Title: Primary cerebral and cerebellar astrocytes display differential sensitivity to extracellular sodium with significant effects on apoptosis

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Running Head: Sodium-induced apoptosis depends on brain regions.

Abstract

Central pontine myelinolysis (CPM) is one of the idiopathic or iatrogenic brain dysfunction and the most common cause is excessively rapid correction of chronic hyponatremia. While myelin disruption is the main pathology, as the diagnostic name indicates, a previous study has reported that astrocyte death precedes the destruction of the myelin sheath after the rapid correction of chronic low Na^+ levels, and interestingly, certain brain regions (cerebral cortex, hippocampus, etc.) are specifically damaged, but not cerebellum. Here, using primary astrocyte cultures derived from rat cerebral cortex and cerebellum, we examined how extracellular Na^+ alterations affect astrocyte death and whether the response is different between the two populations of astrocytes. 2x extracellular [Na^+] and voltage-gated Na^+ channel opening induced substantial apoptosis in both populations of astrocytes, while, in contrast, 1/2 [Na^+] prevented apoptosis in cerebellar astrocytes, in which the Na^+-Ca^{2+} exchanger, NCX2, was highly expressed, but not in cerebral astrocytes. Strikingly, the rapid correction of chronic 1/2 [Na^+] exposure significantly increased apoptosis in cerebellar astrocytes, but not in cerebral astrocytes. These results indicate that extracellular [Na^+] affect astrocyte apoptosis and the response to alterations in

[Na^+] are dependent on the brain region from which the astrocyte is derived.

Key notes: astrocyte, sodium ion, apoptosis, hyponatremia, central pontine myelinolysis; schizophrenia

INTRODUCTION

Central pontine myelinolysis (CPM) has routinely been viewed as a symptom associated with alcoholism or malnutrition [1, 2], however, it is now widely recognized that CPM is an iatrogenic disorder that most probably develops in patients suffering from chronic hyponatremia that is followed by an overly rapid correction of the serum sodium ion concentration [3]. CPM is also recognized as one of the more devastating pathologies that can occur in the clinical psychiatric field since patients with schizophrenia tend to suffer from polydipsia and chronic hyponatremia [4-6]. Although myelin disruption in the pons is the main pathologic finding of CPM, a recent study revealed that rapid correction of Na^+ concentration after chronic hyponatremia induces astrocyte apoptosis prior to myelin disruption in vivo [7], suggesting that astrocytes could be more vulnerable to alterations in the extracellular Na^+ concentration than oligodendrocytes. In light of these findings, a primary question arises as to whether altered Na^+ concentrations could be a trigger for cell death. Early studies have revealed that a critical trigger in apoptosis is the release of cytochrome c from mitochondria and calcium ions (Ca^{2+}) from endoplasmic reticulum [8, 9]. However, recent

investigations of brain hypoxia or ischemia has shown that an increase in intracellular sodium (Na^+) may also induce neuronal apoptosis [10-12] indicating the possibility that [Na^+] itself could be a trigger for apoptosis of neurons, and that surprisingly, astrocyte death can occur after Na^+ overload [13]. Although astrocytes have traditionally been considered more resistant to 'excitotoxic' stimuli than neurons [14], it has recently been shown that astrocyte death precedes neuronal death following ischemic insults [15, 16]. Since Na^+ influx in neurons occurs earlier following ischemia than Ca^{2+}, and can be followed by neuronal death, we hypothesized that the astrocyte death that occurs prior to neuronal death following ischemia could be a result of Na^+ overload, indicating a potentially critical role for [Na^+] changes in the onset of astrocyte death.

Since there have been a limited number of studies on the response of astrocytes to alterations in extracellular Na^+ concentration, we investigated the impact of alterations in extracellular Na^+ concentration on primary astrocyte cultures in vitro. Furthermore, we also sought to determine whether there is a brain region-specificity in the response of astrocytes to alterations in extracellular Na^+ concentration since clinical findings suggest that astrocyte apoptosis ensues only in select parts of brains such as the cerebral cortex and

hippocampus, but not in cerebellum [7].

