Acidotoxicity and acid-sensing ion channels contribute to motoneuron degeneration

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Amyotrophic lateral sclerosis (ALS) is a fatal neurological condition with no cure. Mitochondrial dysfunction, Ca^{2+} overloading and local hypoxic/ischemic environments have been implicated in the pathophysiology of ALS and are conditions that may initiate metabolic acidosis in the affected tissue. We tested the hypothesis that acidotoxicity and acid-sensing ion channels (ASICs) are involved in the pathophysiology of ALS. We found that motoneurons were selectively vulnerable to acidotoxicity *in vitro*, and that acidotoxicity was partially reduced in *asic1a*-deficient motoneuron cultures. Cross-breeding of *SOD1*^{G93A} ALS mice with *asic1a*-deficient mice delayed the onset and progression of motor dysfunction in *SOD1* mice. Interestingly, we also noted a strong increase in ASIC2 expression in motoneurons of *SOD1* mice and sporadic ALS patients during disease progression. Pharmacological pan-inhibition of ASIC channels with the lipophilic amiloride derivative, 5-(*N,N*-dimethyl)-amiloride hydrochloride, potently protected cultured motoneurons against acidotoxicity, and, given post-symptom onset, significantly improved lifespan, motor performance and motoneuron survival in *SOD1* mice. Together, our data provide strong evidence for the involvement of acidotoxicity and ASIC channels in motoneuron degeneration, and highlight the potential of ASIC inhibitors as a new treatment approach for ALS.

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative condition where motoneurons in the spinal cord and brain stem die, resulting in paralysis and eventual death. Despite the significant recent advances in understanding the pathophysiology and genetics of ALS,¹ there is no cure for this condition. Currently, the only FDA-approved, disease-modifying drug used for the treatment of ALS is riluzole, which inhibits neuronal glutamate, releases and stimulates glutamate uptake into astrocytes.² The use of riluzole is based on the hypothesis that overactivation of Ca^{2+} -permeable AMPA receptors in motoneurons results in excitotoxicity, and that this process contributes to motoneuron death in ALS.³ Nevertheless, the disease-modifying effects of riluzole therapy are moderate and vary among patients.⁴

Acid-sensing ion channels (ASICs) represent a group of ion channels activated by protons. They belong to the epithelial sodium (Na^+) family of amiloride-sensitive cation channels, and allow for Na^+ and Ca^{2+} entry into neurons. Of the six ASIC subunits cloned, ASIC1a, ASIC2a and ASIC2b are expressed in the brain and spinal cord neurons.⁵ ASIC1a and ASIC2s are found in the brain regions with high synaptic density and facilitate excitatory synaptic transmission.^{6,7} ASIC1a in particular is involved in nociception and fear behavior triggered by hypercapnia.^{8,9} ASICs have also been investigated as new targets for the treatment of ischemic stroke and cerebral hypoxia¹⁰ on the premise that activation of

ASIC1a during ischemia may cause neuronal cell death through toxic Ca^{2+} and Na^+ influx.^{10,11}

Metabolic acidosis can occur as a result of lactate accumulation when tissue perfusion is inadequate, or when mitochondrial respiration is inhibited.¹² Moreover, mitochondrial dysfunction has been shown to manifest as lactic acidosis in patients with ALS.¹³ Of note, mitochondrial dysfunction and Ca^{2+} overloading, as well as a local hypoxic/ischemic environment, have been implicated in the pathophysiology of ALS.^{14–17} In the present study, we therefore investigated the involvement of acidotoxicity and ASIC channels in motoneuron degeneration, and explored whether pharmacological inhibition of ASIC channels represents a new approach for the treatment of ALS.

Results

Motoneurons are highly vulnerable to acidotoxicity. We first addressed the question whether motoneurons were vulnerable and/or intrinsically sensitive to acidotoxic injury. Acidotoxic stress was produced *in vitro* by exposure of mixed motoneuron cultures to media, pH 6.5 for 4 h, followed by 24-h recovery, as described previously.¹⁰ As mixed motoneuron cultures from mouse spinal cord ventral horns contain both motoneurons and non-motoneurons, we used smi-32, a marker that is expressed preferentially in motoneurons, and

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Abbreviations: ALS, amyotrophic lateral sclerosis; ASIC, acid-sensing ion channel; DMA, 5-(*N,N*-dimethyl) amiloride hydrochloride; DIV, days *in vitro*; PaGE, paw grip endurance test; SOD1, superoxide dismutase 1

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NeuN, a general neuronal marker, to evaluate if there was an enhanced vulnerability in motoneurons to acidotoxicity. This short-term acidotoxic stress was sufficient to cause a significant reduction in neuron counts (28.1%; $P < 0.01$) compared with controls. Although the general neuronal population appeared vulnerable to acidotoxicity, there was a higher vulnerability in motoneurons to acidotoxic stress (61.5% reduction in motoneuron survival; $P < 0.01$, Figure 1a). We next tested whether ASIC1a was involved in mediating this toxicity using two distinct approaches—*asic1a* deletion and a specific ASIC1a blockade with the toxin PcTx1. *wt* primary motoneuron cultures treated with PcTx1 demonstrated a significant increase in motoneuron survival

following acidotoxic stress ($P < 0.01$; Figure 1b). Interestingly, the protection afforded by *wt* cultures treated with PcTx1 following acidotoxic stress was comparable to that observed in *asic1a*-deficient cultures ($P < 0.01$; Figure 1b). These data suggested that motoneurons were more vulnerable to acidotoxicity than other neuronal populations, and that *asic1a* contributed to acidotoxic-induced cell death in motoneurons.

