Metabolic Acidosis-Induced Alterations on motor and cognitive function and acid-sensing ion channels 1 and 2a in the brains of young and aged male mice

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Metabolic acidosis is a condition in which there is a disruption in the acid/base balance of the body due to an excess of hydrogen ions. This study investigated if in vivo metabolic acidosis lead to brain dysfunction, whether these potential effects were exacerbated with age, and if brain alterations were associated with changes in acid-sensing ion channel (ASIC) 1 and 2a expression. We found that mild chronic acidosis induced by ammonium chloride supplementation resulted in decreased weight in old mice, reversed age-related impairments in motor and cognitive function, and improved spatial learning and memory of young mice. No major alterations were observed in expression of ASIC1 or 2a.
METABOLIC ACIDOSIS-INDUCED ALTERATIONS ON MOTOR AND COGNITIVE FUNCTION AND ACID-SENSING ION CHANNELS 1 AND 2a IN THE BRAINS OF YOUNG AND AGED MALE MICE

Thesis

Presented to the Graduate Council of the Graduate School of Biomedical Sciences University of North Texas Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

Masters of Science

By

Robbyn S. Kindle, RDN
June 2015
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6.0 INTRODUCTION

Metabolic acidosis is a condition in which there is a disruption in the physiological acid/base balance due to an excess of H⁺ ions in the plasma serum. This can be caused by a variety of conditions including diabetic ketoacidosis (DKA), hyperchloremic acidosis as a result of severe diarrhea, d-lactic acidosis, kidney disease, severe dehydration, and declining kidney function as a result of age (1-5). According to the National Kidney Foundation and the American Diabetes Association, approximately 26 million and 29.1 million Americans, or approximately 10% of the population, have been diagnosed with either chronic kidney disease and/or diabetes respectively (2), furthermore, studies regarding cognitive decline in patients with end-stage renal disease (ESRD) have found that an increase in uric acid in blood serum corresponds to alterations of cortical excitability, and when treated with therapies such as dialysis or kidney transplantation these effects were reversed (2, 6). However, it is not only important to recognize these types of cognitive alterations exist in individuals suffering from physiological complications causing acidosis, but to also determine the specific mechanism causing the alteration in order to better treat individuals with these disorders. By inducing metabolic acidosis in mice, this study will elucidate what effects, if any, acidosis has on the central nervous system, as well as investigate the effects on specific postsynaptic proton receptors.

Specifically, metabolic acidosis is defined as a reduction of serum pH below 7.4 due to the inability of the kidneys to remove an adequate proportion of hydrogen protons (H⁺), and can happen as a result of an increase in serum hydrogen ions (high anion gap), or a decrease in serum
bicarbonate ions (hyperchloremic or normal anion gap) (7, 8). Acid-base homeostasis is regulated through buffer systems which include the lungs by exhalation of CO₂, and the kidneys by excretion of H⁺ protons in the urine. Impairment of one or more of the following mechanisms of the renal system can induce metabolic acidosis: excretion of excess hydrogen protons, reabsorption of bicarbonate ions, and/or the use of titratable acids such as ammonia, phosphate, and citrate (7, 8).

Detrimental effects of extracellular acidosis include impairment of GABAergic neurons through elevation of threshold potentials and prolongation of refractory periods, and also induction of excitotoxicity by overexcitation of cortical pyramidal neurons through a reduction of threshold potential and shortened refractory periods (9, 10). Other studies have concluded that excessive release of acidic vesicles during episodes of high neuronal activity, such as that which occurs during epileptic seizure activity, causes extracellular acidosis leading to neuronal cell death (11). In Alzheimer’s disease, cerebral acidosis may occur due to impaired oxidative energy metabolism and inflammation (12). Lactic acidosis within the extracellular neuronal spaces has also been observed in individuals with Parkinson’s disease and Huntington’s disease, as well as within areas surrounding cells affected by ischemic stroke (13-15). Recent studies indicate that extracellular localized acidosis that occurs during neuroinflammatory episodes in conditions such as traumatic brain injury, and multiple sclerosis can also induce neuronal degeneration (16, 17).