MATERIALS AND METHODS

Astrocyte culture

Primary astrocyte cultures were prepared from rats on embryonic day 18 (E18). Fetuses were removed from deeply anesthetized Wistar rats on day 18 of gestation by laparotomy under sterile conditions. The cortex and cerebellum of embryos were removed and immediately placed in cold Dulbecco's modified Eagle's medium (DMEM) (Sigma) followed by removal of associated meninges, followed by mechanical disruption by trituration using a Pasteur pipet. Cell suspensions were diluted with DMEM/F12 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin. After filtration through a 70 µm pore size cell strainer (Falcon), the cell suspension was centrifuged at 100 × g for 5 min. Cells were seeded into 75-cm^2 flasks at a concentration of 1 × 10^7 cells in 20 ml of medium. Astrocytes were cultured to confluence (10 days) in an incubator with 5 % CO_2 at 37°C. The media were changed every 2–3 days. Upon reaching confluence, cells were

detached using 0.05 % trypsin/0.02 % EDTA. The dissociated cells were then washed and collected by centrifugation (100 × g, 5 min) and suspended in growth medium, and plated at 2 × 10^5 cells/plate in tissue culture plates pre-treated with poly-L-lysine and cultured to confluency over approximately 7 days. Cells were then trypsinized and reseeded at 5 × 10^4 cells in 35 mm tissue culture dishes pre-treated with poly-L-lysine and cultured in growth medium for 2 days. Growth medium was aspirated and replaced with experimental media (1/2 Na^+; 76 mM, normal Na^+; 153 mM, 2x Na^+; 306 mM) and the cells incubated for 60 hr. Following the exposure to varied [Na^+], the medium was replaced with normal Na^+ medium and the cells cultured for an additional 24 hr.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in PBS for 1 hr and then incubated in a blocking buffer containing 0.1% Triton X-100 and 5% bovine serum albumin in PBS for 1 hr at room temperature. Cells were incubated with primary antibodies, anti-glial acidic fibrillary protein (GFAP) (1:1000, Calbiochem) and anti-cleaved caspase-3 (1:1000, Cell Signaling Technology), overnight at 4°C. After washing, the cells were incubated with the secondary antibodies, Alexa Fluor 488 goat

anti-mouse IgG (H+L) (1:1500, Life Technologies) and Alexa Fluor 546 goat anti-rabbit IgG (H+L) (1:1500, Life Technologies) for 40 min at room temperature followed by counterstaining with DAPI in VECTASHIELD (Vector Laboratories).

Counting of astrocyte death

Cells were examined at 200X magnification using epifluorescence microscope (Leica Microsystems). For quantification, six random fields were captured from each dish and labeled cells were manually counted by a researcher blinded to the treatments. The rate of astrocyte apoptosis was calculated as the ratio of caspase-3-positive cells to the total number of GFAP+/DAPI+ cells.

Quantitative reverse-transcription PCR

RNA was isolated using Qiazol (Qiagen) following the manufacturer's protocol. RNA quantity was determined by absorbance at 260 nm. First-strand cDNA was synthesized from 500 ng of RNA using an iScript kit (Bio-Rad Laboratories). Real time PCR was performed using SYBR Green in reactions run on real time PCR system (Applied Biosystems).

Statistics

Samples were analyzed for statistical significance by either the student t-test or by one-way analysis of variance followed by Newman-Keuls multiple comparison test.

RESULTS

Differential effects of extracellular Na^+ concentration on apoptosis rate in cultured cerebral and cerebellar astrocytes in vitro

After 60 hours incubation in 2x Na^+ media, the number of cleaved caspase-3 positive cortical and cerebellar astrocytes (GFAP+, cleaved caspase-3+, DAPI+) was significantly higher than in normal Na^+ media (Figure 1A, B). A previous study had indicated that Na^+-dependent neuronal death is through voltage-gated Na^+ channel [17], therefore, we examined whether high Na^+-induced astrocyte apoptosis is through voltage-gated Na^+ channels by treating cultures with the voltage-gated Na^+ channel blocker (tetrodotoxin; TTX). While TTX treatment prevented high Na^+-induced apoptosis in cerebral astrocytes , cerebellar astrocytes were not affected (Figure 1A, B), suggesting that high Na^+-induced apoptosis in cerebral, but not cerebellar, astrocytes occurs at least partially