Genetic deletion of *asic1a* delays disease onset and progression in *SOD1* mice. In light of these observations, we next explored whether *asic1a* was involved in motoneuron degeneration *in vivo* in *SOD1*^{G93A} mice (*SOD1*). The

Figure 1 Acidotoxic stress in motoneurons *in vitro*. (a) Direct counts of neuron survival versus motoneuron survival following acidotoxic stress (neurobasal medium (NBM) pH 6.5, 4 h). $n = 6$ per group. (b) Direct counts of motoneuron survival following acidotoxic stress and co-treatment with ASIC1a blocker, PcTx1 in *wt* and *asic1a*^{-/-} primary motoneuron cultures. * $P < 0.01$, $n = 6$ per group. (c) Photomicrographs of smi-32 (red) and DAPI (blue) immunostained *wt* and *asic1a*-deficient primary motoneuron cultures following acidotoxic stress; scale bar = 125 μ m. Five independent cultures (in triplicate) were used per treatment group in these experiments

Figure 2 Genotyping in double-mutant *SOD1*; *asic1a* mice. (a) PCR confirming *SOD1* genotype in double-mutant *SOD1*; *asic1a* mice. The *SOD* allele is recognized by the presence of a PCR product at 236 bp; the presence of a band at 324 bp alone confirms the absence of the *SOD* allele. (b) Representative western blot of human *SOD1* and *ASIC1* protein expression in double-mutant *SOD1*; *asic1a* mice with actin as loading control

Figure 3 Lifespan analysis and assessment of disease progression in double-mutant *SOD1*; *asic1a* mice. (a) Analysis of survival demonstrated no significant increase in lifespan for tg *SOD1*; *asic1a*^{+/-} and tg *SOD1*; *asic1a*^{-/-} mice compared with tg *SOD1*; *asic1a*^{+/+} mice. (b) PaGE test performance demonstrated a delay in muscle strength decline in tg *SOD1*; *asic1a*^{+/-} and tg *SOD1*; *asic1a*^{-/-} mice; (pnd 105–145, 0.01 > *P* > 0.0001). (c) Grip strength analysis demonstrated a significant improvement in grip strength in tg *SOD1*; *asic1a*^{+/-} and tg *SOD1*; *asic1a*^{-/-} mice (pnd 100–145, 0.001 > *P* > 0.00001). (d) Rotarod test showed an improvement in motor performance in tg *SOD1*; *asic1a*^{-/-} mice (pnd 100–150, 0.01 > *P* > 0.001). Data displayed as mean ± S.D.; *n* = 24 per group

Figure 4 Motoneuron survival in double-mutant *SOD1*; *asic1a* mice. (a) Direct counts of motoneuron survival at pnd 120 and end-stage show an increase in lumbar motoneuron counts in tg *SOD1*; *asic1a*^{-/-} mice compared with tg *SOD1*; *asic1a*^{+/+} mice. *ntg *SOD1*; *asic1a* versus tg *SOD1*; *asic1a* mice, *P* < 0.001; # tg *SOD1*; *asic1a*^{+/+} versus tg *SOD1*; *asic1a*^{-/-}, *P* < 0.01; † ntg *SOD1*; *asic1a*^{+/+} versus ntg *SOD1*; *asic1a*^{-/-}, *P* < 0.01, *n* = 6/group. Data displayed as mean ± S.D.; *n* = 6/group. (b) Photomicrographs of Nissl-stained ventral horn motoneurons at pnd 120 in double-mutant *SOD1*; *asic1a* mice. Scale bar = 100 μm

SOD1 mouse is one of the most studied and best-characterized mouse models of ALS and carries the transgene (tg) of a mutant allele human SOD1 containing the Gly93 → Ala (G93A) substitution. A double-mutant breeding program was carried out to generate a mouse model of ALS deficient for *asic1a*. Cross-breeding *SOD1* mice with *asic1a*-deficient mice did not alter the *SOD1* genotype (Figure 2a), nor did it alter the protein levels of SOD1 protein or ASIC1 protein in double-mutant *SOD1*; *asic1a* mice (Figure 2b). Analysis of lifespan in double-mutant mice demonstrated that the deletion of *asic1a* did not extend the lifespan in tg *SOD1*; *asic1a*^{+/-} (160.9 ± 5.1 days) and tg *SOD1*; *asic1a*^{-/-} (162.9 ± 5.0 days) mice compared

with tg *SOD1*; *asic1a*^{+/+} mice (160.6 ± 3.1 days; Figure 3a). However, functional analysis showed a significant delay in disease progression and an increase in motor ability in double-mutant *SOD1*; *asic1a* mice. Disease progression was monitored using functional assessments of motor performance. The paw grip endurance test (PaGE), used to monitor muscular strength and motor neuron integrity of the forelimbs and hindlimbs, showed that the performance of tg *SOD1*; *asic1a*^{+/+} mice began to decline from 100 days onwards (Figure 3b). A delay in muscle strength decline was observed in tg *SOD1*; *asic1a*^{-/-} and tg *SOD1*; *asic1a*^{+/-} mice when compared with tg *SOD1*; *asic1a*^{+/+} counterparts (postnatal day (pnd) 100–150, 0.01 > *P* > 0.0001). Grip

Figure 5 Expression of *asic1*, *asic2* and *asic3* during ALS disease progression. (a) Co-staining of lumbar spinal cords from tg *SOD1*- and *asic1a*-deficient mice with anti-ASIC1/ASIC2 (red) and anti-NeuN/GFAP (green) antibodies. Arrowheads show co-localization of ASIC1 and ASIC2 with NeuN on cell soma of neurons with motoneuron morphology. Scale bar = 20 μ m. (b) *asic1*, -2 and -3 mRNA expression in the transgenic (tg) *SOD1* mouse; lumbar spinal cord lysates show a significant upregulation of the *asic1a* and *asic2a* mRNA; **P* < 0.01. Data displayed as mean $\pm$ S.D.; *n* = 6/group. (c) Co-staining of lumbar spinal cords across disease progression in the tg *SOD1* mouse with anti-ASIC1/ASIC2 (red) and anti-NeuN/smi-32 (green) antibodies. Co-localization of ASIC antibodies with motoneurons is indicated by arrowheads. Scale bar = 15 μ m. (d) An increase in ASIC1 and ASIC2 protein levels in the tg *SOD1* mouse; **P* < 0.01. Data displayed as mean $\pm$ S.D.; *n* = 6/group. (e) A representative image of ASIC1 and ASIC2 protein (double band) levels by western blotting, *n* = 3 experiments with tubulin as loading control. (f) ASIC2 immunohistochemistry in ventral motoneurons of lumbar spinal cord sections from ALS and non-ALS patients. *n* = 3/group. Scale bar = 50 μ m