One potential mechanism by which acidosis could exhibit these deleterious effects on the central nervous system is through activation of specific acid sensing ion channels (ASICs). ASICs are extracellular proton activated sodium-selective channels belonging to the DEG/EnaC superfamily, and are comprised of three subunits of homomeric or heteromeric proteins.
surrounding a central pore (18, 19). They have two transmembrane regions, TM1 and TM2, and large cysteine rich extra-cellular loops with intracellular N and C termini. This extracellular region is the area where protons bind to trigger channel activation, and is also involved in the desensitization/inactivation of the channel (18, 20). There are six known ASIC monomers encoded by four genes: ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4 (20, 21). The isoforms “a” and “b” differ in their N-terminus regions, with ASIC1a having a low permeability to Ca$$^{++}$$, and ASIC1b having no Ca$$^{++}$$ permeability (22). ASICs form trimeric complexes, with homomeric ASIC1a and heteromeric ASIC1a/2a being found to predominate in the mammalian central nervous system (20, 23-25). However, all ASIC subunits can be found in the peripheral nervous system (26). ASIC1 and 2 overlap in expression in higher concentrations in synaptically dense brain regions associated with high synaptic plasticity, and thus have been linked to learning and memory behaviors (27-29). Current research indicates that ASIC2 subunits play a role in targeting and regulation of ASIC’s to the cell membrane surface thus facilitating ASIC activation in synaptically dense regions (30, 31).

Activation of ASICs occur as a result of a rapid decline in extracellular pH. The pH activation range differs for each ASIC channel depending upon subunit makeup (see Figure 1) (25). Homomeric ASIC1a channels have been shown to activate within a pH$$_{50}$$ range of 5.8 to 6.8, with a maximal activation at a pH of approximately 6.0, generating a rapid depolarization and thus possibly enhancing the probability of an action potential (24, 25, 32). Homomeric trimers of ASIC2a differs from homomeric ASIC1a in that in vitro studies have shown activation occurs within a pH$$_{50}$$ range of 4.5-4.9, which is outside of physiological range, and recent studies have found that exogenous peptides can affect its pH sensitivity, thus suggesting that the ASIC2a homomers could be influenced by other modulators in vivo (33-35). Heteromeric ASIC1a/2a
activation occurs in the pH$_{50}$ range of 4.8 to 5.4, and heteromeric ASIC1a/2b occurs at a pH$_{50}$ 6.4 (21, 25, 32, 36).

Desensitization of ASIC’s can occur with a slight lowering of pH between 7.4-6.9, as opposed to a rapid acidification which would in effect activate the channels (18, 21, 22). Additionally, desensitized channels will remain closed despite any further lowering of pH, and also after activation in the continuous presence of protons (22, 25, 32). More specifically, homomeric ASIC1a tetramers desensitize within the pH range of 7.2-7.3 with a time constant of approximately 1.2-3.5 seconds, and recovers from desensitization at a pH or 7.4 with a time constant of approximately 10 seconds (22, 25, 32, 33). Table 1 shows desensitization kinetics of known ASIC channel subunit compositions. The ASIC1a subunit has also been shown to be responsible for current amplitude and channel inhibition, while ASIC2a is the modulatory subunit that plays a role in desensitization, pH sensitivity, recovery from desensitization, and potentiation (24, 37). Desensitization of these channels within the realm of chronic metabolic acidosis is unclear, however, the current study could provide some information that might assist in elucidating this issue.

**Table 1** – Functional properties of ASICs (25, 38):

<table>
<thead>
<tr>
<th></th>
<th>ASIC1a (homomerite)</th>
<th>ASIC1b (homomerite)</th>
<th>ASIC2a (homomerite)</th>
<th>ASIC2b (homomerite)</th>
<th>ASIC1a/2a</th>
<th>ASIC1a/2b</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH$_{50}$ of activation</td>
<td>6.4 – 6.6</td>
<td>6.1 - 6.2</td>
<td>4.5 – 4.9</td>
<td>Does not form pH sensitive channels</td>
<td>4.8 – 5.4</td>
<td>6.4</td>
</tr>
<tr>
<td>pH$_{50}$ of desensitization</td>
<td>7.2 – 7.3</td>
<td>Not determined</td>
<td>Not determined</td>
<td>Not determined</td>
<td>7.3</td>
<td></td>
</tr>
</tbody>
</table>
A pilot study investigated the effects of the ASIC1 blocking diuretic amiloride on the acute sclerotic lesions overexpressing ASIC1 that result in neurodegeneration. The results concluded that those individuals in the treatment group had significantly less neurodegeneration than those who were untreated, therefore concluding that amiloride was in fact neuroprotective by blocking ASIC1 activation (16). Additional studies have shown that the effects of acidotoxicity on in vitro human cortical neurons was partially reduced in ASIC1 deficient neurons, and that inhibiting ASIC channels with a lipophilic amiloride derivative protected these neurons against acidotoxicity and neurodegeneration (39). However, other studies have concluded that overexpression of ASIC1a in hippocampal slices increased the density of dendritic spines suggesting that ASIC1a is important in spine remodeling and synaptic plasticity (40).

