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T-type calcium channels contribute to calcium disturbances in brain during hyponatremia

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Abstract

Disturbance of calcium homeostasis is implicated in the normal process of aging and brain pathology prevalent in the elderly such as Alzheimer's, Parkinson's, and amyotrophic lateral sclerosis. Previous studies demonstrated that applying a hyponatremic iso-osmotic (low-NaCl) artificial cerebrospinal fluid (ACSF) to rodent hippocampus causes extracellular calcium to rapidly decrease. Restoring normonatremia after low-NaCl treatment causes a rapid increase in extracellular calcium that overshoots baseline. This study examined the amplitude, timing, and mechanism of these surprising calcium changes. We also tested whether hyponatremia increased calcium entry into brain cells or calcium binding to chondroitin sulfate (CS), a negatively charged constituent of the extracellular matrix (ECM) that may be occupied by sodium during normonatremia. We report three major findings. First we show that CS does not contribute to extracellular calcium changes during low-NaCl treatments. Second, we show that the time to minimum extracellular calcium during low-NaCl treatment is significantly shorter than the time to maximum extracellular calcium in recovery from low-NaCl treatment. Third, we show that the decrease in extracellular calcium observed during hyponatremia is attenuated by ML 218, a highly selective T-type calcium channel blocker. Together these data suggest that calcium rapidly enters cells at the onset of low-NaCl treatment and is extruded from cells when normonatremia is

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restored. Calcium binding to CS does not significantly contribute to calcium changes in brain during hyponatremia. Differences in timing suggest that extracellular calcium changes during and in recovery from hyponatremia occur by distinct mechanisms or by a multistep process. Finally, partial block of extracellular calcium influx by ML 218 suggests that T-type channels are involved in calcium entering cells during hyponatremia. Given the high prevalence of hyponatremia among elderly patients and the growing understanding of calcium's role in multiple neurologic pathologies, this study promotes a novel approach for studying and potentially preventing the effects of hyponatremia on calcium dysregulation in brain tissue.

Keywords

calcium homeostasis; T-type calcium channels; hyponatremia; extracellular matrix; chondroitin sulfate; hippocampus; aging; elderly patients; neurodegeneration

Introduction

Changes in the intracellular $[Ca^{2+}]$ of neurons mediate a variety of critical processes including signal transduction, neurotransmitter release, transcriptional regulation, and neuronal plasticity (Kawamoto et al., 2012). Under normal conditions, neurons maintain a roughly 10,000:1 gradient between free extracellular $[Ca^{2+}]$ ($[Ca^{2+}]_e$) and intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) using various exchangers, pumps, and intracellular sequestration processes (Kawamoto et al., 2012). However, disturbances of Ca^{2+} homeostasis in brain tissue are common in clinical settings and are now being linked to diseases prevalent among elderly patients including Alzheimer's, Parkinson's, and amyotrophic lateral sclerosis (Nikoletopoulou and Tavernarakis, 2012). One such disturbance of Ca^{2+} , originally reported by Chebabo et al. (1995) in rat hippocampus, occurs at the onset of hyponatremia.

In a healthy individual, the brain is bathed in cerebrospinal fluid with an osmolality of approximately 300 mM, primarily attributable to small molecules and ions. Physiologic $[Na^+]$ in cerebrospinal fluid is 137 – 145 mM (Waxman, 2010). Hyponatremia, or the decrease in $[Na^+]$ below this range, can occur for many reasons including syndrome of inappropriate antidiuretic hormone, restricted NaCl intake, increased water intake, and congestive heart failure (Patel and Balk, 2007). Mannesse et al. (2013) estimated that the prevalence of mild hyponatremia ($[Na^+] < 135$ mM) on the geriatric ward is 22.2%; the prevalence of severe hyponatremia ($[Na^+] < 125$ mM) is 4.5%. Although asymptomatic hyponatremia is treated as a marker of disease, this and previous studies suggest that Ca^{2+} homeostasis in brain might be influenced by $[Na^+]$ in the cerebrospinal fluid.

While studying the effects of hyponatremia on rat brain slices, Chebabo et al. (1995) made the unexpected observation that applying an artificial cerebrospinal fluid (ACSF) with low $[NaCl]$ and normal $[Ca^{2+}]$ to rat brain tissue generated a significant and reversible decrease in $[Ca^{2+}]_e$ that appeared proportional to the reduction in $[Na^+]_e$. These investigators also demonstrated that if normonatremic iso-osmotic ACSF is applied to brain slices after a hyponatremic hypo-osmotic treatment, $[Ca^{2+}]_e$ overshoots baseline concentration. Repeating the same experiments using hyponatremic iso-osmotic ACSF (with reductions of $[NaCl]$ replaced by fructose to maintain osmolality) suggested that changes in $[Ca^{2+}]_e$ observed

during hyponatremia were independent of cell swelling (Chebabo et al., 1995). Based on their findings, Chebabo et al. (1995) speculated that the reductions in $[Ca^{2+}]_e$ observed during hyponatremia represented Ca^{2+} entering brain cells. A subsequent investigation using patch-clamped neurons showed that $[Ca^{2+}]_i$ rapidly increases during hyponatremia in-vitro (Borgdorff et al., 2000). This finding supported Chebabo et al.'s (1995) original interpretation of Ca^{2+} entry into brain cells during hyponatremia. From here onwards, the phrase low-NaCl treatment refers to hyponatremic iso-osmotic (with NaCl replaced with fructose) conditions unless otherwise specified.

The proposed explanations for changes in $[Ca^{2+}]_e$ that occur during hyponatremia focus on Ca^{2+} entry into the cell. However, another potential sink for extracellular Ca^{2+} during hyponatremia is the extracellular matrix of brain tissue (Fig 1). The extracellular matrix (ECM) of brain is a mesh-like network made largely of different glycosaminoglycans and proteoglycans. Glycosaminoglycans (GAGs) are polymers of repeating disaccharide subunits with a large number of negative charges. Proteoglycans are GAGs attached to a protein core. Each proteoglycan can have hundreds of glycosaminoglycans of different types and it has been suggested that variations in proteoglycan structure can greatly affect the functionality of the neurons surrounded by this mesh (Dityatev, 2010). One glycosaminoglycan of particular relevance to this study is chondroitin sulfate (CS), which possesses a large number of negative charges and is abundant in the extracellular matrix of brain tissue (Galtrey and Fawcett, 2007).