Table 1 (A) Characteristics of cases and controls used from ALS and non-ALS patients. (B) Mean staining intensity values for ASIC2 protein in ALS and non-ALS patients

(A)				
Numbers	Age at death	Gender	Diagnosis	Disease onset
Case				
ALS				
1	74	M	Sporadic ALS	Limb
2	49	M	Sporadic ALS	Limb
3	58	M	Sporadic ALS	Limb
Control				
11	79	M	Septicemia	
12	40	M	Renal failure	
13	57	M	Aortic aneurysm	

(B)			
Antibody	Group	Measure	Staining intensity/motoneuron (Intensity/ μm^2)
ASIC-2	Non-ALS	Mean	0.21
		S.D.	0.11
ASIC-2	ALS	Mean	0.53
		S.D.	0.14
		P-value	<0.01

Abbreviations: ALS, amyotrophic lateral sclerosis; ASIC, Acid-sensing ion channel.

strength meter analysis, which measures forelimb strength, displayed a similar, significant improvement in forelimb strength at pnd 110 in tg *SOD1;asic1a*^{+/-} and tg *SOD1;asic1a*^{-/-} mice, which continued until pnd 150 (Figures 3c, 0.01 > P > 0.0001). Rotarod analysis, which measures front and hindlimb performance, demonstrated an improvement in motor performance from pnd 110 to pnd 145 in tg *SOD1;asic1a*^{-/-} mice (Figure 3d, P < 0.01). Together, these assessments demonstrate that *asic1a* deficiency delayed the onset and progression of motor deficits in *SOD1* mice.

Genetic deletion of *asic1a* increases motoneuron survival in *SOD1* mice. We next examined whether *asic1a* deficiency delayed motoneuron degeneration in *SOD1* mice by histologically assessing motoneuron survival in the lumbar spinal cord by Nissl staining. As expected, there was a significant decrease (P < 0.001) in the number of motoneurons surviving in tg *SOD1;asic1a*^{+/+} mice at pnd 120 (Figure 4a; 17.2 ± 1.1 motoneurons) and pnd 160 (18.6 ± 0.7 motoneurons) compared with nontransgenic (ntg) *SOD1;asic1a*^{+/+} counterparts (32.9 ± 2.8 and 30.3 ± 1.9 motoneurons, respectively). However, *asic1a* deficiency rescued ventral horn motoneurons from degeneration at pnd 120 (21.8 ± 2.2 motoneurons) and pnd 160 (23.1 ± 1.6 motoneurons) in tg *SOD1;asic1a*^{-/-} mice compared with tg *SOD1;asic1a*^{+/+} mice (Figures 4a and b, P < 0.01). Interestingly, *asic1a* deletion in nontransgenic *SOD1* mice reduced the baseline number of motoneurons at pnd 120 (23.3 ± 2.3 motoneurons) and pnd 160 (23.1 ± 1.6 motoneurons) compared with nontransgenic *SOD1;asic1a*^{+/+} mice (P < 0.01).

ASIC1 and ASIC2 are increased in the *SOD1* mouse model during disease progression. As a specific role of ASIC channels in motoneurons or in ALS, pathogenesis has

Figure 6 *In vitro* treatment with DMA in mixed motoneuron cultures following acidotoxic stress. Treatment with DMA (black) demonstrated protection in motoneurons against acidotoxic stress-induced cell death *in vitro*, whereas riluzole (gray) did not. *P < 0.01; data displayed as mean ± S.D.; n = 5 per group

not yet been explored previously, we next assessed whether the pathophysiology of motoneuron degeneration was accompanied by changes in ASIC channel expression *in vivo*. We determined ASIC1 and ASIC2 protein levels across disease progression by immunohistochemistry and western blotting and *asic1* and *asic2* mRNA expression by qPCR in the *SOD1* mouse.

We examined co-localization of ASIC1 and ASIC2 with markers for neurons (NeuN) and astrocytes (GFAP). Figure 5a shows staining of lumbar spinal cords from *SOD1* mice with each of the ASIC antibodies. The staining for ASIC1 and ASIC2 is similar in neurons, slightly punctate and of higher intensity over the soma than on dendrites or axons as observed previously.^{18,19} ASIC1 and ASIC2 are enriched in neurons staining positive for the neuronal marker, NeuN. Motoneurons are discerned by their larger size and distinctive multipolar morphology. Overlays of anti-ASIC antibodies and NeuN show co-localization of ASIC1 and ASIC2 with NeuN in the cell bodies of larger motoneurons. ASIC1 and ASIC2 showed very little co-localization within the ventral horn of lumbar spinal cords in *SOD1* mice when overlaid with GFAP.

We detected a moderate but significant increase in *asic1* mRNA and protein levels in tg *SOD1* mouse when compared with nontransgenic *SOD1* mice (Figures 5b–d). This is accompanied by an increase in the expression of ASIC1 on the cell soma in motoneurons at pnd 90 and pnd 120 in the tg *SOD1* mouse (Figure 5c). Interestingly, we found a very pronounced and progressive upregulation of both *asic2* mRNA and ASIC2 protein levels in tg *SOD1* mice (Figures 5a–d; P < 0.01). This is accompanied by an increased staining intensity of ASIC2 protein in motoneurons across disease progression in tg *SOD1* mice, higher than that observed for ASIC1 (Figures 5d and e). As expected, little or no levels of *asic3* mRNA were detected across disease progression in tg *SOD1* mice. Although this data demonstrated moderate changes to ASIC1 across disease progression in ALS, the findings of a pronounced and progressive increase in ASIC2 was unexpected, and suggested that, in addition to the ASIC1, ASIC2 may also have a role in ALS disease progression.