through the activity of the voltage-gated Na^+ channel. To further investigate the function of voltage-gated Na^+ channels in astrocyte apoptosis, we applied the voltage-gated Na^+ channel activator, veratridine, to cultured astrocytes in normal media. Veratridine application significantly increased the apoptosis rate of both cortical and cerebellar astrocytes (Figure 1C, D). Next, we also hypothesized that blocking of voltage-gated Na^+ channel would prevent apoptosis vice vasa. However, voltage-gated Na^+ channel blocker, tetrodotoxin (TTX), failed to decrease apoptosis rate of both cortical and cerebellar astrocytes in normal media (Figure 1C, D). To confirm low Na^+ concentration in astrocytes does not affect apoptosis, astrocytes were grown in DMEM with 1/2 Na^+ for 60 hours and observed. Interestingly, low Na^+ concentration significantly suppressed apoptosis compared to normal Na^+ in cerebellar astrocytes (Figure 1E), but cerebral astrocyte apoptosis was comparable between 1/2 Na^+ and normal Na^+ (Figure 1F).

Exposure to low extracellular Na^+ increases Na^+-Ca^{2+} exchanger expression in cerebellar, but not in cerebral astrocytes

In light of the differences observed above, we wished to define potential cellular

means by which the difference in response between cerebral and cerebellar astrocytes to low extracellular Na^+ occurred? Given the absence of an effect of TTX on astrocyte apoptosis (Figure 1C, D), we hypothesized that Na^+-dependent glutamate transporter (GLAST, GLT1) and/or Na^+-Ca^{2+} exchanger (NCX1, NCX2, NCX3) would be involved in the prevention of low extracellular Na^+-induced apoptosis in cerebellar astrocytes in part because of earlier work reporting a contribution of Na^+-dependent glutamate transporter and Na^+-Ca^{2+} exchanger function in astrocyte death after mechanical injury [13]. First, we measured mRNA expression of GLAST, GLT1, NCX1, NCX2, and NCX3 in both cerebral and cerebellar astrocytes cultured in normal media. Interestingly, GLAST and GLT1 expression levels were much lower in cerebellar astrocytes as compared to cerebral astrocytes (Figure 2A, B). In contrast, NCX2 expression was higher in cerebellar astrocytes than cerebral astrocytes (Figure 2C), while NCX3 expression showed no difference between the two kinds of astrocytes (Figure 2D), and NCX1 could not be detected in either. Next, we examined the effects of 1/2 Na^+ exposure on the expression of GLAST, GLT1, NCX2 and NCX3 in both cerebral and cerebellar astrocytes. While reduced extracellular Na+ caused a significant increase in NCX2 expression in cerebellar astrocytes,

but not in cerebral cortical astrocytes, (Figure 2F), GLAST, GLT1 and NCX3 expression was not different between the two conditions (Figure 2E, F).

Rapid Na^+ correction after low extracellular Na^+ exposure induces apoptosis of cerebellar, not cerebral, astrocytes in vitro

With CPM occurring in many cases as a result of overaggressive correction of hyponatremia leading to astrocyte apoptosis followed by myelinolysis [7], we examined whether Na^+-correction to normal levels would lead to astrocyte apoptosis in vitro as well as in vivo. As expected, rapid correction of 1/2 Na^+ to normal Na^+ levels significantly increased the apoptosis rate in cultured cerebellar astrocytes (Fig. 3B), but unexpectedly, showed no such effect in cultured cerebral astrocytes (Fig. 3A). In addition, TTX application failed to suppress the apoptosis of cerebellar astrocytes (Fig. 3A, B).

DISCUSSION

In the current study, we provide the first evidence that extracellular Na^+ level can be a critical factor in the induction of astrocyte apoptosis. Both exposure to high

extracellular [Na^+] and voltage-gated Na^+ channel opening caused apoptosis in both cerebral and cerebellar astrocytes, while pharmacologic blockade of voltage-gated Na^+ channels prevented enhanced apoptosis with high extracellular Na^+ only in cerebral astrocytes. These findings suggest that high intracellular Na^+ can play a role in the induction of astrocyte apoptosis and that activation of the voltage-gated Na^+ channel, at least partially, may be a critical participant in this pathway. In contrast, low extracellular Na^+ reduced apoptosis in cultured cerebellar astrocytes, without affecting cerebrum-derived cells, and interestingly, TTX application did not diminish apoptosis in either cerebral or cerebellar astrocytes. These results suggest that TTX-sensitive voltage-gated Na^+ channel function is not involved in the causal steps associated with low extracellular Na^+-dependent prevention of cerebellar astrocyte apoptosis, implying the contribution of TTX-resistant voltage-gated Na^+ channel such as Nav1.5, Nav1.8 and Nav1.9 [18-20]. Alternatively, since it has been shown that the Na^+-dependent glutamate transporters, GLAST and GLT1, are differentially expressed in cerebral and cerebellar astrocytes [21], we hypothesized that different responses of GLAST and GLT1 to low extracellular Na^+ would lead to variant consequences in apoptosis. However, neither GLAST nor GLT1