With regard to cognitive behavior studies, ASIC1a knockout mice were shown to have a deficiency in learning and memory tasks (28, 29). Supporting this finding were additional studies that found ASIC1a in the neuronal cell body, dendrites, and postsynaptic spines (28, 40). Conversely, a more recent study has shown that ASIC1a-null mice did not show a deficiency in hippocampal-dependent long-term potentiation and spatial memory (41). Additionally, ASIC2−/− mice exhibited similar behavior to aversive stimuli as ASIC1 +/+ mice, indicating that both subunits potentially play a role in defensive response behavior, but neither subunit knockout had an altered response to less aversive stimuli (27). Therefore, with regard to the mediation of long-term potentiation, it is important to look at other synaptic channels that are also affected by acidotic conditions that have been implicated in learning and neuroplasticity.

N-methyl-D-aspartate receptors (NMDAR) are voltage-dependent ligand-gated ionotropic
glutamate receptors that are found in areas of the brain associated with synaptic plasticity. Heterodimers of NR1 and NR2 form channels that when activated allow the flow of Na\(^+\) and Ca\(^{2+}\) ions into the cell while K\(^+\) flows outward thus mediating long-term potentiation (LTP). Chronic mild acidosis has been shown to reduce the number of NMDA receptors in vitro, however, activation of ASIC1a could alleviate the voltage-dependent Mg\(^{2+}\) blockade and therefore activate the NMDA receptor and facilitate LTP (42, 43).

In addition, due to utilizing NH\(_4\)Cl to induce metabolic acidosis in the current study, it is important to consider the effects of the ammonium ion. Metabotropic glutamate receptor 1 (mGlur1) is a G protein-coupled receptor which forms a homodimer, however, unlike ASICs and NMDARs, they do not form a channel. These receptors indirectly influence learning and memory by modification of other proteins, more specifically for the purposes of the current study, mGluR1 can increase NMDA activity causing a greater influx of Na\(^+\) and Ca\(^{2+}\) ions into the neuronal cell (44). An increase in extracellular ammonium ions have been shown to alter glutamate mediated neurotransmission by either increasing or decreasing glutamate transport activity based on time of exposure and transporter isoforms (45). Therefore, within the context of this study, the effects of the ammonium ion on mGluR1 activity should be taken into consideration as possible factor in functional assessment results.

When considering the large population of individuals affected by metabolic acidosis, it becomes evident that investigating its effects on cognitive function should be studied. An exhaustive literature search failed to locate current published in vivo research directly investigating these issues, nor has the effect of age been considered in this capacity. Therefore, the primary purpose of this study was to determine if in vivo metabolic acidosis leads to brain dysfunction, and whether it was exacerbated with age. Furthermore, the study determined
whether brain alterations were associated with changes in ASICs expression. For induction of metabolic acidosis, a diet supplemented with 2% NH₄Cl was sufficient to decrease serum pH from 7.41 to 7.25 with a corresponding decrease in urine pH (46). After a period of thirty days post-diet initiation, the mice were subjected to a variety of motor and cognitive function tests while remaining on their respective diets. Brain regions were collected after behavioral profiling and preserved at -80°C. Western Blot analyses of ASIC1 and 2a, NMDAR1 and 2b, and mGluR1 were then conducted to determine receptor expression. The results of this study will help to elucidate specific mechanisms altered during conditions involving mild extracellular acidosis which occurs due to metabolic disturbances, therefore assisting in the development of therapies to mitigate these effects or reverse age-related declines.
2.0 MATERIALS AND METHODS