Evidence of increased ASIC2 levels in ALS patients. Given our findings of increased ASIC2 levels across disease progression in the tg *SOD1* mouse, we assessed ASIC2

Figure 7 Lifespan analysis and disease progression in *SOD1* mice following post-symptom onset treatment with DMA. (a) Scatter boxplot of survival, demonstrating a significant increase in DMA-treated *tg SOD1* mice but not Riluzole-treated *tg SOD1* mice; $*P < 0.0001$. (b) PaGE test performance, demonstrating a delay in muscle strength decline in DMA-treated *SOD1* mice; (pnd 100–160, $0.001 < P < 0.01$). (c) Grip strength analysis demonstrated an improvement in grip strength in DMA-treated *SOD1* mice compared with vehicle *SOD1* mice; (pnd 95–160, $0.001 < P < 0.01$). (d) Rotarod test showed improved motor performance in DMA-treated *SOD1* mice compared with untreated *tg SOD1* mice; (pnd 105–160, $0.001 < P < 0.01$). Lifespan analysis and motor function assessments displayed as mean $\pm$ S.D.; $n = 24$ /group

Figure 8 Motoneuron survival in *SOD1* mice, following post-symptom onset treatment with DMA. (a) Direct counts of motoneuron survival at pnd 120 and end-stage in *SOD1* mice following DMA treatment; $*P < 0.01$. $n = 6$ /group. Veh., vehicle. Data displayed as mean $\pm$ S.D.; $n = 6$ /group. (b) Photomicrographs of Nissl-stained ventral horn motoneurons at 120 days in *tg SOD1* mice following DMA treatment. Scale bar = 100 μ m

levels in ALS patients. Postmortem spinal cord cross-sections from sporadic ALS (sALS) and non-ALS patients were immunostained with an ASIC2 antibody (Figure 5f; see Table 1a for case details), and a semi-quantitative analysis of immunostaining intensity was performed. Ventral horn motoneurons from both non-ALS and sALS cases stained positively for ASIC2 (Figure 5f); however, in sALS cases, the intensity of ASIC2 staining was significantly enhanced when compared with non-ALS cases (Table 1b; $P < 0.01$).

Cross-inhibition of ASIC1 and ASIC2 with DMA protects motoneurons against acidotoxic stress-induced cell death *in vitro*. We next tested whether cross-inhibition of both ASIC1 and ASIC2 channels exerted an increased capacity to protect motoneurons against acidotoxic injury *in vitro*. DMA, a lipophilic analog of amiloride, has a high affinity and potency to inhibit ASIC channels over other

sodium transport channels and centrally blocks ASIC1 and ASIC2 *in vivo*.^{20,21} We first determined whether DMA had a greater capacity to protect cultured motoneurons against acidotoxic stress than the specific ASIC1a inhibitor PcTx1. DMA demonstrated protection against acidotoxic stress in motoneuron cultures with a dose-dependent increase in motoneuron survival at 30 and 100 μ M DMA ($17.1 \pm 2.6\%$ and $24.2 \pm 3.6\%$ increase in motoneuron survival, respectively, when compared with controls; Figure 6, $P < 0.01$, $n = 5$ /group). Moreover, while ASIC1a blockade with PcTx1 already demonstrated partial protection against acidosis in motoneurons, an additional increase in motoneuron survival was observed in these cultures following DMA treatment ($36.3 \pm 3.2\%$ and $22.8 \pm 2.9\%$ increase in motoneuron survival at 30 and 100 μ M DMA, respectively, when compared with controls, $P < 0.001$). We also compared the *in vitro* effect of DMA treatment to that of riluzole following

acidotoxicity. Treatment with riluzole ($10 \mu\text{M}$) did not protect motoneurons against acidotoxic stress in motoneuron cultures (Figure 6, $n = 5/\text{group}$).

DMA treatment, post-symptom onset, extends lifespan, delays symptom onset and increases motor performance in *SOD1* mice. We therefore examined the effect of oral administration of DMA in the *SOD1* mouse. *SOD1* mice were age, gender, weight and litter-matched, and given DMA (10 mg kg^{-1} per day in drinking water, *ad libitum*), commencing at day 90, that is, post-symptom onset until end-stage. Survival analysis showed that treatment with DMA significantly increased lifespan in the tg *SOD1* mouse (167.7 ± 3.2 days) compared with untreated tg *SOD1* counterparts (157.5 ± 1.3 days; Figure 7a; $P < 0.0001$). We also compared the *in vivo* effect of DMA treatment to that of riluzole. In this study, treatment with riluzole did not extend lifespan in tg *SOD1* mice (160.2 ± 1.3 days; $P > 0.05$) compared with untreated tg *SOD1* mice (Figure 7a; 157.3 ± 1.8 days).

DMA treatment also led to pronounced improvement in motor performance. PaGE tests demonstrated a delay in muscle strength decline at pnd 100, following DMA treatment in tg *SOD1* mice (Figure 7b; pnd 100–160, $0.001 < P < 0.01$). Grip strength meter analysis displayed an improvement in forelimb strength at pnd 95 through to end-stage in tg *SOD1* mice following DMA treatment (Figure 7c; pnd 95–160, $0.001 < P < 0.01$). Rotarod analysis demonstrated a significant improvement in motor performance from pnd 105 onwards in tg *SOD1* DMA-treated mice (Figure 7d) when compared with vehicle-treated tg *SOD1* counterparts (pnd 105–160; $0.001 < P < 0.01$). Nontransgenic, vehicle-treated mice and DMA-treated mice showed no difference in motor function ability across each of the motor function paradigms. Together, these findings demonstrate that DMA treatment can significantly increase lifespan and improve motor performance in tg *SOD1* mice.

