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Adenine Nucleotide Translocator 1 Deficiency Increases Resistance of Mouse Brain and Neurons to Excitotoxic Insults

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Abstract

The mitochondrial adenine nucleotide translocators (Ant) are bi-functional proteins that transport ADP and ATP across the mitochondrial inner membrane, and regulate the mitochondrial permeability transition pore (mtPTP) which initiates apoptosis. The mouse has three Ant isoforms: Ant1 expressed in heart, muscle, and brain; Ant2 expressed in all tissues but muscle; and Ant4 expressed primarily in testis. Ant1-deficient mice manifest muscle and heart but not brain pathology. Brain Ant1 is induced by stress, while Ant2 is not. Ant1-deficient mice are resistant to death induced by systemic exposure to the brain excitotoxin, kainic acid (KA), and their hippocampal and cortical neurons are significantly more resistant to neuronal death induced by glutamate, KA, and etoposide.. The mitochondrial membrane potential of Ant1-deficient brain mitochondria is increased and the mtPTP is more resistance to Ca^{++} induced permeability transition. Hence, Ant1-deficiency may protect the brain from excitotoxicity by desensitizing the mtPTP and by blocking the pro-apoptotic induction of Ant1 by stress.

Keywords

Adenine nucleotide translocator; mitochondria; mitochondrial permeability transition; excitotoxicity; apoptosis

INTRODUCTION

The mitochondrial adenine nucleotide translocator (Ant or ADP/ATP translocase) is the most abundant mitochondrial inner membrane protein whose primary function is to exchange ADP and ATP across the mitochondrial inner membrane [1]. Humans have three main ANTs plus a testis isoform, while mouse has two main isoforms, Ant1 and Ant2, plus a

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testis isoform, Ant4. In mouse, Ant1 is expressed in the skeletal muscle, heart, and brain, while Ant2 is expressed in all tissues except skeletal muscle[2][3][4][5][6][7].

The Ants interact with a multi-polypeptide complex which spans the mitochondrial inner and outer membranes, the mitochondrial permeability transition pore (mtPTP)[8–10]. While the Ants do not appear to provide the actual ion channels of the mtPTP, they play an important role in regulating the mtPTP in response to calcium and adenine nucleotides [11]

Mice in which the Ant1 gene (*Ant1*) is insertionally inactivated (Ant1-deficient mice) develop mitochondrial myopathy associated with ragged-red fibers and abnormal mitochondria and a hypertrophic cardiomyopathy [12]. The muscle of *Ant1* $-/-$ mice exhibit an induction of mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) oxidative phosphorylation (OXPHOS) gene expression, antioxidant defense proteins, and anti-apoptotic factors [13][14][15]. Yet, Ant1-deficient mice show no overt evidence of neurological disease.

To determine why the muscle and heart are affected in Ant1-deficient mice but the brain is not, we examined the response of Ant1-deficient mice to excitotoxic KA brain insult and Ant1-deficient cultured mouse neurons to excitotoxins (glutamate and KA) and the DNA damaging agent (etoposide). Ant1-deficient mice were as prone as wild type mice to KA induced seizures, but were more resistant to KA-induced death. Ant1-deficient neurons were resistant to glutamate and KA induced death and their mitochondria had increased membrane potential, decreased ATP, and partially stabilized mtPTP. These results suggest that the loss of Ant1 stabilizes the mtPTP against Ca^{++} activation. Therefore, Ant1-deficient mice may be resistant to neurodegeneration because the mtPTPs of their neurons are less prone to permeability transition and to stress-induced induction of Ant1 and initiation of apoptosis.

MATERIALS AND METHODS

Ant1-Deficient Mice, Brain Expression of Ants, and Kainic Acid Toxicity

Mice and Mouse Handling—Ant1-deficient mice on C57BL/6J2z background were previously prepared by the insertional inactivation of the *Ant1* gene by in-frame insertion of the βgeo cassette encompassing a β-galactosidase-neomycin fusion protein (*βgeo*) [12]. Mice were maintained at 20–23°C on a 13-hr light/11-hr dark cycle using the procedures mandated by the National Institutes of Health guidelines and the University of California Institutional Animal Care and Use Committee.

Regional Brain Expression of Ant1—The regional expression of Ant1 was investigated by staining brain sections of heterozygous $Ant1^{\beta geo}$ mice for expression of β-galactosidase transcribed from the *Ant1* gene promoter [12]. Mouse brains were serially cryostat sectioned (15 μm) in either the coronal or the sagittal plane, the sections fixed in 2% formaldehyde and 1% glutaraldehyde in saline buffer, the sections rinsed and stained with X-gal solution for 24 hours at 37°C, and then counterstained with Nuclear Fast Red [16].