It has been shown that Ca^{2+} interacts with CS on the ECM of brain tissue (Hrabetova et al., 2009), but the specifics of this interaction have yet to be fully characterized. One possibility is that the interaction is rapid and tenuous, as suggested by Hrabetova et al. (2009). Under this model, decreases in $[Ca^{2+}]_e$ represent an increase in $[Ca^{2+}]_i$ exclusively. However, it has been suggested that Na^+ might screen larger divalent cations like Ca^{2+} from binding to the matrix (Magdelenat et al., 1974). Based on these studies, it is possible that when $[Na^+]_e$ is decreased, additional binding sites for Ca^{2+} become available on the ECM of brain. As $[Na^+]_e$ is decreased further, more cation binding sites are made available. This would explain why $[Ca^{2+}]_e$ decreased proportionally with decreases in $[Na^+]_e$ here (see results) and previously (Chebabo et al., 1995). Under this model, decreases in $[Ca^{2+}]_e$ would represent both an increase in $[Ca^{2+}]_i$ and an increase in Ca^{2+} binding to the extracellular matrix (Fig 1).

Building on the findings of the Chebabo et al. (1995) and Borgdorff et al. (2000), we hypothesize that negative charges on CS sequester extracellular Ca^{2+} during hyponatremia. We test this idea by superfusing low-NaCl solutions over mouse brain slices treated with chondroitinase ABC enzyme, which cleaves negative charges on CS, and measuring $[Ca^{2+}]_e$ using ion-selective microelectrodes. We also test the mechanism of Ca^{2+} entry into brain cells using the highly selective T-type channel blocker ML 218.

Materials and Methods

Mouse Brain Tissue Preparation

All experiments were conducted at SUNY Downstate Medical Center in accordance with the NIH guidelines and approved by the local Institution Animal Care and Use Committee. For this report, a total of 20 adult mice (strain C57BL/6; 20.5 – 37 g) were anaesthetized with sodium pentobarbital (50 mg/kg intraperitoneal) and decapitated. During each dissection, the mouse brain was removed from the skull and cooled in ice-cold artificial cerebrospinal fluid (ACSF; see below). Coronal mouse brain slices of 400 μ m thickness were prepared following a procedure previously described (Xiao and Hrabetova, 2009) using a vibrating blade microtome (VT 1200S; Leica Microsystem GmbH, Nussloch, Germany). All slices were submerged in a room temperature ACSF recovery chamber for at least 1 hr prior to experimentation.

Composition of Regular Artificial Cerebrospinal Fluid (ACSF) and Treatment of Brain Slices

The regular ACSF used prior to and between all test treatments (see below) was prepared as previously described (Hrabetova et al., 2009; Xiao and Hrabetova, 2009). The composition of regular ACSF was (in mM): NaCl 124, KCl 5, NaHCO₃ 26, NaH₂PO₄ 1.25, D-glucose 10, MgCl₂ 1.3, CaCl₂ 1.5. The ACSF was gassed with 95% O₂ - 5% CO₂ to buffer pH at 7.4.

The composition of hyponatremic treatments was based on the description provided by Chebabo et al. (1995). Hyponatremic hypo-osmotic treatments were made by omitting 40, 60, or 90 mM NaCl from the regular ACSF described above. Hyponatremic iso-osmotic treatments, referred to as low-NaCl treatments, were made by substituting fructose for omitted NaCl. Specific hyponatremic hypo-osmotic solutions are referred to in this paper as H-40, H-60, and H-90 while the corresponding low-NaCl treatments are referred to as F-40, F-60, and F-90 following the convention originally used by Chebabo et al. (1995).

The osmolality of all regular ACSF and treatments was confirmed by a freezing point-depression osmometer (Osmette A, model no. 5002; Precision Systems Inc., Natick, MA, USA). Regular ACSF and low-NaCl treatments had an osmolality of about 300 mosmol/kg. Hypo-osmotic treatments with 40, 60, and 90 mM NaCl omitted had osmolalities of about 220, 190, and 135 mosmol/kg respectively.

All treatments were administered as previously described (Chebabo et al., 1995). For both low-NaCl and hyponatremic hypo-osmotic treatments, brain slices were exposed to 3 test solutions (40, 60, and 90 mM NaCl omitted) lasting 30 min each in order of decreasing [NaCl]. Between and after each treatment, slices were allowed to recover in regular ACSF for 30 min (referred to as the recovery period). Each low-NaCl condition was repeated in at least 4 brain slices from 3 mice.

In 5 of the trials reported (3 control slices and 2 brain slices that underwent cleavage of CS using chondroitinase ABC (chABC) enzyme; all 5 involving fructose substitution), treatment was stopped after recovery from F-60 (F-90 was not applied). Likewise, in 5 of the

trials reported (3 control slice and 2 brain slices that underwent cleavage of CS using chABC enzyme; all 5 involving fructose substitution), only F-90 was applied. Data from these trials was consistent with data from comparable brain slices that experienced all three treatments successively.

In a separate control experiment, 7 brain slices from 4 mice were exposed to the F-60 test solution repeatedly. Between and after each treatment, slices were allowed to recover in regular ACSF for 30 min as above. During these trials, $[Ca^{2+}]_e$ was continuously monitored before and during the application of each test solution.

Incubation of Brain Slices with Chondroitinase ABC and Confirmation of Cleavage by Immunochemistry

Cleavage of CS on ECM with chABC was performed as described previously (Bukalo et al., 2001; Hrabetova et al., 2009). To summarize, after brain tissue preparation, including 1 hr recovery in regular ACSF, several slices were transferred into a chamber with room temperature ACSF containing chABC (0.2 U/mL, Cat # C3667, Sigma-Aldrich, St. Louis, MO, USA). These slices were then incubated at room temperature for 3 hr. Prior to $[Ca^{2+}]_e$ recording, treated slices were returned to a normal ACSF bath at room temperature. A control group underwent the same preparation without the addition of chABC.