expression was altered after exposure to low extracellular Na^+. On the other hand, the expression of NCX2, but not NCX3, was increased after low extracellular Na^+ exposure. Since GLAST and GLT1 expression is much lower and NCX2 expression is higher in cerebellar astrocytes than cerebral astrocytes (Figure 2A, B, C), Na^+-Ca^{2+} exchanger might play a more pronounced role in Na^+-dependent astrocyte apoptosis rather than Na^+-dependent glutamate transporter in cerebellar astrocytes, particularly in view of the increased expression of NCX2 in cerebellar astrocytes after 1/2 Na^+ exposure. Mechanistically, increased expression of NCX2 in cerebellar astrocytes might exclude more Ca^{2+} in media with 1/2 Na^+ than in cerebral astrocytes, which might suppress astrocyte apoptosis.

Central pontine myelinolysis is known as a fatal condition of myelin impairment ensuing after overly rapid serum Na^+ correction, whereas myelination in other brain regions such as cerebral cortex, hippocampus, even including cerebellum, can also be damaged in additional similar conditions in humans, referred to as extrapontine myelinolysis [22-25]. Clinically, there appears to be region-specific differences in myelinolytic susceptibility in each human case [25]. In a published rat model, there also was brain

region-specificity in both astrocyte and myelin disturbances; cerebral cortex and hippocampus being damaged, but not the cerebellum, implying that cerebellar astrocytes and myelin are relatively resistant to extracellular Na^+ alteration in vivo. In contrast, our findings suggest that cerebellar astrocytes are more susceptible to extracellular Na^+ alteration than cerebral astrocytes in vitro. This controversy might be due to differential expression of Na^+-related channels or transporters when astrocytes are cultured. In fact, GLT1 is predominantly expressed in cerebral cortex and hippocampus, and GLAST is expressed in cerebellum in vivo [21], whereas our current study revealed much lower expression of both transporters in cerebellar astrocytes than cerebral ones in vitro. Although the mechanism of differential astrocyte apoptosis in vivo needs to be studied, it is clear that cerebral and cerebellar astrocytes display differential sensitivity to extracellular Na^+ alteration. Here, we have found that chronic 1/2 Na^+ exposure increases NCX2 expression only in cerebellar astrocytes already intrinsically expressing relatively high NCX2, which suggests that further potentiated NCX2 function might lead to low 1/2 Na^+-induced apoptosis prevention and might also be involved in the marked astrocyte death that occurs after rapid Na^+ correction of chronic hyponatremia in vitro, and possibly in vivo.

The mechanism in which even normal [Na^+] increases apoptosis rate of cerebellar astrocytes remains elusive. Since the direction of Na^+-Ca^{2+} exchanger (Forward; Na^+_{out}-Ca^{2+}_{in}, Reverse; Na^+_{in}-Ca^{2+}_{out}) can be reversed by Na^+ influx [26], rapid correction of [Na^+] even to normal level might reverse Na^+-Ca^{2+} exchanger direction (Na^+_{out}-Ca^{2+}_{in} to Na^+_{in}-Ca^{2+}_{out}) leading to Ca^{2+} influx, and the subsequent apoptosis together with elevated expression of NCX2. Although further studies are needed, these findings could shed light on important approaches for the prevention or mitigation of central pontine myelinolysis.

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Figure Legend

Figure 1

Extracellular 2x Na^+ exposure for 60 hours markedly increased the number of caspase3+/GFAP+/DAPI+ cells in cerebral and cerebellar astrocytes compared to normal Na^+ (A, B). 2x Na^+-dependent apoptosis was partially blocked by TTX in cerebral astrocytes, but not in cerebellar astrocytes (A, B). TTX application for 60 hours in itself had no effect on apoptosis in either cerebral or cerebellar astrocytes in normal media (C, D). Veratridine application for 60 hours substantially increased astrocyte apoptosis in cerebral and cerebellar astrocytes in normal media (C, D). Extracellular 1/2 Na^+ exposure for 60 hours reduced astrocyte apoptosis in cerebellar astrocytes, but not in cerebral astrocytes (E, F). *; $p<0.05$, **; $p<0.01$, ***; $p<0.001$