Kainic Acid Excitotoxicity—The tendency of Ant1-deficient and control mice to develop seizures and to die in response to the brain excitotoxic agent kainic KA was investigated by a single intraperitoneal injection of 35 mg/kg KA into age-matched Ant1-deficient and control animals. Mice were then monitored continuously for 3–4 h. At 2 hours they were evaluated for the onset and extent of seizure activity and animal survival. Seizure activity was rated as immobility, stage 1; forelimb and/or tail extension and rigid posture, stage 2; repetitive movements and head bobbing, stage 3; rearing and falling, stage 4;

continuous rearing and falling, stage 5; severe tonic–clonic seizures, stage 6 [17]. Survival was assessed at 2 and 48 hours.

Western Blot Analysis of Ant1 and Ant2 Protein Levels—Relative protein levels were assessed by Western blots prepared from 50 μg solubilized protein using as primary antibodies rabbit polyclonal antibodies against Ant1 and Ant2 [12], and a mouse porin 31HL monoclonal antibody (CalBiochem, 89–173/016; 1:10000 dilution). Western blots were immunoreacted with primary antibodies overnight at 4°C, stained with horse radish peroxidase (HRP)-conjugated secondary antibody (the species origin of the secondary antibodies varied depending on the origin of the primary antibody), the antibody staining detected by chemiluminescence (Amersham), and quantified by densitometric analysis.

Primary Neuronal Cell Cultures: Treatments and Cell Death

Preparation of Primary Neuronal Cultures—Primary mouse embryonic brain cell cultures were established from dissociated 18 day embryos [18]. The hippocampus and cortex were removed and incubated for 15 min in calcium and magnesium-free Hank's Balanced Saline Solution (Mediatech, Inc.) containing 0.25% trypsin. The cells were dissociated and plated into poly-D-Lysine-coated plastic or glass-bottom culture dishes containing Neurobasal Medium supplemented with 10% B27, 2 mM L-glutamine, and 25 μ M glutamate. Following cell attachment, the culture medium was replaced with medium without glutamate. Toxicity experiments were performed after 7 days in culture (7 DIC), a time during which neurons are vulnerable to excitotoxic and apoptotic insults [19, 20].

Quantification of neuron survival in hippocampal cell cultures [21] involved pre-marking microscope fields for the number of undamaged neurons, challenge of these cells with the excitotoxic agent, and monitoring the number of surviving cells over time. Neurons with intact neurites and a cell body that was smooth and round to oval in shape were considered viable, whereas neurons with beaded or fragmented neurites and a cell body that was shrunken and rough in appearance were considered nonviable. Overall cell viability was assessed by the release into the medium of lactate dehydrogenase assay (LDH kit, Roche), performed in a 96 well assay format. The LDH activities were normalized as the percentage of control of DMSO-treated control groups.

Measurement of Cellular ATP Levels—Neuronal cell ATP levels were assayed using a luciferin/luciferase-based assay. Neuronal cell cultures were rinsed with phosphate buffered saline and lysed with 0.2 ml lysis reagent (Roche, Mannheim, Germany). The ATP concentrations were quantified using the ATP Bioluminescence Assay Kit CLS II (Roche, Mannheim, Germany) with a Sirius-Luminometer (Berthold Detection Systems). Experimental values were compared to an ATP standard curve with values reported as nmole ATP per mg protein [22].

Rhodamine 123 Evaluation of Mitochondrial Membrane Potential and mtPTP Activation—Mitochondria from the forebrain (whole brain minus the cerebellum and brain stem) were isolated following euthanasia by cervical dislocation. The forebrains were homogenized with six hand strokes of a glass-Teflon dounce homogenizer containing 10 ml of isolation buffer (225 mM mannitol, 75 mM sucrose, 10 mM MOPS, 1 mM EGTA, 0.5% BSA, pH 7.2). The homogenate was then centrifuged for 5 min at 4°C at 1000 × *g*, the supernatant retained and centrifuged for 15 min at 4°C at 8000 × *g*, and the pellet resuspended in approximated 300 μl of isolation buffer. Protein was measured by pelleting the mitochondria in 4 μl aliquots, 2 min at 10000 × *g*, resuspending the pellet in 200 μl of isolation buffer without BSA by sonication and quantified using the Bradford method. Mitochondrial membrane potential and permeability transition were measured using the

fluorescent probe rhodamine 123 [23] with a Perkin Elmer LS50B luminescent spectrophotometer set at 490 nm excitation and 535 nm emission with stirring. One mg of mitochondrial protein was added to 3 ml of reaction buffer (250 mM sucrose, 10 mM MOPS, 2 mM K_2HPO_4, 5 mM succinate, pH 7.2) at 30°C. Fluorescence was allowed to stabilized, and then $CaCl_2$ was added in sequential aliquots of 15 or 25 nmol, each associated with a transient increase in fluorescence. Activation of the mtPTP, and collapse of the electrochemical gradient was signaled by the maximum rise and stabilization of fluorescence.