2.1 Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee at UNT Health Science Center at Fort Worth. Sixteen four-month-old and sixteen twenty-month-old male C57BL/6J mice were obtained from the National Institute on Aging. One week after arrival to the UNT Health Science Center vivarium, the animals were injected subcutaneously with a unique identification chip (Biomark HPT8, Boise, Idaho). The mice were housed individually in clear polycarbonate cages (28 x 17 x 12.5 cm) and had ad libitum access to food and water. The ambient temperature in the colony room was maintained at 23 ± 1°C, under a 12h light-dark cycle beginning at 0700 hr. After 2 weeks of acclimation, the mice were randomly assigned to one of two treatment groups (n=8/age/treatment group (see section 2.5 Statistical Analysis for justification of power)): the control diet (LabDiet R&M 5LG6/Irr 4F, cat #: 5S84 from Test Diet, Richmond, IN) or the control diet supplemented with ammonium chloride ((2% NH₄Cl cat# 12125-02-91 from Sigma), cat #: 5W07-5LG6 from Test Diet, Richmond, IN). The 2% NH₄Cl diet has been previously shown to induce metabolic acidosis by Tashima, et. Al, Nowick, et. Al and Hafner et. Al, with a decline in urine pH corresponding with decline in serum pH as noted by Hafner et. Al, as seen in Table 2 (46-48):
**Table 2** – Blood and serum analysis of pH for control and NH$_4$Cl fed mice after 7 days (48).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NH$_4$Cl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum pH</td>
<td>7.35 m pH}</td>
<td>7.23 m pH}</td>
</tr>
<tr>
<td>Urine pH</td>
<td>6.7 ne pH}</td>
<td>5.730.14}</td>
</tr>
</tbody>
</table>

Individual weights, food intake, and a urine samples were obtained and recorded three mornings per week for the duration of the study. Urine was collected, transferred by pipette into a clean tube, and the pH immediately measured with a benchtop/portable pH meter (IQ Scientific Instruments Model IQ240, Carlsbad, CA).

After 4 weeks on their respective diets, the mice were subjected to a series of behavioral tests during which they remained on their respective diets. The behavioral tests were conducted over a period of 4 weeks in the following order: locomotor activity, reflexive musculoskeletal responses, bridge walking, discriminated avoidance, and spatial learning and memory (49-51). By the end of testing, the mice were euthanized and brains were dissected into 6 regions (cortex, striatum, hippocampus, midbrain, hindbrain and cerebellum) and stored at -80°C for future biochemical measurements.

2.2 Neurobehavioral measures

2.2.1 Locomotor activity – Spontaneous locomotor activity was measured using a Digiscan apparatus (Omnitech Electronics, model RXYZCM-16, Columbus, OH), as previously described (47). Each testing box consisted of a clear acrylic chamber (40.5 x 40.5 x 30.5 cm) surrounded by a metal frame lined with photocells, inside a sound-attenuating chamber (Model 71-ECC, Omnitech Electronics, Columbus, OH). The ambient noise within the chambers was set at 64dB and testing was done under dim illumination (31.8 ± 1.5 lux). Each mouse was
placed in a chamber for 4 consecutive sessions, each 4 minutes in duration for a total of 16 minutes, where the mice were allowed to freely move around the chamber. Movements in the horizontal and vertical plane (7.6 cm above the floor) were detected by the photocells and processed by photocell-based system (Superflex, Model 71-CCPX, Omnitech Electronics, Columbus, OH) to yield different variables (distance, vertical activity, and spatial components). This test measures arousal, ambulation, rearing, and anxiety/fear levels which corresponds to striatal and midbrain (limbic) areas of the brain.

2.2.2. Reflexive musculoskeletal responses- Over 4 consecutive daily sessions, the mice were tested on four simple reflex tests (maximum testing latency was set at 60 seconds). Walk initiation – This test consisted of placing the mouse on a flat smooth surface and recording the latency to initiate movement equivalent to one body length. The latency to walk was averaged over the four daily sessions. This is a test of arousal which corresponds to various brain regions including the striatum and midbrain. Alley turn – For this test, the mouse was gently placed in a 3.5-cm wide, 14-cm long black acrylic dead-end alley and the latency to reverse direction was measured. The latency to turn was averaged over the four daily sessions. This test measures reflexive and motor planning skills which correspond to cortical regions of the brain. Negative geotaxis – In this test, the mouse was placed facing downward on a flat surface tilted at 45° and the latency to turn 90° either direction was recorded. The latency to turn was averaged over the four daily sessions. This test is an orienting and reflex battery which measures reflex, arousal, visual, somatosensory, and hemispatial neglect which corresponds to the cortical orienting circuitry and sense organs. Wire suspension – The mice were allowed to grip a horizontal wire with the front paws while suspended 40 cm above a padded surface. The latency to tread (grab
the wire with their hind legs), and the latency to fall was recorded and averaged over 4 consecutive daily sessions (2 trials/day). This test measures muscle grip strength and coordination with corresponds to skeletal reflex circuitry in the cerebellum and striatum.