DMA treatment, post-symptom onset, increases motoneuron survival in *SOD1* mice. We finally examined whether DMA treatment delayed motoneuron degeneration across disease progression in *SOD1* mice. As expected, there was a significant decrease in the number of motoneurons surviving in tg *SOD1* mice at 120 days (23.8 ± 1.9 motoneurons) and end-stage of the disease (23.1 ± 2.1) when compared with nontransgenic *SOD1* mice (15.7 ± 1.2 and 19.3 ± 0.9 motoneurons, respectively; Figure 7a, $P < 0.01$). Interestingly, DMA treatment not only increased motoneuron survival but restored motoneuron morphology in treated tg *SOD1* mice at 120 days compared with untreated *SOD1* mice (Figures 8a and b; $P < 0.01$). Motoneuron survival at end-stage was not significantly increased in tg *SOD1* mice following DMA treatment (Figure 8a).

Discussion

Here we provide evidence in support of a role of acidotoxicity and ASICs in the pathogenesis of ALS, and for ASICs as a viable target for the treatment of ALS. Our results

demonstrate ASIC channel upregulation during ALS disease progression, demonstrate ASIC1a-dependent and -independent roles in motoneuron acidotoxicity, and provide evidence for a neuroprotective effect of the ASIC channel blocker, DMA, in prolonging survival and improving motor performance in the *SOD1* mice, an established *in vivo* model of ALS.

Motoneuron degeneration in ALS has been increasingly linked to conditions that favor metabolic tissue acidosis.¹³ What are the possible causes of acidosis during motoneuron degeneration in ALS? First, previous findings have implicated the occurrence of local hypoxia/ischemia and blood–spinal cord barrier leakage in the pathophysiology of ALS,¹⁴ events that may lead to metabolic acidosis in the affected tissues. Reduced angiogenesis may also contribute to the pathophysiology of ALS: mutations in hypoxia inducible factor angiogenin are associated with familial and ‘sporadic’ forms of ALS in humans,²² while a deletion in the hypoxia response element of the *vascular endothelial growth factor (VEGF)* gene is sufficient to cause motoneuron degeneration in mice.²³ Second, ALS has been increasingly linked to mitochondrial dysfunction and disturbances in bioenergetics. Mitochondrial abnormalities have been identified in post-mortem tissue derived from ALS patients,²⁴ with ALS-linked *SOD1* mutations shown to associate with mitochondria and disturb their bioenergetic capacity through an interaction with VDAC1.²⁵ *In vivo* autoradiography studies in *SOD1* mice have also demonstrated that glucose utilization is impaired in motor tracts before any pathologic alterations and that this is accompanied by ATP depletion.²⁶ Metabolic alterations favouring lactacidosis may also be triggered directly as a consequence of Ca^{2+} -mediated excitotoxicity and glutamate excitotoxicity.¹⁶ Metabolic acidosis may be furthermore reinforced during disease progression by respiratory failure and subsequent respiratory acidosis, a condition that correlates with poor prognosis in ALS patients.¹²

Interestingly, we found that ALS disease progression is accompanied by an increase in ASIC1 levels and specifically, ASIC2 mRNA and protein levels, in *SOD1* mice. ASIC1a, the ASIC1 isoform expressed in the brain, is required for high-affinity sensing of acidosis,⁶ and is known to have a causative role in neuronal damage induced by prolonged acidosis.^{10,27} ASIC channels are activated in response to a marked decline in pH.²⁸ Moreover, gene deletion of *asic1a* has demonstrated neuroprotection in mouse models of stroke and multiple sclerosis.^{10,11,29} Physiologically, ASIC1a has been implicated in neurotransmission and synaptic physiology underlying synaptic plasticity, learning, and memory.^{6,8} Our data demonstrate that *asic1a* deletion protected motoneurons from degeneration in tg *SOD1* mice, delayed disease onset, and lead to an improved motor performance. Interestingly, tg *SOD1* mice heterozygous for *asic1a* showed improvement in grip strength and PAGE in a pattern similar to tg *SOD1* mice homozygous for *asic1a* deletion. As these tests are sensitive indicators of early motor signs in tg *SOD1* mice, these data suggest that tg *SOD1* mice heterozygous for *asic1a* express *asic1a* levels below the threshold required to elicit a phenotype similar to that observed in wt *SOD1* mice.

Interestingly, while *asic1a* deficiency was neuroprotective to motoneurons in the tg *SOD1* mouse, we did not expect to observe smaller motoneuron counts in *asic1a*-deficient ntg

SOD1 mice, suggesting a physiological role for ASIC1a in motoneuron development. Moreover, *asic1a* deficiency in tg *SOD1* mice overwhelmed the detrimental effect on motoneuron survival caused by *asic1a* deletion in ntg *SOD1* mice. Despite these alterations, *asic1a*-deficient ntg *SOD1* mice showed no impairment in motor function, suggesting a remarkable neuronal plasticity. In contrast, a reduction in motoneuron number may not be compensated for during the process of age-related motoneuron degeneration as seen in tg *SOD1* mice.

ASIC2 displayed a pronounced and progressive increase in expression across disease progression *in vivo* and in ALS patients, at levels significantly higher than that observed for ASIC1a in the tg *SOD1* mouse. ASIC2a and ASIC2b are isoforms of ASIC2 abundantly expressed in central neurons in a similar pattern to ASIC1a.³⁰ Although ASIC1a-containing channels are activated in response to subtle changes in acidification, ASIC2 channels usually require severe acidification (< pH 4.0) for their activation when expressed *in vitro*.³¹ Identifying a role for ASIC2 activation is complicated by the fact that extremely acidic pH does not usually occur in the central nervous system (CNS). However, ASIC2a and ASIC2b channels also form heteromers with ASIC1a channels that exhibit different electrophysiological properties.³² ASIC2a can form heterodimeric channels with ASIC1a (ASIC2a/1a), which sense more acidic pH than ASIC1a homomeric channels and are PcTx1 insensitive.²⁷ While ASIC2b homomeric channels do not sense subtle changes in acidity,³³ recent data indicates that ASIC2b/1a heterodimeric channels may also contribute to acidosis-induced neuronal death.³² Given our findings of increased ASIC2 levels in the ALS spinal cord, it is tempting to speculate that ASIC2/1a heterodimeric channels contribute to acidotoxicity in motoneurons, previously thought to be mediated by ASIC1a alone. The specific role of ASIC2 in ALS pathogenesis warrants further investigation.