Rhodamine 123 concentrations were quantified using a standard curve, and membrane potentials were calculated by the Nernst equation with the concentration outside being measured directly by the minimum level of fluorescence after the addition of mitochondria ($F_{outside}$), and the concentration inside being the difference between the total concentration (maximum fluorescence obtained after $CaCl_2$ addition, F_{total}) and the concentration outside. The mitochondrial volume was assumed to be 1 μl /μg [24].

RESULTS

Expression of Ant1 in Brain and Hippocampal Neurons

The regional distribution of Ant1 expression in the brain was determined by staining brain sections from heterozygous *Ant1*$^{\beta geo}$ mice with X-gal to detect β-galactosidase transcribed from the endogenous *Ant1* promoter. Ant1 was found to be expressed throughout the adult *Ant1*$^{\beta geo}$ brain in both neurons and glial cells. It was highly expressed in the external and internal pyramidal neurons and in cortical layers III and V. Higher staining was seen in the reticular thalamic nucleus and lateral ventricles. Ant1 expression was also seen in the hippocampal pyramidal neurons (CA1, CA2, CA3 and hilus), but not in the granule cells in the dentate gyrus (Fig. 1A). However, the absence of β-galactosidase staining in the granule cells in the dentate gyrus is probably the result of suppression of expression of the *βgeo* fusion protein in these cells rather than lack of transcription from the *Ant1* promoter [25]. Sagittal sections of cerebellum showed that Ant1 was highly expressed in the Purkinje and granule cell layers, and in the fastigal nuclei (Fig. 1B).

To confirm that neurons express Ant1, primary cultured hippocampal neurons differentiated from the embryonic brains of *Ant1*$^{\beta geo}$ mice were stained with X-gal and found to be strongly positive. Moreover, the fusion protein was localized to the neuronal mitochondria, presumably targeted there by the first third of the Ant1 protein that was included in the fusion protein (Fig. 1C) [12].

Previously, we observed that Ant1 is up-regulated in response to cortical lesions resulting in glial scaring [26] and that this is mediated through TGFβ-Smad signal transduction pathway [27]. To determine if Ant1 is also inducible in neurons, we used Western blots to examine the levels of Ant1 and Ant2 in culture hippocampal neurons, following 24h exposure to 100 μM glutamate. After glutamate exposure, Ant1 increased in the neurons, but Ant2 levels were unaffected (Fig. 2A). This was also true for the whole brain since mice injected with the neuronal excitotoxin KA showed an increase in Ant1 protein, but not in Ant2 protein (Figure 2B).

Ant1-deficient Mice are More Resistant KA Induced Mortality

To determine if Ant1-deficiency affects the brain's susceptibility to excitotoxic challenge, we injected age-matched Ant1-deficient and control mice with a single dose of KA and then monitored the seizure activity and mortality of the mice at 2 hours and then 48 hours. Two hours after injection, 90% of the Ant1-deficient (9 of 10) mice and 100% of the control mice exhibited stage 3 or above seizure activity (Table 1). However, 92% (11 of 12) of the control

mice died, while only 40% (4 of 10) of the ANT1-deficient mice died, a significant difference ($P < 0.01$) (Table 1). This mortality difference was significant even when only mice showing stage 3 seizure activities or above were included in the analysis with 4/9 stage 3 Ant1-deficient mice versus 10/12 stage 3 control mice having died. By 48 hours post-injection an additional Ant1-deficient mouse had died, yet the difference in mortality was still significantly different, $P < 0.02$ (Chi Square) or $P < 0.05$ (Fisher Exact Test). Thus, Ant1-deficiency it is strongly protective of animal mortality following KA excitotoxicity, though Ant1-deficiency is not protective for KA-induced seizures..

Ant1-deficient Neurons are More Resistant to Glutamate-Induced Excitotoxicity

To examine the role of Ant1 in neuronal survival after excitotoxic challenge, we exposed cultured hippocampal and cortical neurons to increasing concentrations of glutamate. Glutamate binds to the NMDA receptor, resulting in the influx of Ca^{++} into the cells and leading to increased ROS production and excitotoxic cell death [28]. After 24 hours of 20 to 400 μM glutamate exposure, about twice as many of the Ant1-deficient hippocampal neurons survived as undamaged neurons in pre-marked microscopic fields as wild type neurons (Fig. 3A). This cell survival data was confirmed by cell lysis release of LDH into the medium (data not shown). Increased glutamate resistance was also seen in Ant1-deficient cortical neurons (Fig. 3B). Therefore, Ant1-deficient neurons are significantly more resistant to glutamate excitotoxicity than the wild type neurons, suggesting that the loss of Ant1 is neuroprotective.