2.2.3 Bridge walking – Each mouse was placed on one of 4 bridges mounted 45 cm above a padded surface. The bridges differ in diameter (small vs. large) and shape (round vs. square) providing 4 levels of difficulty. Each bridge was presented 3 times and the measure of performance was the average latency to fall (up to a maximum of 60 seconds) across all bridges. This test measures balance and coordination which corresponds to the cerebellar and striatal brain regions.

2.2.4 Discriminated avoidance – This task was carried out using a T-maze with a stainless steel floor wired for scrambled shock to the feet. During the first session, an initial preference trial was given wherein a shock was initiated with the mouse in the stem of the maze and then terminated upon entry by the mouse into either goal arm. The correct goal arm was then defined as the arm opposite to the one chosen. On each trial thereafter, shock was initiated 5 seconds after the opening of the start box door (or immediately upon entry into the incorrect goal arm) and was terminated after entry into the correct goal arm (maximum time was 60 seconds). As an animal learns the task, the latency to enter the correct goal arm (escape latency) should decrease. The training session continued until the mouse made a correct avoidance on four out of five consecutive trials, with the last two being correct. A correct avoidance is considered when the mouse reaches the correct goal arm within the 5 second time frame during
which shock is not delivered. Performance was scored as the number of trials it took a mouse to reach the avoidance criterion. The mice received 3 sessions in one day, and on each subsequent session the correct arm was opposite from the one learned in the previous session. Mice were given a maximum of 25 trials to reach criterion. This test measures conditioned fear, cognitive flexibility, and working memory which corresponds to the frontal cortex and midbrain (limbic system) brain regions.

2.2.5 Spatial learning and memory- The swim maze test involved acquisition of an ability to swim to a hidden platform from different starting locations in the presence of only distal cues as to the location during a series of nine sessions. The apparatus was a plastic tank (120 cm in diameter and 60 cm in depth) filled with water opacified with nontoxic white paint and maintained at 24±1°C. A computerized tracking system (ANY-MAZE-Stoelting, Wood Dale, IL) was used to record the position of the mice. Testing was conducted in four phases: (1) a pretraining phase (straight alley) in which the mouse learned the simple response components of swimming and standing on the platform hidden below the water surface, (2) a place discrimination acquisition phase in which the mouse must learn and remember the location of the platform, (3) a probe trial retention phase in which the mouse was tested for retention of the learned behavior after one week of no exposure to the apparatus. Straight alley pretraining: During these training sessions, a black curtain was placed above the tank to remove visual cues. The animals were placed at the end of the straight alley, and swam to reach the hidden platform at the opposite end within 60 seconds. Once the mouse reached the platform, it was allowed to stay for 10 seconds before being placed back in the holding apparatus. The mice received 5 trials per session with an intertrial interval of 5 minutes, with one session per day for 2 days (ex:
Place discrimination acquisition: This phase began the day after the last session of training with the straight alley (ex: Tuesday). The curtain was removed and the mice were allowed to use distal cues to locate the platform in the tank. The mice were lowered in the water at one of four starting locations and given 90 seconds to find the platform. Once the mouse reached the platform, it remained on the platform for 30 seconds prior to removal to the holding apparatus, for a 90 second intertrial interval. Each mouse was given 4-5 platform trials per session, with one session a day for a total of 9 sessions (ex: Tuesday-Friday, Sessions 1-4 and Monday-Friday, Sessions 5-9). The measures of performance for this phase were the distance traveled by the mouse to reach the platform (path length), speed and duration. From the path length measures, a learning index was generated as the averaged performance for sessions 2, 3, and 4. As part of sessions 2, 4, 5, 7, and 9, a probe trial was given as the 5th trial instead of a platform trial. During this trial, the platform was inaccessible to the mice for 30 seconds. Once the trial was over, the platform was raised and the mice were given 60 seconds to find it. On this trial, spatial bias for the platform location was evaluated in terms of the percentage of time spent within 40-cm diameter annuli surrounding the platform location. The purpose of the reappearing platform was to ensure that each trial ends with a successful location of the platform which decreases the probability of the extinction of the learned behavior and minimizes the potentially disruptive effect of the probe trial upon subsequent performance. This test measures visual and spatial discrimination, explicit reference learning and memory, and executive function and planning which corresponds to the hippocampal and frontal cortical regions of the brain.
2.3 Biochemical measurements