Confirmation of CS cleavage from chondroitin sulfate proteoglycan (CSPG) was conducted using immunochemistry as previously described (Hrabetova et al., 2009; Brückner et al., 1998). Enzymatic treatment and immunostaining were performed in brain slices in-situ without cell permeabilization. This approach achieved an extracellular space-selective detection of CS cleavage. Antibodies used recognize CSPG “stubs” that appear after cleavage of full length CS on CSPG by chABC enzyme (Caterson et al., 1985). Briefly, slices treated with chABC (described above) and comparative control slices were fixed in 4% paraformaldehyde in 1 M PBS at 4 °C post experimentation. Hippocampus was then cut out and wet tissue was sliced into 50 µm sections using a vibrating blade microtome (VT 1200S; Leica Microsystem GmbH, Nussloch, Germany). Sections were then blocked in 2% bovine serum albumin (BSA) in 1 M PBS for 1 hr at room temperature. A primary antibody targeting CSPG “stubs” (C-4-S, Cat # 1042009, MD Bioproducts, St Paul, MN, USA) was diluted with 2% BSA in 1 M PBS (1:200) and allowed to incubate overnight at room temperature. Sections were then washed 3 times for 10 min, using 0.1% BSA in 1 M PBS at room temperature for each rinse. Following washing, slices were incubated for 1 hr in a goat anti-mouse IgG secondary antibody labeled with Alexa Fluor 488 (Cat # A-11001, Life Technologies, Carlsbad, CA, USA) diluted with 1% BSA in 1 M PBS (1:400). Sections were then washed 3 times for 10 min, each rinse with 0.1% BSA in 1 M PBS at room temperature, and mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) for imaging under an epifluorescence microscope.

In 1 trial, an additional goat anti-mouse immunoglobulin (GAM IgG) antibody (Jackson ImmunoResearch, West Grove, PA, USA) was applied to optimize specific binding. In this trial, sections were washed 3 times in 1 M PBS (10 min per rinse) immediately following sectioning. Sections were then incubated in GAM IgG (diluted with 2% BSA in 1 M PBS to 0.035 g/L IgG) overnight at 4 °C. All other steps prior to and following sectioning, including

blocking, washing, primary antibody, secondary antibody, and mounting were as described above. Both GAM and non-GAM procedures resulted in similar contrast between cleaved and control slices.

In 2 of the 4 chABC cleavage trials, visual comparison under 40 X magnification using an epifluorescent microscope of 6 control sections and 5 sections from slices incubated in chABC enzyme was used to confirm cleavage. Stratum radiatum of CA1 region of hippocampus was used for confirmation of cleavage. The intensity of fluorescent staining (including those presented below) was standardized using ImageJ (Rasband, 1997–2012) to allow for direct visual comparison of control and treated sections.

T-type Ca^{2+} Channel Blocker

T-type calcium channels were blocked using the selective inhibitor ML 218 hydrochloride (Cat# 4507; Bio-Techne, Minneapolis, MN, USA). For these experiments, all brain slices were exposed to ACSF containing ML 218 for 30 min, then F-60 containing ML 218 for 30 min, followed by ACSF containing ML 218 for 30 min. Throughout these experiments, ML 218 was maintained at 3 μM dissolved in 0.1% dimethyl sulfoxide (DMSO) and either regular ACSF or F-60 solution. Working concentration of ML 218 was chosen based on a previous study (Xiang et al., 2011) that characterized ML 218 in brain.

Prior to ML 218 treatment, each slice was subjected to a control treatment. For these treatments, slices were superfused with ACSF containing 0.1% DMSO for 20 min, F-60 containing 0.1% DMSO for 30 min, and ACSF containing 0.1% DMSO for 30 min. Ca^{2+} signal obtained during the above served as paired control for subsequent ML 218 treatments.

Ca^{2+} Ion-Selective Microelectrodes (ISM)

Ca^{2+} -ISMs were prepared from double-barreled theta glass (TG200-4; Warner Instruments Corp., Hamden, CT) as described previously (Nicholson, 1993; Hrabetova et al., 2009). The ion-detecting barrel of the ISM was filled with 150 mM CaCl_2 and used Ca^{2+} ionophore I, cocktail A (Cat # 21048, Sigma-Aldrich, St. Louis, MO, USA) in the tip for a semipermeable membrane. The reference barrel was filled with 150 mM NaCl and voltage on this barrel was continuously subtracted from that on the ion barrel during testing. In a few trials, the reference barrel on the Ca^{2+} -ISM showed some instability during pre-testing calibration. In these cases, a reference microelectrode was used in place of the reference barrel on the Ca^{2+} -ISM. Reference microelectrodes were created using the same method described above save that both barrels on reference microelectrodes were filled with 150 mM NaCl and no ionophore was added to either barrel.

Both Ca^{2+} -ISMs and reference microelectrodes were calibrated in a set of standard solutions (0.375, 0.75, 1.5, 3, or 6 mM Ca^{2+} all in 150 mM NaCl) prior to and immediately after experimentation. Results from Ca^{2+} -ISM pre-test calibrations were fitted to the Nikolsky equation to obtain slope and interference (Nicholson, 1993). Changes in the slope of Ca^{2+} -ISM during testing were small, less than 10% in all trials except 1, in which the slope of the ISM changed by 13%.

Measurement of $[Ca^{2+}]_e$ in Extracellular Space

For $[Ca^{2+}]_e$ recording, each brain slice was placed in a submersion recording chamber (Warner model RC-27L; Harvard Apparatus, Holliston, MA) and superfused with ACSF at flow rate of 2.0 mL/min delivered by peristaltic pump. The ACSF temperature was maintained at 34 ± 1 °C by a dual automatic temperature controller (Warner model TC-344B; Harvard Apparatus) operating an in-line heater (Warner model SH-27A; Harvard Apparatus) in tandem with the heating element of the recording chamber. The recording chamber was placed on the fixed stage of a compound microscope (BX51WI; Olympus America, Melville, NY) equipped with a water immersion objective (Olympus UMPlanFI, 10 X, numerical aperture 0.3) and infrared differential interference contrast optics for visualization of the specimen and microelectrodes. The glass microelectrode for recording $[Ca^{2+}]_e$ was held in a robotic manipulator (MP 285; Sutter Instrument, Novato, CA), which ensured high precision of movement and stability. All $[Ca^{2+}]_e$ recordings were performed in the stratum radiatum of hippocampal CA1 region.