Ant1-Deficient Neurons are More Resistant to DNA Damage-Induced Cell Death

To determine if Ant1-deficiency protected neurons to other initiators of cell death, we exposed cortical and hippocampal Ant1-deficient and wild type neurons to the topoisomerase II inhibitor, etoposide [29]. Cell lysis was quantified through the release of LDH into the medium after 24 hours. Following etoposide exposure, wild type cortical and hippocampal neurons released almost twice as much LDH as did the Ant1-deficient neurons (Figure 4). This etoposide-induced neuronal death was associated with PARP cleavage, detected using Western blots (data not shown). Thus, Ant1-deficient cells were more resistant to etoposide induced cell death than wild type neurons (Figure 4).

Ant1-deficient Neurons have Reduced Intracellular ATP levels

To determine if Ant1-deficiency reduced cellular ATP levels, we used a luciferase assay to measure ATP concentrations in cell lysates. This revealed that the ATP levels were decreased by approximately 20% in the embryonic cortical neurons ($p < 0.05$) and 67% in hippocampal neurons ($p < 0.001$) derived from Ant1-deficient brains (Fig. 5). The residual levels of ATP sustained by the Ant1-deficient neurons probably reflect continued ADP - ATP exchange through Ant2 and ATP generation by glycoysis.

Ant1-deficient Neuronal Mitochondria are Resistant to Ca^{++}-Induced mtPTP Opening

Mitochondria isolated from the brains of Ant1-deficient and wild type mice were assayed for the mitochondrial inner membrane electrochemical potential and sensitivity of the mtPTP to Ca^{++} activation, both monitored by the uptake and release of rhodamine 123 in energized mitochondria. Forebrain mitochondria from Ant1-deficient mice were found to have a membrane potential of 193 mV while wild type mitochondria had a membrane potential of 184 mV (Fig. 6A). This approximately 9 mV/mg protein increase in mitochondrial inner membrane potential is highly significant and may reflect a reduction in the proton leak through the inner membrane due to loss of the Ant1 [30].

To measure the potential of the Ant1-deficient mtPTP to undergo permeability transition, the amount of Ca^{++} was quantified that was required to cause an irreversible release of rhodamine 123 from the mitochondria due to the collapse of the electrochemical gradient (Figure 6B). The Ant1-deficient mitochondria required 72 nmole of Ca^{++} to initiate mtPTP transition while wild type mitochondria required 60 nmoles, approximately 20% less ($P < 0.05$) (Figure 6C). Hence the mtPTPs of the Ant1-deficient mitochondria are significantly more resistant to Ca^{++}-induced permeability transition. Still, the increased resistance of the partially Ant1-deficient brain mitochondria (Ant1 null, Ant2 positive) to Ca^{++} induced permeability transition was much less than that required to activate the completely Ant1-deficient liver mitochondria (Ant1 null, Ant2 null) [11], suggesting that both Ant1 and Ant2 may contribute to the Ca^{++} sensitivity of the mtPTP.

DISCUSSION

We had previously shown that Ant1 is strongly expressed in mouse skeletal muscle, heart, and brain [5, 12]; and have now determined the regional expression of Ant1 in the brain and confirmed that Ant1 is strongly expressed in neurons. Even so, while inactivation of *Ant1* in mouse [12] and *ANT1* in humans [31] results in severe muscle and heart pathology, neither mice of humans show an overt neurological phenotype. This is particularly remarkable since Ant1-deficiency in the mouse cortical and hippocampal neurons results in a 1/5 and 2/3 reduction in ATP levels, respectively. This reduction in cellular ATP levels is not a product of reduced OXPHOS coupling, since the mitochondrial inner membrane potential of Ant1-deficient mitochondria is significantly higher than that found in wild type mitochondria, a result consistent with our previous observation that Ant1 makes a significant contribution to the mitochondrial inner membrane basal proton leak which inactivation Ant1 blocks [30].

The Ant1-deficient mouse is also significantly more resistant to KA-induced neuronal excitotoxicity and death than wild type mice, and Ant1-deficient cortical and hippocampal neurons are more resistance to glutamate and KA induced excititoxic cell death as well as etoposide-induced cell death. Isolated Ant1-deficient brain mitochondria are also significantly more resistance to Ca^{++} induced mtPTP permeability transition than controls [21, 32–35]. Since both etoposide-induced cell death and Ca^{++}-induced permeability transition of cultured rodent embryonic neurons has been shown to be inhibited by cyclosporin A [36] and ectoposide induced cell death shown to be associated with increased cell reactivity to annexin V [36], it would seem that Ant1 is important in excitotoxic Ca^{++}-induced mtPTP permeability transition leading to cell death by apoptosis.