Figure 2

GLAST and GLT1 expression was much higher in cerebral astrocytes than in cerebellar astrocytes (A, B). In contrast, NCX2 expression in cerebellar astrocytes was 3 times as high as in cerebral astrocytes (C). NCX3 expression

was comparable between cerebral and cerebellar astrocytes (D). In these experiments, astrocytes were cultured in normal media (A-D). While 1/2 Na^+ exposure for 60 hours had no effect on the expression of GLAST, GLT1, NCX2 and NCX3 in cerebral astrocytes (E), only NCX2 was significantly increased in cerebellar astrocytes, but not GLAST, GLT1 or NCX3 (F). *; $p<0.05$, **; $p<0.01$, ***; $p<0.001$

Figure 3

Rapid correction of 1/2 Na^+ exposure for 60 hours (1/2 Na^+ to normal Na^+) had no effect on the number of caspase3+/GFAP+/DAPI+ cells in cerebral astrocytes (A). The number of caspase3+/GFAP+/DAPI+ cells in cerebellar astrocytes was remarkably increased after rapid correction of 1/2 Na^+ exposure for 60 hours (B). TTX application had no effect on the rapid correction-induced apoptosis in either cerebral or cerebellar astrocytes (A, B). **; $p<0.01$

Figure 1

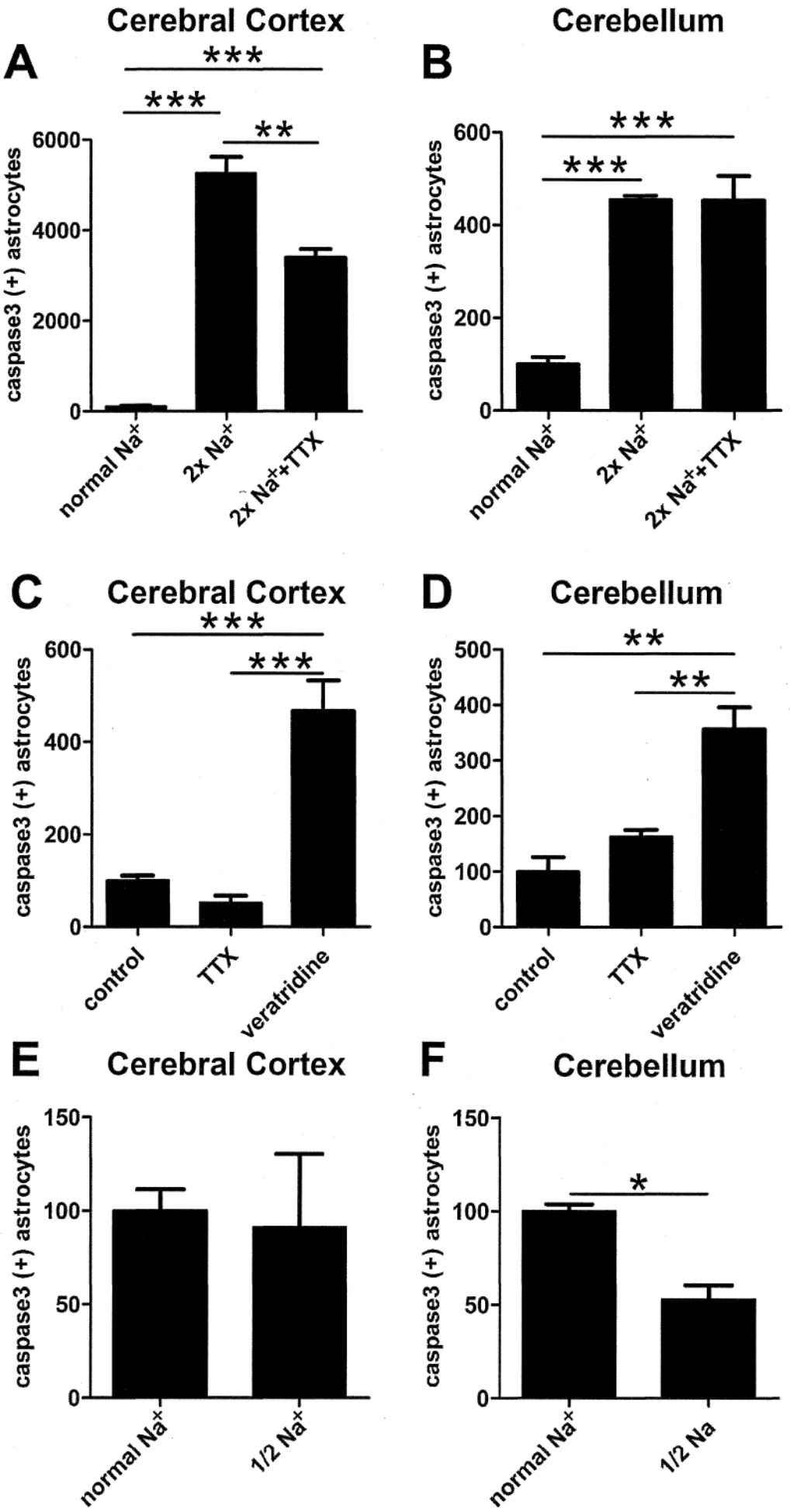
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Figure 2

