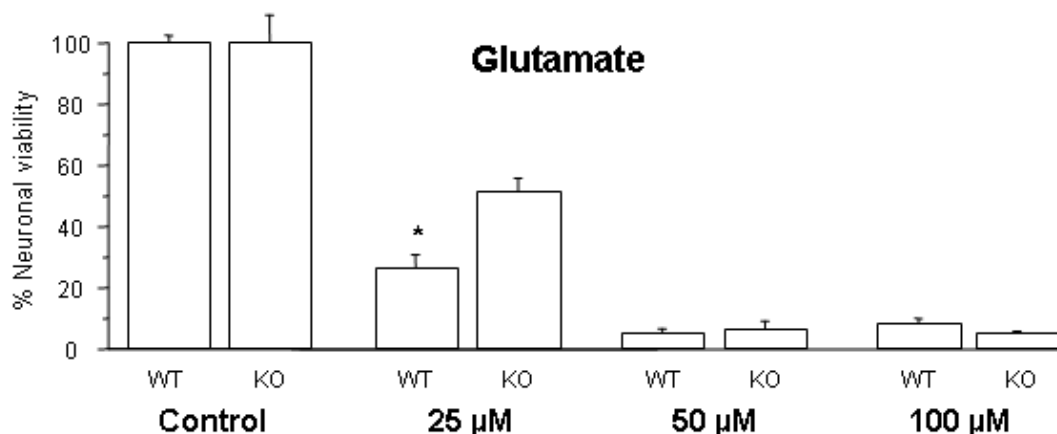


Fig. 3 - Glutamate dose response experiment using cortical neuronal cultures derived from wildtype and NCX3 knockout mice. MTS data were expressed as percentage neuronal viability with control (no insult) taken as 100% viability. Data are means \pm SE; N = 4 wells; * $P < 0.05$ compared to corresponding treatment in NCX3 knockout neuronal cultures.

4. Discussion

Our study is the first to assess the effect of NCX3 knockout on brain infarct volume following permanent focal cerebral ischemia in the mouse. A previous study by Molinaro et al. (2008) subjected NCX3 knockout mice to transient focal cerebral ischemia and reported increased infarct volumes in knockout mice compared to wildtype mice when assessed at 24 hours. Similarly, antisense down regulation of NCX3 prior to permanent MCAO in the rat resulted in increased infarct volumes compared to controls (Pignataro et al., 2004). However, unlike previous NCX3 knockdown studies, we did not detect any difference in infarct volumes in NCX3 knockout and wildtype mice, even though we used a 12 hour end point. We believe that the lack of an observable differential effect of NCX3 knockout in our model was likely due to the near maximal infarct volumes induced by permanent MCAO. Taken together these findings suggest that the beneficial effects of NCX3 activity observed following milder forms of cerebral ischemia are overridden if cerebral ischemia is severe. Interestingly, heterozygous NCX1 +/- mice expressing 40% less NCX1 than wildtype mice, did not display differences in infarct volumes compared to wildtype mice after transient focal ischemia (Luo et al., 2007). Moreover, NCX2 knockout mice, but not antisense NCX2 treated rats, displayed increased infarct volumes compared to control animals following transient and permanent focal ischemia respectively (Pignataro et al., 2004; Jeon et al., 2008).

With respect to NCX3 activity in the ischemic core following MCAO, it is likely that, because of the lack of reperfusion, subsequent recovery in ATP levels and restoration of sodium gradients, the exchanger protein will have limited benefit. While it could be argued that reduced exchanger activity would reduce neuronal calcium influx during ischemia and thereby lessen the ischemic insult this does not appear to be the case. This is not surprising because during cerebral ischemia, NCX3 can be proteolytically inactivated (essentially causing NCX3 knockdown) and several additional important calcium influx pathways are activated, which all contribute to calcium overload and ischemic brain injury (Pringle, 2004; Bano and Nicotera, 2006; Mac Donald et al., 2006). While it is possible that the assessment of brain injury at an earlier time point may have given some indication of differences in ischemic vulnerability between NCX3 knockout and wildtype mice, this would require the application of more sensitive histological staining techniques. TTC staining at a 3 hours end point did not reveal any significant infarct maturation (data not shown). Moreover, even if differences in sensitivities of NCX3 knockout and wildtype mice to permanent cerebral ischemia could be detected at an earlier time point, its clinical relevance would be limited as any benefit would be short-term.

The lack of any detectable difference in infarct volume in NCX3 knockout and wildtype mice following permanent focal ischemia has clinical relevance with respect to NCX as a therapeutic target. Since a large

proportion of ischemic stroke is not associated with reperfusion, our data indicate that therapies aimed at inhibiting NCX calcium entry mode activity may be of limited benefit. In contrast, therapies aimed at increasing NCX calcium exit mode activity following cerebral ischemia may be beneficial, as this would enable neurons to better extrude and regulate intracellular calcium, however further experimental studies are required to confirm this hypothesis.

Interestingly, our *in vitro* experiments have revealed that neuronal cultures established from NCX3 knockout mice appear more tolerant to the effects of ischemia and glutamate insults than neuronal cultures established from wildtype mice. These results indicate that NCX3 in wildtype neurons is operating in calcium entry mode during and/or after the *in vitro* ischemic and glutamate insults and is contributing to calcium induced neurotoxicity. Similarly, Luo et al. (2007) reported cortical neuronal cultures derived from NCX1 +/- mice were more tolerant to oxygen-glucose deprivation than wildtype cultures, and others (Hoyt et al. 1998; Dietz et al., 2007; Kiedrowski et al., 2007; Wu et al., 2008) have shown that exchanger calcium entry mode activity contributes to neuronal intracellular calcium increases associated with anoxia and glutamate receptor activation. In contrast, Molinaro et al. (2008) reported increased cell death in NCX3 knockout cortical cultures exposed to oxygen-glucose compared with wildtype cortical cultures.

The reason for the discrepancy in sensitivity of NCX3 knockout derived neuronal cultures to *in vitro* ischemia-like insults is unknown, but could be related to the milder three hour oxygen-glucose deprivation conditions used by Molinaro et al. (2008), compared to shorter more severe deprivation periods (25 - 50min) used in our model. The longer and milder oxygen glucose deprivation insult used by Molinaro et al. (2008) is likely to be related to the use of plastic culture vessels which are known to store oxygen (Meloni et al., 2001) and thus supplying oxygen to neurons for energy generation and maintenance of ionic gradients. In this setting, which may be compared with the environment in the ischemic penumbra in focal ischemia (Tortiglione et al., 2007), NCX3 activity may be contributing to calcium efflux, enabling neurons to better survive oxygen-glucose deprivation. Conversely, during more severe insults, like in our oxygen glucose deprivation model performed in glass wells (which do not store oxygen) and glutamate model, and which more closely resemble conditions in the ischemic core following focal ischemia, NCX3 activity may be contributing to calcium influx and cell death.

In conclusion, we have shown that NCX3 protein knockout has no obvious impact on infarct volume in our permanent focal ischemia model, and that in cortical neuronal cultures following severe ischemic and excitotoxic insults, NCX3 knockdown appears to reduce cell death. These findings have important implications for the use of the NCX protein as a therapeutic target following cerebral ischemia, because depending on the environmental setting, exchanger activity may promote or disrupt calcium homeostasis and ultimately contribute to neuronal recovery or demise.

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