2.3.1 Tissue homogenization – Ten percent (cortex and cerebellum) or 13.3% (all other regions) homogenates were prepared using an antioxidant buffer (5mM Na phosphate, pH 7.4, 5mM EDTA, 2mM BHT) containing protease inhibitors (#04 693 159 001, Roche, Indianapolis, IN). The samples were then centrifuged at 1000 rpm for 5 min at 4°C, after which the supernatant was reserved for protein concentration and Western blot analyses. Protein concentration was determined according to the BCA method.

2.3.2 Antibodies – The following previously validated primary antibodies were used: rabbit anti-ASIC1 (1:200, #ASC-012) and rabbit anti-ASIC2a (1:200, #ASC-014) from Alomone Labs (Jerusalem, Israel); rabbit anti- β-Actin (1:1000, #13E5), rabbit anti-NMDAR1 (1:800, #D65B7), rabbit anti-NMDAR2B (1:1000, #D8E10), and rabbit anti-mGluR1(1:1000, #D5H10) from Cell Signaling Technology (Beverly, MA, USA). Secondary antibody was goat anti-rabbit IgG-HRP (1:7000, #sc-2004), from Santa Cruz Biotechnology (Dallas, TX, USA).

2.3.3 Semi-quantitative immunoblotting – Forty micrograms of total proteins were loaded and electrophoretically separated for 50 min. at 200 mV then transferred to polyvinylidene difluoride (PVDF) membrane (Bio Rad USA, Hercules, CA, 0.45µm, #162-0015) for 60 min at 4°C at 100mV. The membranes were blocked with 5% BSA in a 1X TBS buffer solution containing 0.1% Tween 20 (TBST), then incubated on a rotator for 18h at 4°C with the primary antibodies (in 5% BSA/TBST). After washing with TBST, the membranes were incubated for 2h with the secondary antibody (in 5% BSA/TBST). Membranes were imaged on a Biospectrum Imaging
System (UVP) (Upland, CA) with ECL western blotting detection reagents (#RPN2232, Amersham, Pittsburgh, PA). Quantification of band density was performed by densitometry using ImageJ Software (NIH, Bethesda, MD, USA), and values were corrected for background and normalized for β-actin expression.

2.4 Statistical Analyses

Data analysis of the behavioral and biochemical datasets involved two-way analysis of variance (ANOVA) with Age and Diet as between group factors. Planned individual comparisons between different age groups (young vs. old) and diet groups (control vs. NH₄Cl supplemented) were performed using a single degree-of-freedom F tests involving the error term from the overall ANOVA. Performances were also considered in three-way ANOVAs with Sessions or Days on diet as the repeated measures. The α level was set at 0.05 for all analyses. The software used for the analyses was Systat 13 (Systat Software Inc., San Jose, CA, USA).
3.0 RESULTS

3.1 Body weight and urine pH

The effects of age and ammonium chloride supplementation on body weight are shown in Figure 1A. Throughout the study, the young control mice weighed less than the old controls. Interestingly, the treated mice lost weight starting during the first week of implementation of the ammonium chloride diet. The effect was more drastic in the old mice, as they lost almost 10g of body weight. It is noteworthy that weights in all groups were stabilized by the time functional assessment was started. A three-way ANOVA revealed significant main effects of Age, Diet and an interaction between Age and Diet (all ps<0.001). Food intake data indicated no difference in the amount of food eaten from the different age and treatment groups (data not shown, all ps>0.201). Urine pH was measured every 3 days throughout the study and presented in Figure 1B. The urine pH from the young mice was higher than that of the old ones. Mice on the NH₄Cl supplemented diet had lower urine pH than the control mice, however in the old mice it only lasted one month.