Free $[Ca^{2+}]_e$ was measured in stratum radiatum of hippocampal CA1 region as described previously (Hrabetova and Nicholson, 2007; Hrabetova et al., 2009). Briefly, during each trial a Ca^{2+} -ISM was inserted to a depth of 200 μ m into the 400 μ m brain slice using robotic micromanipulator under visual control. The position of the tip was confirmed using water-immersion objective. The Ca^{2+} signal was extracted by continuous subtraction of the potential on the reference barrel from the potential on the ion-detecting barrel using a dual-channel microelectrode preamplifier (model IX2-700; Dagan Corp., Minneapolis, MN, USA). Both Ca^{2+} and DC potentials were amplified and low-pass filtered (6 Hz) using a signal conditioner (CyberAmp 320, Axon Instruments, Molecular Devices, Sunnyvale, CA, USA) and continuously monitored on a chart recorder. In a few experiments, pre-calibration showed that the reference barrel of the Ca^{2+} -ISM might not be stable. In such cases, a separate reference microelectrode was placed 20 μ m from the Ca^{2+} -ISM (also in stratum radiatum of CA1 hippocampus) and the voltage from this microelectrode was used in place of the reference barrel on the Ca^{2+} -ISM. All voltages recorded during experimentation were converted to $[Ca^{2+}]_e$ using slope and interference values obtained from prior calibrations (see above).

Chebabo et al. (1995) previously reported and corrected for shifts in Ca^{2+} -ISM calibration curves during the treatment of slices with hypo-osmotic solutions. Such corrections were not necessary in these trials. When Ca^{2+} -ISMs were switched from a solution containing 1.5 mM Ca^{2+} and 140 mM NaCl to hyponatremic calibration solutions (1.5 mM Ca^{2+} in 90, 60, 40 mM NaCl omitted), the shift in voltage on the ion-detecting barrel (without subtraction) was consistently the same as the shift observed on the reference barrel. Such shifts were corrected for by subtracting voltage on the reference barrel from voltage on the ion-detecting barrel during trials (see above).

Although the slope of the Ca^{2+} -ISM stayed relatively constant, during trials a small amount of drift was observed such that voltages representing $[Ca^{2+}]_e = 1.5$ mM either decreased or increased slightly throughout the trial. To correct for this drift, the Ca^{2+} -ISM (and reference microelectrode when used) was raised 50 μ m above the level of the slice every 30 min (immediately before changing solutions). After a stable recording was observed, the Ca^{2+} -

ISM was reinserted into its original position in the CA1 region of hippocampus. This calibration process took roughly 10 sec and baseline voltage (equivalent to $[Ca^{2+}]_e = 1.5$ mM) in each trial was assumed to be the line connecting the voltages readings when the Ca^{2+} -ISM was above the slice. In the few trials where such calibrations were not done, baseline voltage for the entire trial was taken as the line connecting voltages prior to the first treatment and after the last treatment (at which point Ca^{2+} -ISM and reference microelectrode were consistently raised 50 μ m above the level of the slice) for the entire trial.

Statistical Analysis

All statistics, including the mean and standard error for each group, were calculated using SigmaStat 3.5 (Systat Software, San Jose, CA, USA), with n equal to the number of slices exposed to each test solution (40, 60, 90 mM NaCl substituted; control or cleaved; blocked or paired control). ANOVA was used to compare three or more groups and Student's t-test or Wilcoxon signed rank test to compare two groups. For all tests performed, $p < 0.05$ is considered significant.

Results

Effect of Chondroitin Sulfate Cleavage on $[Ca^{2+}]_e$ Changes During and In Recovery from Hyponatremia

Figure 2A shows a typical record capturing the application of F-40, F-60, and F-90 to a single mouse brain slice. Immediately after regular ACSF is switched to F-40 a rapid decrease in $[Ca^{2+}]_e$ is observed. A few hundred seconds later (without changing the treatment solution), $[Ca^{2+}]_e$ levels return to baseline. The minimum $[Ca^{2+}]_e$ observed during treatment is referred to as $\text{min-}[Ca^{2+}]_e$ and the time to $\text{min-}[Ca^{2+}]_e$ is referred to as T1.

Regular ACSF applied after 30 min of F-40 test solution produces an immediate increase in $[Ca^{2+}]_e$ that peaks and then returns to baseline (Fig 2A). The maximum $[Ca^{2+}]_e$ observed during treatment is referred to as $\text{max-}[Ca^{2+}]_e$ and time to $\text{max-}[Ca^{2+}]_e$ during the recovery period is referred to as T1.RP. The pattern observed during F-40 test solution is repeated when F-60 and F-90 are applied. The pattern exemplified by the record shown in Figure 2A was observed in all trials (hypo-osmotic and low-NaCl) here and previously (Chebabo et al., 1995); the only exception is discussed below.

Figure 2B shows a typical record from the application of the same solutions in the same order as above to brain slices pre-treated with chABC enzyme. Changes in $[Ca^{2+}]_e$ observed in Figure 2B follow the same pattern as above. At the onset of low-NaCl treatment, $[Ca^{2+}]_e$ decreases rapidly and then returns to baseline. During the recovery period, $[Ca^{2+}]_e$ increases before returning to baseline. The similarity supports evidence below demonstrating that cleavage of CS from the ECM of brain tissue has no significant effect on $[Ca^{2+}]_e$ changes during and in recovery from hyponatremia.

Following each trial involving cleavage of CS from the ECM, slices treated with chABC enzyme and control slices from the same mouse were processed by immunohistochemistry to confirm cleavage (see methods). Representative sections taken from stratum radiatum of

CA1 region of hippocampus are shown in Figure 2C. This figure demonstrates that primary antibodies targeting cleaved CS sites have an affinity for slices treated with chABC enzyme but not for control slices. This difference was observed as a fluorescent signal in the chABC treated slices after the application of a fluorescently labeled secondary antibody. The contrast between all slices treated with chABC enzyme ($n = 5$ sections from 2 mice) and their respective controls ($n = 6$ sections from 2 mice) was similar that shown in Figure 2C, confirming the effectiveness of methods described above in cleaving CS from the ECM of mouse brain slices.