Our PcTx1 data demonstrating additive neuroprotection in motoneurons when combined with DMA was suggestive of DMA mediating its protective effect on PcTx1-insensitive ASIC2a/1a channels, and that pan-ASIC inhibition may be required to provide a potent protection against acidotoxicity in motoneurons. We administered DMA post-symptom onset to the *SOD1* mouse to investigate the neuroprotective effect, if any, of a pan-inhibition of ASIC channels. Our data demonstrated that DMA treatment in the *SOD1* mouse delayed disease onset, improved motor performance, increased lifespan and increased motoneuron survival. The effects of DMA treatment in tg *SOD1* mice were generally more pronounced than those observed in *asic1a*-deficient *SOD1* mice. Motoneuron survival, while increased throughout disease progression in *asic1a*-deficient mice, did not remain increased at end-stage in tg *SOD1* mice following DMA treatment. A significantly increased lifespan in tg *SOD1* mice following DMA treatment may explain this finding given that motoneuron survival was assessed at a later end-stage time point to that observed in *asic1a*-deficient tg *SOD1* mice. Conversely, such findings may allude to a nonspecific effect of DMA in tg *SOD1* mice in the late stages of disease. It is also important to note that other properties of DMA may contribute to the responses observed during *in vitro* acidotoxic stress and *in vivo* in the tg *SOD1* mice. For example, amiloride derivatives can have

inhibitory effects on other ion channels such as the Na⁺/H⁺ exchanger.³⁴ Our findings of a superior protective effect of DMA over PcTx1 in motoneurons against acidotoxicity and evidence of an extended lifespan and improved performance in *SOD1* mice following DMA treatment suggest an ASIC2-dependent role of acidotoxicity in motoneuron degeneration.

ALS is a neurodegenerative disorder characterized by the inexorable loss of motoneurons.³⁵ Despite the progress over the last few decades revealing mechanisms of action for ALS pathogenesis, there is presently no effective pharmacological intervention to slow, stop or reverse motoneuron degeneration in ALS. Consequently, disease-modifying therapeutic interventions represent a significant unmet need for ALS. Many drugs tested to date are incapable of increasing a substantial extension in lifespan in animal models of ALS, particularly in a post-symptom onset treatment regimen. For example, we and others failed to observe an increase in lifespan after treatment with riluzole,³⁶ currently the only FDA- or EMA-approved drug for the treatment of ALS. Our study demonstrates a neuroprotective potential of the ASIC inhibitor, DMA, in motoneurons and suggests a prominent therapeutic potential of ASIC channel inhibitors for the treatment of motoneuron disorders. The increase in lifespan and improvement of motor performance by DMA was similar to that reported for dexamipexole;³⁷ this drug has now entered Phase III trials for the therapeutic treatment of ALS.³⁸ Interestingly, the FDA-approved drug amiloride, albeit a less lipophilic ASIC inhibitor, may also be worthy of further investigation for the treatment of ALS on the premise that it would provide a quicker route to the clinic. Our findings advocate the need for further pre-clinical investigation and eventual clinical studies of DMA or equivalent ASIC inhibitors for the treatment of motoneuron disorders.

Materials and methods

Animals. *asic1a*-deficient mice were kindly provided by Drs. Michael Welsh and John Wemmie (University of Iowa, Iowa, USA), and have been described previously;⁸ *asic1a*-deficient mice were generated when a linearized targeting vector for deleting exon1 of *asic1a* was introduced into embryonic stem (ES) cells by electroporation, ES cell lines carrying the disrupted *asic1a* allele were injected into C57BL/6 blastocysts to generate chimeras and mice containing a deletion of exon 1 of *asic1a* were generated by homologous recombination and subsequent genotyping.⁸ Transgenic mice (tg) (*SOD1*^{G93A})1Gur/O, with the incorporation of a G93A mutant form of human superoxide dismutase (*SOD1*), were purchased from the Jackson Laboratory and were fully congenic on a C57BL6 background.³⁹

After weaning on postnatal day (pnd) 28, all pups from litters of the same generation and colony were housed in groups of 3–5 per cage and maintained at 21 ± 1 °C on a 12-h light/dark cycle (0700 hours on; 1900 hours off), with *ad libitum* access to food and water.

***SOD1;asic1a*^{-/-} double-mutant generation.** The *SOD1;asic1a*^{-/-} colony was generated by cross-breeding tg *SOD1* mice with *asic1a*^{-/-} females generating the F1 generation of *SOD1;asic1a*^{+/-} mice. From the F1 generation, nontransgenic (ntg) *SOD1;asic1a*^{+/-} females were crossed with tg *SOD1;asic1a*^{+/-} males. The F2 colony generated six genotypes, ntg *SOD1;asic1a*^{+/+}, ntg *SOD1;asic1a*^{+/-}, ntg *SOD1;asic1a*^{-/-}, tg *SOD1;asic1a*^{+/+}, tg *SOD1;asic1a*^{+/-} and tg *SOD1;asic1a*^{-/-}, with all genotypes generated equal in incidence and in a ratio consistent with Mendelian inheritance. The *SOD1;asic1a*^{-/-} colony was backcrossed for eight generations and cross-breeding confirmed by genotyping for *SOD1* and *asic1a* genes. *Asic*-deficient *SOD1* mice did not differ in development, fertility or size to *SOD1* mice.

Drugs. 5-(N,N-dimethyl) amiloride hydrochloride (DMA; Sigma-Aldrich, St. Louis, MO, USA) and riluzole (25 mg ml⁻¹ in dimethyl sulfoxide) were dissolved

in ultrapure H₂O for *ad libitum* treatment at final concentrations of 10 and 22 mg kg⁻¹ per day, respectively.