3.2 Locomotor Activity

The effects of age and diet on spontaneous locomotor activity are shown in Figure 2. The total distance traveled (Figure 2A) was lower in old mice than in young mice. While in the young groups, the treated mice had lower distance travelled, in the old groups, the treated mice had higher distance. These observations were supported by a two-way ANOVA leading to a
main effect of Age and an interaction between Age and Diet (all ps < 0.045). Rearing activity (Figure 2B) was also lower in old mice compared to young ones. The treated mice reared similarly to the control ones. A two-way ANOVA solely revealed a main effect of Age (p=0.045). Time spent in the center (Figure 2C) was lower in the old mice though only significantly in the treated mice when compared to their diet-matched young groups. There was no effect of treatment. A two-way ANOVA did not yield any significant main effect of Age, Diet or an interaction between Age and Diet (all ps > 0.059).

### 3.3 Reflexive musculoskeletal responses

The effects of age and diet on musculoskeletal reflex responses are shown in Figures 3 and 4. Overall, old mice took longer latencies to move one body length compared to the young ones (Figure 3A). Treated mice took similar latencies when compared to the controls. A two-way ANOVA revealed a main effect of Age (p=0.04) but no effect of Diet or interaction between Diet and Age (all ps > 0.142). Old mice also took longer latencies to turn in a dead-end alley compared to the young ones (Figure 3B). Regardless of age, the treated mice seemed to take less time to turn compared to their age-matched controls. A two-way ANOVA yielded a significant main effect of age (p=0.047) but did not support the observed effect of diet (p=0.117). Latency to turn 90 degrees was higher in old mice compared to young mice, and was lower in treated mice compared to controls (Figure 3C). A two-way ANOVA revealed a main effect of Age (p=0.04) but no main effect of Age or an interaction between Age and Diet (all ps < 0.099).

Strength and reflexes were measured using a wire suspension test and presented in Figure 4. Old mice took longer latencies to tread compared to young ones (Figure 4A). Old treated
mice took shorter time to tread compared to the old control mice, while there was no effect of treatment in the young mice. A two-way ANOVA revealed a significant interaction between Age and Diet, and main effects of Age and Diet (all ps <0.001). Similar effects were observed for the latency to fall supported by a significant interaction between Age and Diet (p=0.04) (Figure 4B).

3.4 Bridge Walking

The effects of age and diet on strength on performance in the bridge walking test are shown in Figure 5. Old control mice fell faster from the bridges compared to young controls. Treated mice took longer latencies to fall than control mice, however it only reached significance in the old group. A two-way ANOVA yielded significant main effects of Age and Diet (all ps<0.002).

3.5 Discriminated Avoidance

The effects age and diet on learning (Session 1) and cognitive flexibility (Sessions 2 & 3) are shown in Figure 6. In the learning phase, the treated mice regardless of age seemed to take less number of trials to reach criterion than the control ones. However, a two-way ANOVA did not yield any significant effect of Diet or Age (all ps > 0.109). In session 2 which is indicative of cognitive flexibility, old control mice took more trials to reach criterion than their young counterparts while the old treated mice took the same number of trials as the young mice. This was supported by a significant interaction of Age and Diet from a two-way ANOVA (p=0.014). In session 3, while there was an age difference between the young treated and old treated mice,
there was no effect of diet on the performance of the mice. These observations were supported by a two-way ANOVA yielding a main effect of Age (p=0.028) but no effect of Diet or interaction between Age and Diet.