Initial tests of $[Ca^{2+}]_e$ changes during hyponatremia were conducted without osmotic compensation. However, spreading depression (SD) was observed repeatedly in H-90 trials ($n = 3$ slices from 3 mice), a finding consistent with Chebabo's initial investigation (Chebabo et al., 1995). A typical SD episode from H-90 is shown in Figure 2D. During SD, there was a large shift in DC potential and a dramatic decrease in $[Ca^{2+}]_e$; this is typical for SD (Nicholson et al., 1977). Observing SD during H-90 trials is not surprising, as hypotonic solutions are routinely used to artificially increase brain region susceptibility to SD (Nicholson and Kraig, 1981). Because $[Ca^{2+}]_e$ changes induced by SD would make data analysis in H-90 difficult, we did not use H-solutions beyond our initial experiments. No SD was observed in F-solutions.

In Figure 3A, the average min- $[Ca^{2+}]_e$ observed during F-40, F-60, and F-90 is plotted for control slices ($n = 4$ slices from 3 mice for F-40, F-60, and F-90) and slices treated with chABC enzyme ($n = 4$ slices from 3 mice for F-40, F-60; $n = 4$ slices from 4 mice for F-90). Figure 3A also shows the average max- $[Ca^{2+}]_e$ that occurred during recovery periods in these slices. The recovery periods following F-40, F-60, and F-90 are referred to as F-40.RP, F-60.RP, and F-90.RP herein forward. We found no statistically significant differences between control slices and slices treated with chABC enzyme ($p = 0.55$ for F-40, $p = 0.46$ for F-60, $p = 0.96$ for F-90, $p = 0.10$ for F-40.RP, $p = 0.17$ for F-60.RP, and $p = 0.37$ for F-90.RP; all comparisons by Student's t-test). This data suggest that cleavage of CS from the matrix of mouse brain tissue has no significant effect on $[Ca^{2+}]_e$ changes during and in recovery from low-NaCl treatments.

Figure 3B shows both the average T1 and average T1.RP for control slices ($n = 4$ slices from 3 mice for F-40, F-60, and F-90) and slices treated with chABC enzyme ($n = 4$ slices from 3 mice for F-40, F-60; $n = 4$ slices from 4 mice for F-90). We found no statistically significant differences between control slices and slices treated with chABC enzyme ($p = 0.73$ for F-40, $p = 0.61$ for F-60, $p = 0.71$ for F-90, $p = 0.69$ for F-40.RP, $p = 0.50$ for F-60.RP, and $p = 0.36$ for F-90.RP; all comparisons by Student's t-test). This data indicates that the application of chABC enzyme does not have a significant effect on T1 or T1.RP.

One possible confounding variable in our results was that a longer time from surgery might influence the amplitude of Ca^{2+} changes during low-NaCl treatment. Slices treated with chABC enzyme were tested roughly 4 hr post-surgical isolation whereas many of the comparison control slices were tested at 1 hr post-surgical isolation. To rule-out this possible confounding variable, F-60 was applied to brain slices between 5 and 8 hr after surgical isolation. The average min- $[Ca^{2+}]_e$ in this group was $1.25 \text{ mM} \pm 0.01 \text{ mM}$ (mean $\pm$ SEM, n

= 3 slices from 3 mice). The same measurement from the chABC treatment group was also $1.25 \text{ mM} \pm 0.02$ (mean $\pm$ SEM, $n = 4$ slices from 3 animals), which was not statistically different from the late treated control slices ($p = 0.96$, Student's t -test).

[Ca²⁺]_e Changes during Hyponatremia are Dependent on NaCl Concentration

Data from chABC treated and control slices were pooled and the average min-[Ca²⁺]_e that occurred during F-40, F-60, and F-90 was used for statistical analysis in Figure 4A ($n = 8$ slices from 6 mice for F-40 and F-60; $n = 8$ slices from 7 mice for F-90). Mean values among the 3 groups were significantly different ($p < 0.05$, ANOVA). A multiple comparison procedure showed that min-[Ca²⁺]_e observed in F-40 and F-60 were statistically different from that observed during F-90 ($p < 0.05$, Holm-Sidak method). However there was no statistical difference between min-[Ca²⁺]_e during F-40 and F-60 ($p = 0.09$, Holm-Sidak method). The average decrease in [Ca²⁺]_e during the F-40, F-60, and F-90 trials was 13%, 18%, and 34% respectively.

Also using pooled data, the average max-[Ca²⁺]_e that occurred during F-40.RP, F-60.RP, and F-90.RP is plotted in Figure 4B ($n = 8$ slices from 6 mice for F-40 and F-60; $n = 8$ slices from 7 mice for F-90). Mean values among the three groups were significantly different ($p < 0.05$, ANOVA). As above, max-[Ca²⁺]_e during F-40.RP and F-60.RP were significantly different from max-[Ca²⁺]_e during F-90.RP ($p < 0.05$, Holm-Sidak method); however max-[Ca²⁺]_e during F-40.RP and F-60.RP were not different from each other ($p = 0.28$, Holm-Sidak method). The average increase in [Ca²⁺]_e during the F-40.RP, F-60.RP, and F-90.RP trials was 11%, 14%, and 24% respectively. The pattern observed in min-[Ca²⁺]_e and max-[Ca²⁺]_e suggests that changes in [Ca²⁺]_e during and in recovery from low-NaCl treatment are concentration dependent. In other words, when fructose is substituted for NaCl in the ACSF applied to mouse brain slices, the less NaCl present in the ACSF the greater the changes in [Ca²⁺]_e observed during and in recovery from the treatment (within the range of 40 – 90 mosmol NaCl omitted).

One possible explanation for the concentration dependent decrease in [Ca²⁺]_e during low-NaCl treatment was that brain slices exposed to a low-NaCl treatment were sensitized to subsequent treatments. To rule out this possibility, brain slices were exposed to a F-60 treatment solution repeatedly and min-[Ca²⁺]_e was measured. The min-[Ca²⁺]_e during treatments 1, 2, and 3 was 1.12 ± 0.05 , 1.17 ± 0.04 , and 1.19 ± 0.02 mM respectively ($n = 7$ slices from 4 mice for 1st and 2nd treatments; $n = 6$ slices from 4 mice for 3rd treatment). Whereas successive treatment of brain slices with increasing levels of hyponatremia (F-40, F-60, F-90) resulted in decreasing min-[Ca²⁺]_e, there was no statistical differences in min-[Ca²⁺]_e when slices were repeatedly exposed to a F-60 treatment ($p = 0.067$, repeated one-way ANOVA).