Primary motoneuron cultures. Primary motoneuron cultures (mixed cultures enriched for motoneurons) were prepared from E13 wild-type (wt) and *asic1a*-deficient mouse embryos, as described previously.¹⁴ Spinal cord ventral horns were dissected from individual embryos and the tissue was cut into <1-mm slices and incubated for 10 min in 0.025% trypsin in Neurobasal media (NBM; Invitrogen, Strathclyde, UK). Cells were transferred into NBM containing 0.1 mg ml⁻¹ DNase1 (Sigma-Aldrich, Dublin, Ireland), and gently dissociated. Cells were seeded onto poly-D, L-ornithine/laminin-coated cell culture wells and maintained at 37 °C and 5% CO₂. Primary motoneuron cultures yielded mixed population of cells with glial (60%), neuronal (35%) and non-neuronal morphology (5%); 50% of the neuronal population were motoneurons.

Acidosis, DMA treatment and motoneuron viability. Mixed motoneuron cultures were maintained at 37 °C in 5% carbon dioxide for 7 days *in vitro* (DIV). Acidosis was generated by adjusting NBM to pH 6.5 with addition of 1 M hydrochloric acid (1:80 dilution); the optimal osmolarity for motoneurons of 235 mOsm was maintained at pH 6.5. Injury by acidosis was produced by exposure of motoneurons to NBM, pH 6.5 for 4 h, followed by 24-h recovery. Controls were treated with NBM at pH 7.4. wt- and *asic1a*-deficient motoneuron cultures were treated with 30 μM or 100 μM DMA or with the selective ASIC1a inhibitor, tarantula toxin *Psalmotoxin cambridgei* (psalmotoxin 1, [PcTx1, 100 ng ml⁻¹], Abcam, Cambridge, UK). wt motoneuron cultures were also treated with riluzole (10 μM). Motoneuron survival was assessed using the trypan blue (Sigma-Aldrich, Dublin, Ireland) exclusion method and motoneuron-specific marker, smi-32, as described previously.^{14,17} Following the treatment, cultures were incubated in trypan blue (Sigma-Aldrich, Dublin, Ireland) for 5 min, washed in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in 0.1 M PBS. Cells were immunostained with antibodies to smi-32 (1:500, Abcam, Cambridge, UK), for motoneuron survival counts, and the neuron-specific marker, neuronal nuclear antigen (NeuN, 1:400; Millipore, Cork, Ireland), for total neuronal counts. Neurons stained with either NeuN only or both NeuN and smi-32 containing no trypan blue were considered viable and counted. Criteria used for the identification of motoneurons were smi-32 immunoreactivity of the cell body (>25 μm in diameter), the axon and the dendritic extensions on a multipolar structure. Values were expressed as smi-32-positive and smi-32-negative percentages of sister cultures. The numbers of smi-32-positive motoneurons and NeuN-positive neurons were counted in a blinded fashion across an area of five fields (0.125 mm²) on each coverslip (three coverslips per treatment). Five independent cultures per treatment group were used. Statistical significance was assessed using a Mann–Whitney's *U*-test, *P* < 0.05.

Analysis of mRNA expression. Total RNA was extracted from lumbar spinal cord homogenates using Trizol Reagent (Invitrogen, Strathclyde, UK). First-strand complementary DNA (cDNA) synthesis was carried out according to the manufacturer's instruction using 20 μg Moloney murine leukemia virus reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed using the LightCycler (Roche Diagnostics, Basel, Switzerland) and the QuantiTect SYBR Green PCR kit (Qiagen, Sussex, UK). Sense and antisense primers, respectively, were as follows: *asic1*, 5'-CTGTACCATGCTGGGGAAC-3' and 5'-GCTGCTTTTCATCAGCCATE-3', *asic2* 5'-TGACATTGGTGGTCAATGG-3' and 5'-ATCATGGCTCCCTTCTCTT-3', *asic3* 5'-AGGGAGAAGTCCCAAGCAT-3' and 5'-GACACTCCATCCAGGAGA-3' and *β-actin* 5'-AGGTGTGATGGTGGGAATGG-3' and 5'-GGTTGGCCTTAGGGTTTCAGG-3'. *Asic1* and *asic2* primer pairs recognized all isoforms of either the *asic1* or *asic2* subunit. *Asic3* expression was also determined to examine a central role, if any, of this gene in response to acidosis. Data were normalized to *β-actin* and relative RNA expression levels were determined using LightCycler software, version 5.0 as described previously.¹⁵ Statistical significance was analyzed by analysis of variance (ANOVA) and *post-hoc* analysis by Tukey's multiple comparison tests using PASW18 Software.

Western blotting. Using lumbar spinal cord lysates from pnd 90 and pnd 120 *SOD1* mice, equal concentrations of denatured protein homogenates (20 μg per lane) were loaded and resolved on SDS-polyacrylamide gel as described previously.¹⁷ Preliminary experiments indicated this amount to be within the linear range of detection for all proteins assessed. Membranes were incubated with the following primary antibodies: polyclonal ASIC1 antibody (1:1000, a kind gift from Dr. John Wemmie), polyclonal ASIC2 antibody (1:500, Novus Biologicals,

Colorado, USA), polyclonal SOD1 antibody (1:500 Sigma-Aldrich, Dublin, Ireland), monoclonal tubulin antibody (1:5000, Sigma-Aldrich, Dublin, Ireland) and polyclonal actin antibody (1:5000, Abcam, Sussex, UK). Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000, Jackson, Suffolk, UK), followed by detection using enhanced chemiluminescence (ECL) detection reagent (Amersham Biosciences, Buckinghamshire, UK). Band densities were normalized against the optical density of *β-actin* for that sample and the mean relative expression for each protein was calculated. Where double bands were present (ASIC1), the optical density for both bands was determined, averaged and normalized as outlined above. Statistical significance was analyzed by ANOVA and *post-hoc* analysis by Tukey's multiple comparison test using PASW18 Software.