These observations lead to the question, why are Ant1-deficient brains resistance to excitotoxic insult? We have previously shown that Ant1, but not Ant2, is induced by brain trauma, medicated by TGFβ and the Smad pathway [26, 27]. Furthermore, it has been reported that increased expression of Ant1 achieved by transfection of cultured cells is pro-apoptotic [37]. Therefore, we might hypothesize that Ant1 is bi-functional in the brain. Under normal circumstances, Ant1 functions to export mitochondrial ATP into the nucleus-cytsol compartment. When the brain is stressed, TGFβ and the Smads are activated, inducing Ant1 to transport out more mitochondrial ATP to the nuclear-cytosol to facilitate repair and recovery. However, if the damage to the cell is sufficient to compromise mitochondrial ATP production, then induction of Ant1 can not increase ATP levels and does not resolve the cellular problem. Since the accumulation of such energetically defective cells would erode organ function, the continued induction of Ant1 ultimately activates the mtPTP, resulting in the elimination of the defective cell by apoptosis. While this is an optimal strategy at the individual cell level, generalized brain insults such as those resulting from KA excitotoxicity or ischemic stroke can result in pan-neuronal apoptosis, causing

destruction of the brain function and death. Inactivation of Ant1 or inhibition of the mtPTP [38] may then block this catastrophic outcome.

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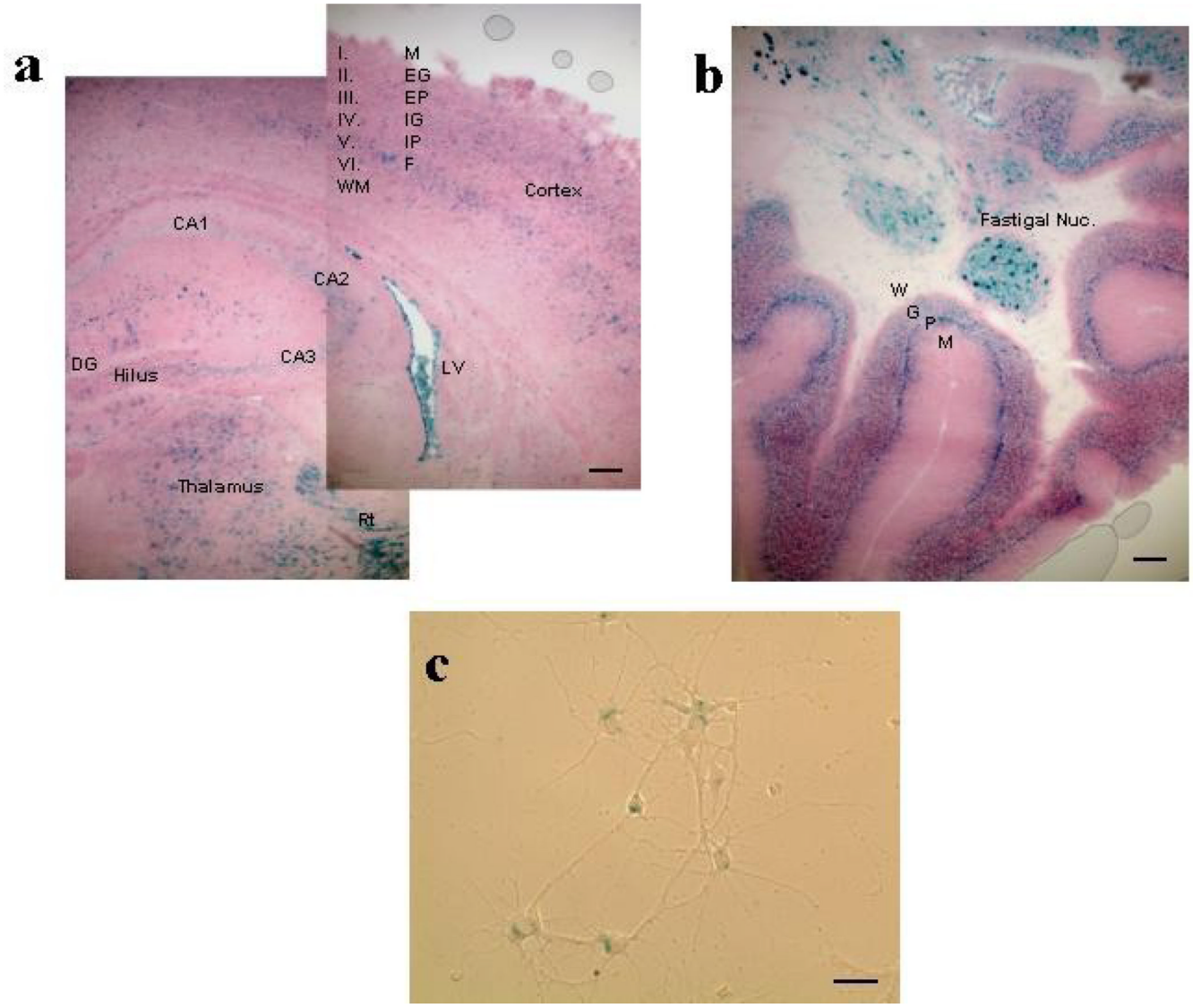