Characterizing Time Scales of [Ca²⁺]_e Changes

T1 and T1.RP are graphed in Figure 4C ($n = 8$ slices from 6 mice for F-40 and F-60; $n = 8$ slices from 7 mice for F-90). As in previous figures, data is pooled from slices treated with chABC and untreated slices (see above results for verification of method). When T1 and T1.RP at all three levels of hyponatremia tested (F-40, F-60, and F-90) are considered as a

single group, two-way ANOVA with interaction shows that the degree of hyponatremia does not significantly affect the T1 and T1.RP ($p = 0.311$, Holm-Sidak method). However, within the same group T1 times are significantly shorter than T1.RP times ($p < 0.001$, ANOVA) and this difference is independent of the level of hyponatremia ($p = 0.82$, ANOVA). The implications of these findings are elaborated upon in the discussion section that follows.

Blocking Min- $[Ca^{2+}]_e$ with ML 218 T-type Ca^{2+} Channel Inhibitor

Figure 5A shows the $[Ca^{2+}]_e$ changes during a representative ML 218 T-type Ca^{2+} channel blocker treatment. As above, $[Ca^{2+}]_e$ decreases sharply in response to F-60 treatment and recovers to baseline shortly afterwards under both the blocked and unblocked conditions. Also as above, $[Ca^{2+}]_e$ overshoots baseline during a normonatremic recovery period. Figure 5B plots the average min- $[Ca^{2+}]_e$ and max- $[Ca^{2+}]_e$ from ML 218 T-type Ca^{2+} channel blocker treatments and paired controls ($n = 5$ slices from 5 mice). Analysis demonstrates that min- $[Ca^{2+}]_e$ is attenuated significantly by ML 218 treatment ($p < 0.05$, paired Student's t -test). On average, the decrease in $[Ca^{2+}]_e$ during hyponatremia after ML 218 treatment is 20% less than paired controls. ML 218 had no effect on max- $[Ca^{2+}]_e$ ($p = 0.313$, Wilcoxon signed rank test), T1, or T1.RP (data not shown).

Discussion

This study is the first to analyze $[Ca^{2+}]_e$ changes in a recovery period following hyponatremia and to test the mechanisms of $[Ca^{2+}]_e$ decreases in brain during hyponatremia. After measuring $[Ca^{2+}]_e$ during and in recovery from low-NaCl treatments, we report three major findings. First, we show that CS does not significantly contribute to $[Ca^{2+}]_e$ changes during low-NaCl treatments. Second, we show that T1 and T1.RP are significantly different. Third, we show that ML 218, a T-type calcium channel blocker, attenuates min- $[Ca^{2+}]_e$ by 20% without significantly changing max- $[Ca^{2+}]_e$, T1, and T1.RP. We also confirm that $[Ca^{2+}]_e$ changes occurring during low-NaCl treatment are dependent on $[NaCl]$. Together, these data suggest that Ca^{2+} rapidly enters brain cells at the onset of low-NaCl treatment and is extruded from brain cells while recovering from low-NaCl treatment. These processes most likely occur by distinct mechanisms or by multi-step processes. The data presented add to the current understanding of how disturbances of Na^+ , a pervasive finding in clinical practice, might influence Ca^{2+} homeostasis in brain.

Changes in $[Ca^{2+}]_e$ Recorded During and After Hyponatremia are Robust and Reproducible

The trials conducted here confirm that rodent brain slices respond in predictable ways to changes in $[NaCl]$. Rodent brain slices exposed to a low-NaCl treatment for 30 min experience a significant decrease in $[Ca^{2+}]_e$ that lasts a few hundred seconds followed by a return of $[Ca^{2+}]_e$ to baseline levels. Applying regular ACSF to brain slices after a 30 min low-NaCl treatment produces an initial increase in $[Ca^{2+}]_e$ followed by a return of $[Ca^{2+}]_e$ to baseline (Fig 2). The return to baseline of $[Ca^{2+}]_e$ observed during treatments and recovery periods probably represents an equilibration of $[Ca^{2+}]_e$ with the solutions (F-40, F-60, F-90, or regular ACSF) continually washed over the slices, all of which had a $[Ca^{2+}]$ of 1.5 mM. The only exception to the pattern above is the frequent spreading depression observed in trials reported here and previously by Chebabo et al. (1995) during H-90 trials.

While the results of this study and the report by Chebabo et al. (1995) on Ca^{2+} changes during hyponatremia in brain tissue are strikingly similar, there were three noteworthy differences. First, changes in $[\text{Ca}^{2+}]_e$ during application of test solutions and recovery periods occurred over different time periods. In our trials average T1 time was a few hundred seconds (Fig 4); Chebabo et al. (1995) reported a T1 time of roughly 10 min. Second, Chebabo et al. (1995) did not observe a complete recovery from min- $[\text{Ca}^{2+}]_e$ during low-NaCl treatments. Third, Chebabo et al. (1995) reported more dramatic changes in $[\text{Ca}^{2+}]_e$ during low-NaCl treatments than those reported here (Fig 4).

These seeming discrepancies can be attributed to differences in the tissue chamber used to superfuse brain slices. Whereas all data presented here was collected using a submersion chamber, Chebabo et al. (1995) used an interface chamber. In our experiments, mouse brain slices were completely surrounded by superfused solution (versus being partially surrounded in Chebabo's interface setup). This leads to a rapid equilibration of the entire brain slice (including recording site) with superfused solution. A slower equilibration with bath solution likely delayed T1 times in Chebabo et al.'s (1995) experiments compared to data presented here. Furthermore, the faster exchange between bath and brain slice in experiments here probably washed off $[\text{Ca}^{2+}]_e$ changes faster than the interface chamber used by Chebabo et al. (1995), blunting the min- $[\text{Ca}^{2+}]_e$ observed previously.

Despite major differences in experimental setup and the discrepancies above, there is remarkable consistency between the pattern of $[\text{Ca}^{2+}]_e$ changes during and after hyponatremia reported here and previously by Chebabo et al. (1995). Although Chebabo et al. (1995) used a different recording chamber and a different rodent species (rat), they also observed a decrease in $[\text{Ca}^{2+}]_e$ following a low-NaCl treatment and an increase in $[\text{Ca}^{2+}]_e$ that overshoots baseline if regular ACSF is applied to brain slices during a recovery period. Such consistency suggests that the findings of this and Chebabo et al. (1995) original study are both robust and highly reproducible.