Immunohistochemistry. *SOD1* mice were anesthetized with 5% sodium pentobarbitone, perfused with PBS and lumbar spinal cords removed, fixed and cryoprotected as detailed previously.¹⁴ Frozen sections (20 μm) were processed for immunofluorescence histochemistry. Sections were blocked and incubated overnight at 4 °C with their respective primary antibody (polyclonal ASIC1 (1:1000), Abcam, UK; polyclonal ASIC2 (1:200), Millipore, Cork, Ireland) in PBS/0.05% Triton X-100/5% non-fat dried milk, followed by incubation with the following primary antibodies (monoclonal NeuN (1:500), Chemicon, Temecula, USA; monoclonal smi-32 (1:500), Abcam, Cambridge, UK; monoclonal GFAP (1:500), Sigma, Wicklow, Ireland) in PBS/1.0% Triton X-100/5% non-fat dried milk. After several washes with the same buffer, sections were incubated with anti-rabbit or anti-mouse secondary antibodies (1:500) labeled with Alexafluor (Molecular Probes, Invitrogen, Dublin, Ireland). Permeabilization steps (Triton X-100) were omitted for the blocking and overnight incubation of *SOD1* mice samples with ASIC antibodies to allow staining of plasma membrane ASIC proteins only.

Spinal cord sections of sporadic ALS and non-ALS patients were obtained from the MRC brainbank, London (<http://www.mrc.ac.uk>) for this study. The intensity of ASIC2 immunostaining in human lumbar spinal cord sections was assessed as follows. Sections were deparaffinized in xylene and antigen retrieval was performed using citrate buffer. Sections were incubated with a polyclonal ASIC2 antibody (1:200, Alomone Labs, Jerusalem, Israel), biotinylated secondary antibody (1:500; Jackson Laboratories) and visualized using Sigma fast 3,3'-diaminobenzidine tablets. The mean staining intensity (mean intensity per μm²) of ventral horn motoneurons for ASIC2 immunoreactivity was calculated for each case. Statistical analysis was assessed using the Fisher's exact test.

DMA Treatment of *SOD1* post-symptom onset. *SOD1* mice were given DMA (10 mg kg⁻¹ per day in drinking water), riluzole (22 mg kg per day in drinking water) or vehicle commencing at day 90, that is, post-symptom onset when disease features have manifested, until end-stage of the disease. *SOD1* mice were trained to use motor function analysis equipment from pnd 70. Preliminary investigations on the frequency of drinking and volume of water drank by *SOD1* mice was assessed to determine final DMA and riluzole concentrations in drinking water. Administration of DMA to ntg *SOD1* littermates for 75 days had no effect on mouse viability, breeding behavior or motoneuron survival.

Assessment of lifespan and disease progression *in vivo*. For lifespan and motor function analyses, animals (*n* = 24 per group) were age, gender (12 males and 12 females), weight and litter-matched in accordance with recent ALS guidelines for the generation of preclinical data.⁴⁰ Assessments were performed blind by a single observer, twice weekly (*n* = 24/group). PaGE analysis measures forelimb and hindlimb integrity and the time to fall from an inverted cage top; the cutoff period was 60 s. The grip strength meter (Ugo Basile, Italy) assesses forelimb ability and measures the force applied in grams, as mice instinctively grasp a trapeze to stop involuntary backward movement. Rotarod analysis (Stoelting, IL, USA) measures performance of fore and hind limb on a rotating rod at a constant acceleration for 180 s. Rotarod measures stopping rotations per minute (r.p.m.), distance traveled and latency period. End-stage of ALS disease progression was determined by a 20% reduction in weight and the loss of righting reflex when mice were placed on their side after 20 s. Statistical significance for motor function assessments was determined by ANOVA and *post-hoc* analysis by Tukey's multiple comparison tests using PASW18 Software. Survival statistics were analyzed by Kaplan–Meier survival using Prism 4.0 software (GraphPad Software, San Diego, CA).

Assessment of motoneuron survival *in vivo*. Motoneuron survival was assessed by Nissl staining at mid-(pnd 120) and end-stage of disease in *SOD1*

mice ($n = 6/\text{group}$). *SOD1* mice were anesthetized with 5% sodium pentobarbitone, perfused with PBS and lumbar spinal cords removed, fixed and cryoprotected, as detailed previously.¹⁴ Lumbar spinal cord samples were sectioned at $20\ \mu\text{m}$ and Nissl stained with cresyl violet (0.1%). Motoneuron survival was assessed by counting Nissl-positive ventral horn motoneurons on every third section between the L1 and L5 levels of the spinal cord. Inclusion criteria used for the identification of Nissl-stained motoneurons was the presence of a large cell body $> 30\ \mu\text{m}$ in diameter, dark cytoplasm, present nucleolus and a multipolar structure. Statistical significance was assessed using a Mann–Whitney's *U*-test, $P < 0.01$.

Conflict of Interest

The authors declare no conflict of interest.

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Retraction

Acidotoxicity and acid-sensing ion channels contribute to motoneuron degeneration

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The authors wish to retract the above paper. The journal has recently been notified by a reader who expressed concerns about some of the figures in this paper. Dr. Áine Behan, co-investigator of this study, has acknowledged sole responsibility for errors affecting certain panels in Figures 2b and 5, in which data were processed with the result that the figure panels do

not correctly reflect the original data. Dr. Behan and co-author Dr. Bridget Breen have acknowledged responsibility for clerical errors in Figures 8b and 2a, respectively. Dr. Behan and Dr. Breen regret these errors, of which the co-authors Marion Hogg, Ina Woods, Karen Coughlan, Mollie Mitchem and Jochen Prehn were completely unaware. We thank the reader for bringing this to our attention and sincerely apologize to the scientific community for any confusion or adverse consequences resulting from the publication of these data.