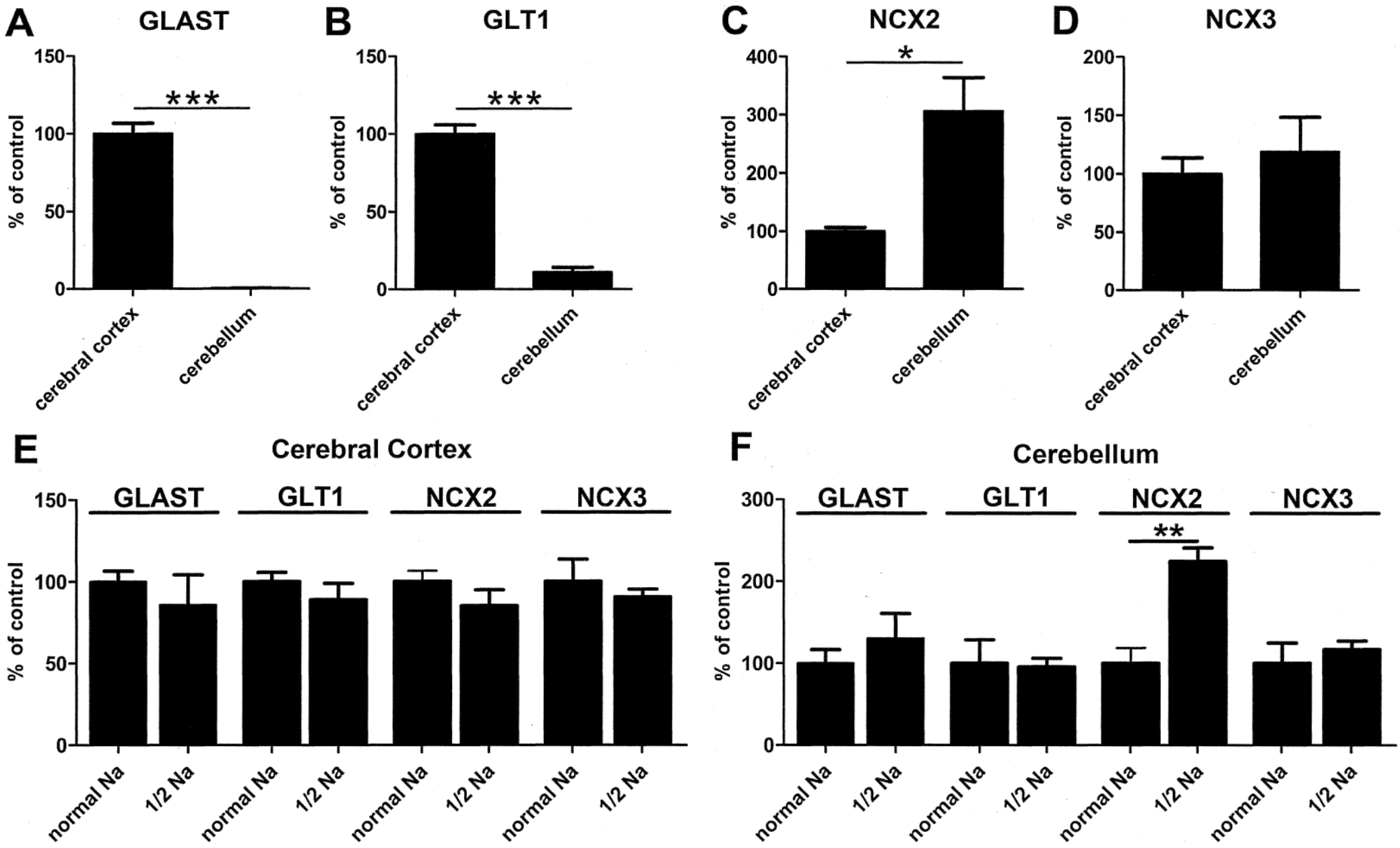
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