Cleavage of Chondroitin Sulfate Does Not Significantly Affect $[\text{Ca}^{2+}]_e$ Changes During and In Recovery from Hyponatremia

One aim of this study was to better characterize the interaction between Ca^{2+} and CS in the extracellular space of brain cells. Prior modeling of CS's interaction with cations in the ECM suggested that the high concentrations of Na^+ in the extracellular space of normal brain tissue might shield larger, divalent cations from the negative charges on CS (Magdelenat et al., 1974). The potential for a robust electrostatic interaction between CS and Ca^{2+} was further supported by Hrabetova and with co-investigators' finding that cleavage of CS from the ECM of brain tissue increased the rate of diffusion of Ca^{2+} through the extracellular space (Hrabetova et al., 2009).

If CS sequestered a significant amount of Ca^{2+} for longer than a few seconds during hyponatremia, we would expect a significant change in min- $[\text{Ca}^{2+}]_e$ and T1 observed after cleavage of CS from the ECM. However, we found no difference in min- $[\text{Ca}^{2+}]_e$, max- $[\text{Ca}^{2+}]_e$, T1, or T1.RP between slices treated with chABC enzyme and control slices (Fig 3). For this reason, we now suggest that as Ca^{2+} ions diffuse through the extracellular space of brain tissue, their interaction with the CS is both fast and tenuous.

[Ca²⁺]_e Changes During and In Recovery from Hyponatremia are Dependent on [NaCl]

Chebabo et al. (1995) originally described the decrease in [Ca²⁺]_e following low-NaCl treatments as dependent on the degree of hyponatremia. The results reported in Figures 4A and 4B support this conclusion. Although statistically significant differences were not found between all degrees of hyponatremia tested during and in recovery from hyponatremia, the differences measured suggest a step-wise concentration dependence in both min-[Ca²⁺]_e and max-[Ca²⁺]_e. Assuming larger differences between min-[Ca²⁺]_e and baseline represent increased Ca²⁺ entry into cells, the concentration dependence observed here might have clinical implications in defining cut-off values for the treatment of hyponatremia induced Ca²⁺ dysregulation (see clinical implications).

Multiple Lines of Data Suggest Distinct Mechanism for [Ca²⁺]_e Changes During and In Recovery from Hyponatremia

Prior to this study, all mechanistic explanations of [Ca²⁺]_e changes during hyponatremia were purely speculative. Chebabo et al. (1995) originally proposed that changes in [Ca²⁺]_e during and in recovery from a low-NaCl treatment were due to a reduced or reversed driving force (and thus activity) of a Na⁺-Ca²⁺ exchanger (NCX). This hypothesis was later refuted by Borgdorff et al. (2000), who showed that applying low-NaCl treatments with Na⁺ substituted by choline⁺, which cannot ride the exchanger, failed to produce increases in [Ca²⁺]_i. We also tested Chebabo's original hypothesis that hyponatremia might reverse NCX activity by applying NCX blocker KB-R7943 mesylate (Cat #1244; Bio-Techne, Minneapolis, MN, USA). In these experiments, we found no changes in min-[Ca²⁺]_e, max-[Ca²⁺]_e, T1, or T1.RP during and in recovery from hyponatremia (unpublished data).

Borgdorff et al. (2000) suggested that total reduced ionic strength might contribute to changes in [Ca²⁺]_e observed during hyponatremia and implicated voltage-dependent Ca²⁺ channels in this process. Based on this suggestion, we used ML 218 to test whether voltage dependent T-type calcium channels are involved in Ca²⁺ entering cells at the onset of hyponatremia. For the first time, we show that decreases in [Ca²⁺]_e induced by hyponatremia can be attenuated by a T-type Ca²⁺ channel blocker, strongly implicating these channels in the influx of Ca²⁺ following hyponatremia (Fig 5B). ML 218 did not affect max-[Ca²⁺]_e, T1, or T1.RP.

Taken along with differences between T1 and T1.RP, these data suggest that separate mechanisms are responsible for the changes in [Ca²⁺]_e during and in recovery from hyponatremia. Based on the data presented and the current understanding of Ca²⁺ regulation in brain, we suggest that the activity of voltage gated Ca²⁺ channels contributes to the influx of Ca²⁺ during hyponatremia. Although the mechanism of extrusion was not experimentally addressed here, we speculate that Ca²⁺ is extruded during the recovery period by the activation of plasma-membrane Ca²⁺ ATPase (PMCA) and/or the activation of NCX.

An alternative explanation for why T1 is significantly shorter than T1.RP might be that the extrusion of Ca²⁺ from cells during the recovery period is delayed by the release of Ca²⁺ from the endoplasmic reticulum, mitochondria, and/or other intracellular stores of Ca²⁺. According to this explanation, [Ca²⁺]_e decreases more rapidly during low-NaCl treatment

than it increases during the recovery period because in the former we are measuring the activity of a single Ca^{2+} channel while in the latter we are measuring the activity of at least two intracellular processes. This explanation raises the question of how Ca^{2+} release from intracellular stores is triggered during the recovery period.

Based on this study, we cannot differentiate between the multiple possible mechanisms for Ca^{2+} extrusion during hyponatremia. Kawamoto et al. (2012) outline two major mechanisms for Ca^{2+} extrusion, the PMCA and the NCX. By showing that $[\text{Ca}^{2+}]_i$ does not change when patch-clamped cells are exposed to low-NaCl solutions in which Na^+ is replaced by choline⁺, Borgdorff et al. (2000) demonstrated that reversing the driving gradient on NCX is not sufficient to cause the influx of Ca^{2+} observed during hyponatremia. However, this does not exclude NCX's involvement in the efflux of Ca^{2+} following hyponatremia. Future investigation of $[\text{Ca}^{2+}]$ changes during and in recovery from hyponatremia using blockers while recording intracellular and extracellular changes in $[\text{Ca}^{2+}]$ might further increase our understanding of this important process.

Clinical Implications

We report that hyponatremia is associated with a concentration dependent decrease in $[\text{Ca}^{2+}]_e$, which most likely represents Ca^{2+} entering brain cells to a degree that could produce CNS dysfunction (Nedergaard and Verkhratsky, 2010). Hyponatremia is one of the most common electrolyte abnormalities in clinical practice and current treatment is directed at modulating water and NaCl intake and excretion (Patel et al., 2007). However, the consequences of Ca^{2+} disturbances during and in recovery from hyponatremia are not well understood and not treated in clinical settings.