Fig. 1. Ant1 Expression in the Mouse Brain and Hippocampal Neurons

X-gal stained brain and hippocampal neurons taken from homozygous *Ant1*$^{\beta geo}$. (a) A coronal section of adult mouse brain and (b) a sagittal section of cerebellum were fixed and stained with X-gal, then counterstained with Nuclear Fast Red. I. Molecular layer (M), II. External Granular layer (EG), III. External Pyramidal layer (EP), IV. Internal Granular layer (IG), V. Internal Pyramidal layer (IP), VI. Fusiform layer (F), WM: white matter, Rt: reticular thalamic nucleus, DG: dentate gyrus, W: white matter, G: granular layer, P: purkinje cell layer, M: molecular layer. (C) Hippocampal neurons cultured from embryonic brains of Ant1 βgeo mice and stained with X-gal. Scale bar: 100 μm (A, B), 20μm.

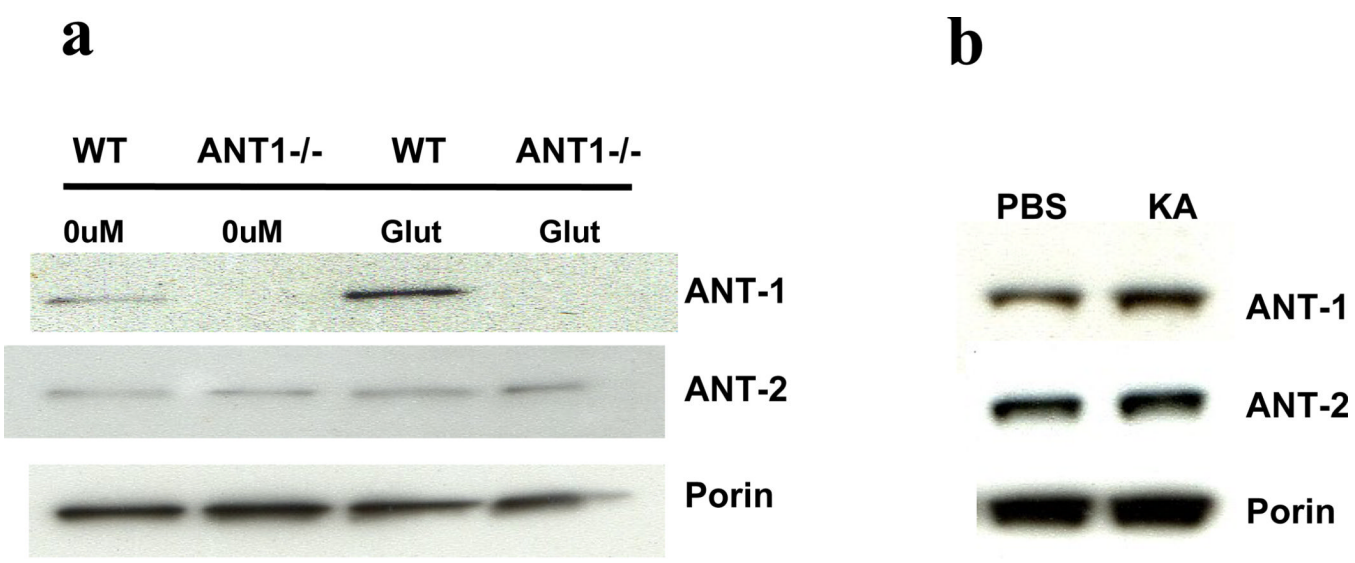

Figure 2. Excitatory Challenge Induces Ant1 but not Ant2 Protein Levels in Cultured Neurons and Brains

(a) Cultured hippocampal neurons were challenged with glutamate, and Ant1 and Ant2 protein levels assessed by Western blot relative to porin. (b) Mice were challenged with KA, the hippocampi isolated, and the levels of Ant1 and Ant2 assessed by Western blot relative to porin. The data were confirmed by three independent experiments

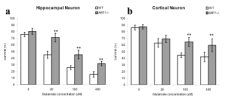

Figure 3. Ant1-Deficiency Protects Cultured Hippocampal Neurons from Excitotoxic Death

Cultured (A) hippocampal and (B) cortical neurons were treated for 24 hrs with glutamate, and neuronal survival assessed by phase-contrast photomicrographs (four to five fields per culture dish, approximately 20–40 neurons present in each field). Results are the mean ± SD of 5 to 6 cultures. (**P < 0.01 compared to the wild type).