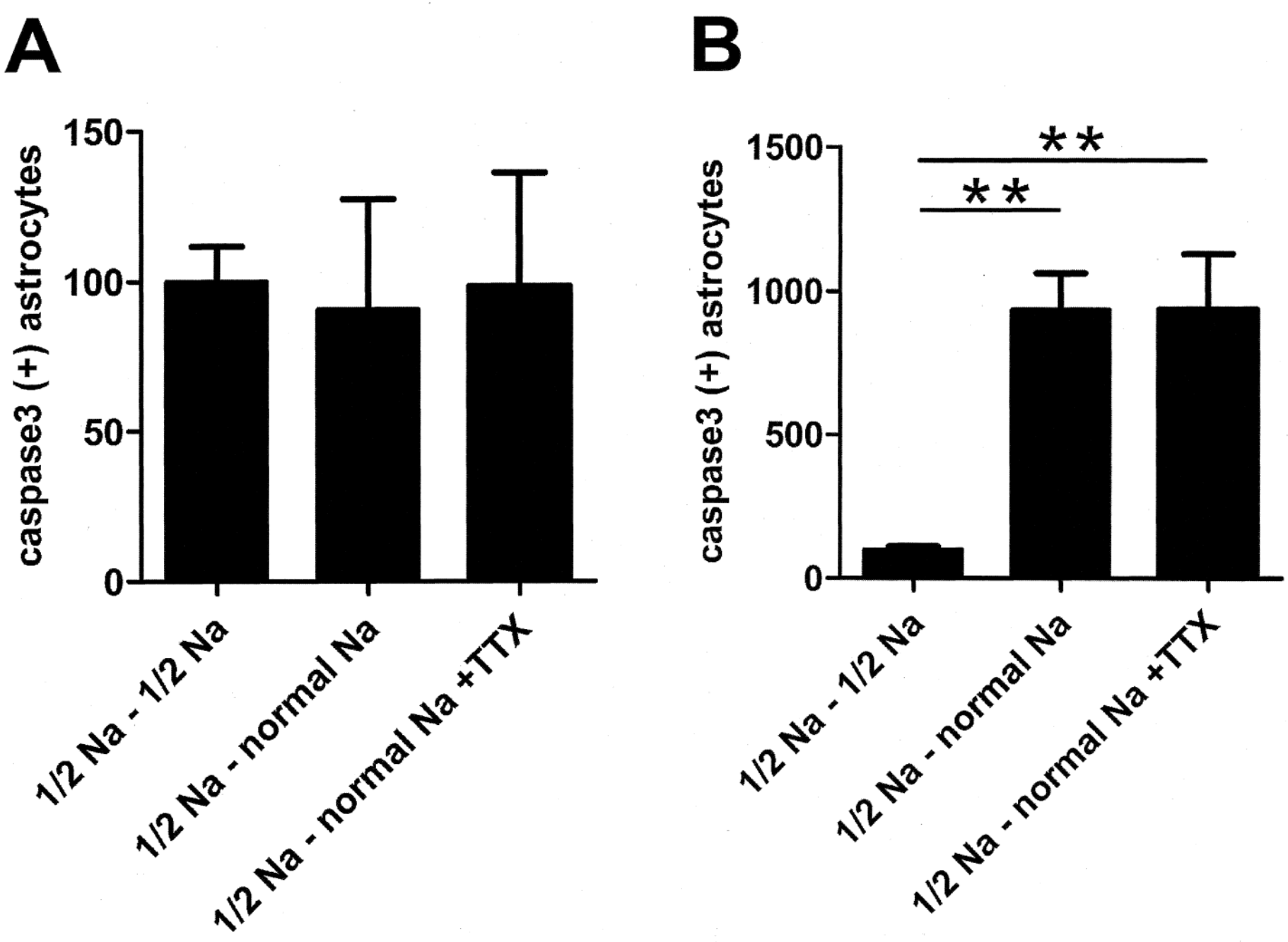
Figure 3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