Recent associations between aging, neurodegenerative disease, and dysregulation of brain Ca^{2+} homeostasis highlight the importance of investigating how Ca^{2+} homeostasis changes during hyponatremia. Given Ca^{2+} 's role in maintaining normal brain function and the changes in Ca^{2+} homeostasis reported during hyponatremia, it is possible that chronic hyponatremia, which is currently treated as a marker for underlying conditions, might contribute to normal aging and brain pathology. Furthermore, the concentration dependence of $[\text{Ca}^{2+}]_e$ changes during low-NaCl treatment shown above suggests that the degree of hyponatremia might correlate with the degree that Ca^{2+} homeostasis is disrupted in brain tissue. Since current clinical practice does not evaluate for Ca^{2+} disturbances during and in recovery from hyponatremia, confirmation of any of the above associations would have profound implications on how elderly patients with hyponatremia are managed.

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Abbreviations

ACSF	artificial cerebrospinal fluid
BSA	bovine serum albumin
[Ca²⁺]_e	extracellular calcium
[Ca²⁺]_i	intracellular calcium
chABC	chondroitinase ABC
CS	chondroitin sulfate
CSPG	chondroitin sulfate proteoglycan
ECM	extracellular matrix
GAG	glycosaminoglycan
GAM IgG	goat anti-mouse immunoglobulin
ISM	ion-selective microelectrode
low-NaCl	hyponatremic iso-osmotic
NCX	Na ⁺ -Ca ²⁺ exchanger
RP	recovery period
PMCA	plasma-membrane Ca ²⁺ ATPase
SD	spreading depression

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Highlights

- Hyponatremia abruptly decreases brain $[Ca^{2+}]_e$ in a concentration dependent manner
- Restoring normonatremia gradually increases $[Ca^{2+}]_e$ above physiologic levels
- Cleavage of chondroitin sulfate does not affect $[Ca^{2+}]_e$ in hyponatremia
- T-type Ca^{2+} channel blocker attenuates $[Ca^{2+}]_e$ decrease during hyponatremia
- Study links hyponatremia, common in elderly, and Ca^{2+} dysregulation in brain

Figure 1. Research Model

Diagram of possible sinks for $[\text{Ca}^{2+}]_e$ during hyponatremia. As illustrated, we hypothesized that $[\text{Ca}^{2+}]_e$ decreases during low-NaCl treatment due to (1) the entry of Ca^{2+} into brain cells and (2) the binding of Ca^{2+} to negative charges on the ECM.

Figure 2. Changes in $[Ca^{2+}]_e$ After Low-NaCl Treatment in Control and chABC Pre-treated Slices

(A) $[Ca^{2+}]_e$ during treatment of a single brain slice with low-NaCl test solutions. The F-40 test solution begins at $T \sim 30$ min and continues until $T \sim 1$ hr, when regular ACSF is applied. $Min-[Ca^{2+}]_e$ and $max-[Ca^{2+}]_e$ were measured assuming the baseline voltage represents a $[Ca^{2+}]_e$ of 1.5 mM, shown in the figure as a dashed line. Non-specific signals from the ISM were filtered out. (B) As in (A), except that the brain slice was pre-treated with chABC. (C) Immunohistochemical confirmation of chondroitin sulfate cleavage with chABC. Primary antibodies targeting cleaved CS sites have an affinity for slices treated with chABC enzyme but not for control slices. (D) $[Ca^{2+}]_e$ (left) and DC potential (right) changes during a spreading depression event in a brain slice treated with H-90.

Figure 3. Comparison of Min-[Ca²⁺]_e, Max-[Ca²⁺]_e, T1, and T1.RP in Control and chABC Pre-treated Slices

(A) The average min-[Ca²⁺]_e and max-[Ca²⁺]_e observed during and in recovery from low-NaCl treatments plotted for control slices and slices pre-treated with chABC enzyme. There were no statistical differences between control slices and slices treated with chABC enzyme (see text for statistics), suggesting treatment with chABC enzyme does not significantly effect [Ca²⁺]_e changes during hyponatremia. (B) The average T1 and T1.RP observed during and in recovery from low-NaCl treatment plotted for control slices and slices treated with

chABC enzyme. There were no statistically significant differences between control slices and slices treated with chABC enzyme (see text for statistics). This data indicates that the application of chABC enzyme does not have a significant effect on either T1 or T1.RP. For each treatment, n = 4 slices from 3 mice except for the F-90 treatment in chABC group, where n = 4 slices from 4 mice. Error bars show standard error of the mean for each group.

Figure 4. Characterizing [Ca²⁺]_e Changes During Hyponatremia Using Pooled Data

(A) The average min-[Ca²⁺]_e observed during F-40, F-60, and F-90 for all slices tested. (B) The average max-[Ca²⁺]_e observed during F-40.RP, F-60.RP, and F-90.RP for all slices tested. These data suggest that changes in min-[Ca²⁺]_e are dependent on [NaCl]_e (see text for statistics). (C) The average T1 and T1.RP calculated for all slices tested. The degree of hyponatremia does not significantly affect the T1 and T1.RP. However, T1 times are significantly shorter than T1.RP times and this difference is independent of the level of hyponatremia (see text for statistics). For each treatment, n = 8 slices from 6 mice except for the F-90 treatment in chABC group, where n = 8 slices from 7 mice. Error bars show standard error of the mean for each group.

Figure 5. Partial Block of $[Ca^{2+}]_e$ Decrease During Hyponatremia by T-type Channel Blocker ML 218

(A) Shows a typical record from a ML 218 treatment including unblocked F-60 for paired control. The pattern of $[Ca^{2+}]_e$ change during treatment occurs as previously described under both blocked and unblocked conditions. (B) Plots the average min- $[Ca^{2+}]_e$ and max- $[Ca^{2+}]_e$ from ML 218 T-type Ca^{2+} channel blocker treatments and paired controls. ML 218 significantly reduces the min- $[Ca^{2+}]_e$ that occurs during F-60 while having no significant effect on max- $[Ca^{2+}]_e$ (see text for statistics). The average reduction in min- $[Ca^{2+}]_e$ after ML 218 treatment was 20%. For each treatment, $n = 5$ slices from 5 mice. Error bars show standard error of the mean for each group.