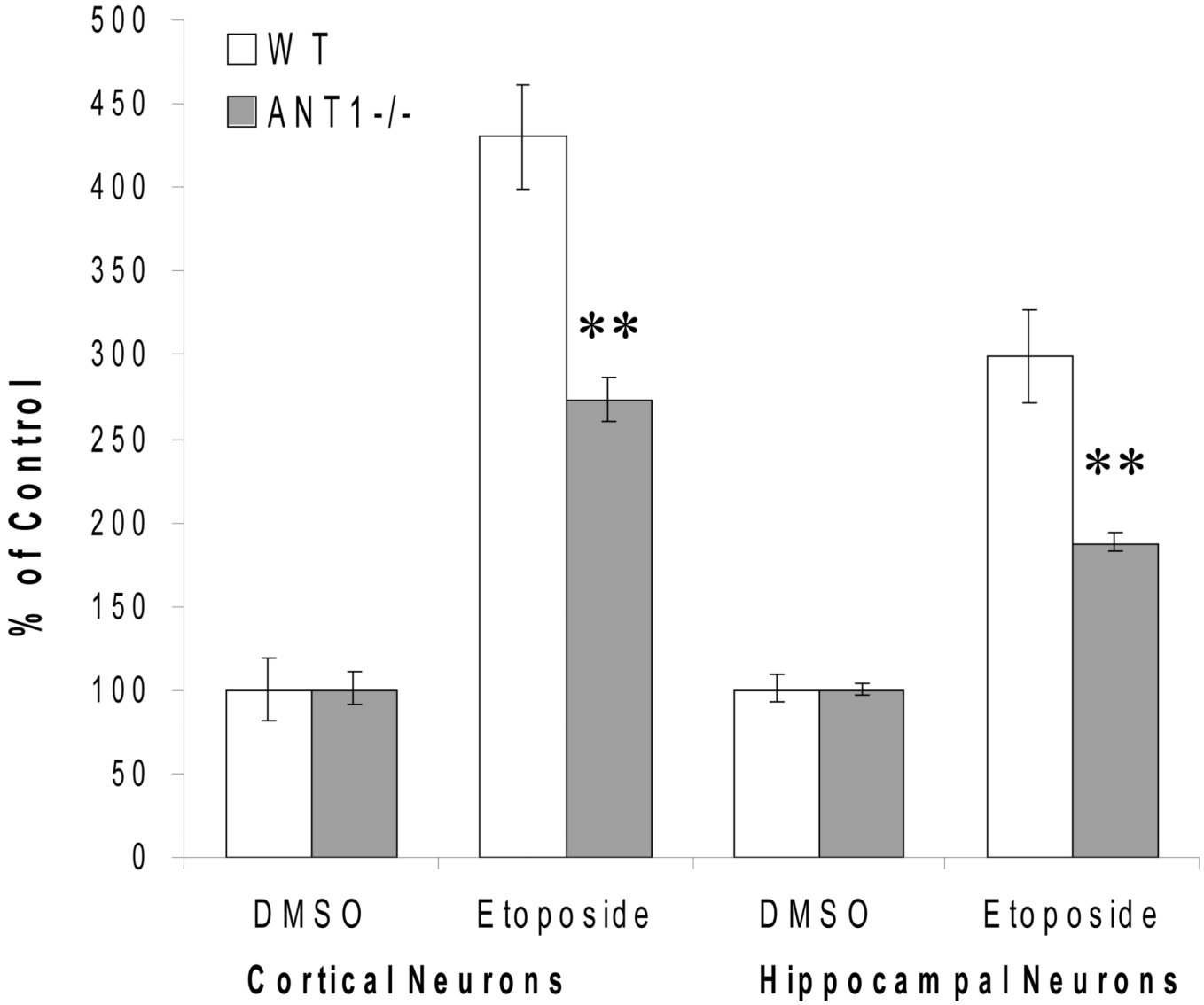

Figure 4. Ant1-Deficiency Protects Cultured Neurons from Death Induced by DNA Damage

Neurons were exposed to 10 μM ectoposide for 24 hrs, and cell viability was assessed by LDH release. Values are the means and SD of determinations made in six different cultures. **P < 0.01 compared to corresponding value for cultures from wild type mice.

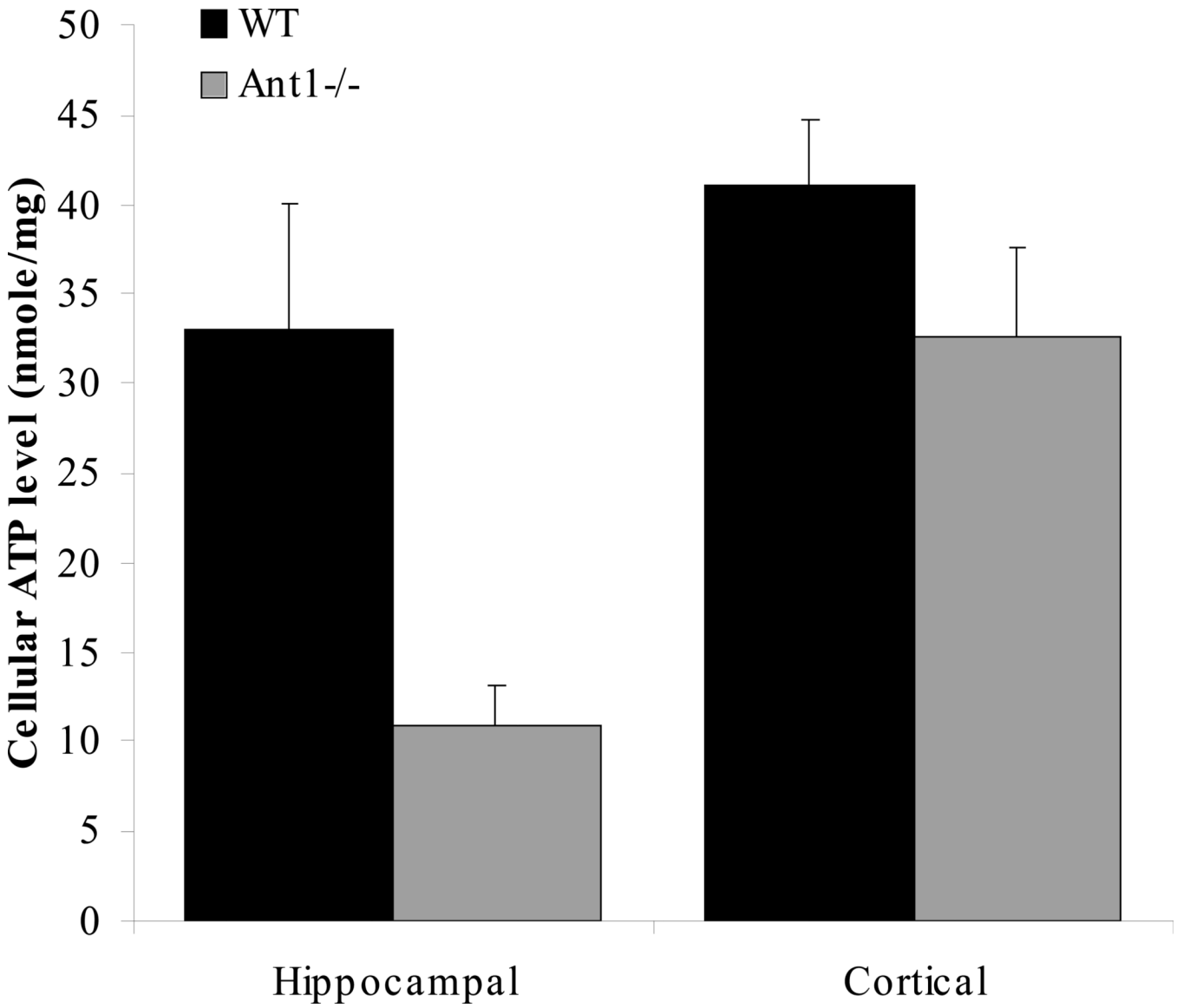

Figure 5. Lower Intracellular ATP Levels in Cortical and Hippocampal Neurons of ANT1-Deficient Mice

Values are the mean and SD of determinations made in 4 different cultures. Ant1-deficient cortical neurons, $P < 0.05$ and Anti-deficient hippocampal neurons ($P < 0.001$) relative to wild type neurons.

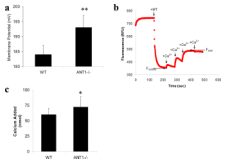

Fig. 6. Monitoring Membrane Potential and mtPTP Transition in Response to Ca^{++} Additions and mtPTP Transition

(A) Mitochondrial membrane potentials of Ant1-deficient and wild type brain mitochondria ($P < 0.0001$). (B) Additions of mitochondria (MT) and $CaCl_2$ (Ca^{++}) are indicated as are the points at which the fluorescence readings, $F_{outside}$ and F_{total}, were made. (C) The Ca^{++} required to initiate mtPTP transition in mitochondria isolated from Ant1-deficient and wild type brains $P < 0.05$. Results are means and SD, n = 12 per genotype.

Table I

Seizure Activity and Mortality in Mice Two Hours After Systemic KA Administration.

Group	Stage 3 or Above Seizures		Mortality	
	#/N	%	#/N	%
Wild type	12/12	100	11/12	92
Ant1-KO	9/10	90	4/10	40[1]
			4/9	44[2]

KA was administered systemically to mice by intra-peritoneal injection of 35 mg/kg. Ant1-KO = Ant1-knockout mice.

[1] mortality of all mice injected with KA, relative to wild type P = 0.01.

[2] mortality of mice showing stage 3 seizure activity or above following KA injection, relative to wild type P = 0.02.