3.6 Spatial learning and memory

Performance of mice was measured as path length taken to reach a hidden platform and presented in Figure 7A. All mice learned to located the platform by the end of the training as evidenced by the path length decreasing across sessions, and supported by a significant effect of Session (p<0.001). Old mice were less efficient at reaching the platform than the young ones by taking longer path length to reach the platform. Treatment improved the performance of the young and old mice, yet differently and session dependently supported by a three-way ANOVA yielding a significant interaction of Session, Age and Diet (p=0.013). The effects of the diet were observed mainly during the steep learning phase of the task. A two-way ANOVA during sessions 1-4 yielded significant interaction of Session, Age and Diet and main effects of Age and Diet (all ps <0.015). There were no effect of Age or Diet in the second week of testing (all ps>0.215). A composite measure representing the steep learning phase of this test was calculated (averaged path length of sessions 2, 3 and 4). The learning index was higher in old control mice and similar in old treated mice when compared with the young controls. However, a two-way ANOVA only yielded a main effect of Age (p=0.003) while the main effect of Diet and interaction between Age and Diet were almost significant (p=0.051 and 0.053, respectively). Interestingly, the young treated mice took shorter path length in the first session indicative of either a faster learning or a difference in initial performance. Therefore, an analysis of session 1 trial by trial was done (Figure 8). While the performance of the young treated mice seemed to be
initially better than the controls, there were no significant differences between any of the groups following a two-way ANOVA on the path length for trial 1 (all ps>0.248). It is noteworthy that the young treated learned rapidly during trials 2 and 3, while the other groups remained relatively stable. A three-way ANOVA revealed an overall main effect of Age (p<0.001), but no effect of Diet or interaction between Age and Diet (all ps>0.143).

The speed of the mice was variable across sessions and was affected by age, with the young mice swimming faster than the old ones (Figure 7B). However, there was no effect of treatment on the swimming speed of young or old mice. A three-way ANOVA revealed significant main effects of Session, and Age (all ps<0.001).

Spatial bias for the platform location was measured by percent of time spent in an area 40-cm around its location during probe trials (Figure 9). As training advanced, the mice developed a bias for the platform location, higher than chance (~12%) evidenced by an increase in percent time spent in the defined area. There was no effect of age on performance and at least in the first 2 probe trials, the young treated mice had higher percent time than any other groups. During the second week, these differences went away. Across all 9 sessions, an ANOVA revealed only a main effect of Session (p<0.001) while all main effects and interactions did not reach significance (all ps >0.052). Across the first 2 sessions (corresponding to the steep learning phase), an ANOVA revealed a main effect of Diet (p=0016), and significant interaction of Session with Age and with Diet (all ps <0.005). When tested one week later for retention, there was no effect of age or diet (all ps>0.05)
3.7 Immunoblot Analysis

3.7.1 Determination of ASIC1 and 2a Expression

The effects of age and diet on expression of ASIC1 and ASIC2a in homogenates of six different brain regions are shown in Figures 10 – 13. Noticeably, the level of expression of ASIC1 and 2 were different depending on the brain regions. ASIC1 expression seemed highest in the hindbrain > cortex > cerebellum = hippocampus = midbrain = striatum, while ASIC2a expression seemed highest in the cerebellum > striatum > hindbrain > cortex > hippocampus (the 65kD band was not detectable in the midbrain).

With the exception of the old treated mice having lower expression of ASIC1 in the hippocampus, there was no major effect of age or diet on expression of ASIC1 or ASIC 2a. A two-way ANOVA yielded neither significant main effects of Diet, Age nor an interaction of Age and Diet (all ps>0.207). It should be noted that the trends observed with ASIC1 were the same with ASIC2a, most notably an increased expression in the cerebellum of treated mice and a decreased expression in the cortex of treated mice, regardless of age.

3.7.2 Determination of NMDAR1 Expression

The effects of age and diet on NMDAR1 expression in homogenates from brain regions are shown in Figures 14 – 15. The expression of NMDAR1 was region dependent with the highest expression in the cortex > striatum > hippocampus > midbrain = cerebellum > hindbrain. The effects of age and treatment were also region dependent with hindbrain and midbrain being unresponsive to either. In the cerebellum, the expression of NMDAR1 was higher in old mice than in the young ones, and the treated mice had lower expression than their age-matched
controls. A two-way ANOVA revealed significant main effects of Age and Diet (all ps<0.032) but no interaction between Age and Diet (p=0.612). Age effects were also observed in the cortex and striatum however aged mice had lower expression of NMDAR1, supported by main effects of Age following a two-way ANOVA (all ps <0.029). In the cerebellum, cortex and hippocampus, treated mice had lower expression of NMDAR1, while it was increased in the striatum of old treated mice. A significant main effect of Diet was only found after two-way ANOVAs in the cerebellum (p=0.032).

3.7.3 Determination of NMDAR2 Expression

The effects of age and diet on relative densities, normalized to actin, on NMDAR2b in homogenized brain region tissues are shown in Figures 16 – 17. The expression pattern of NMDAR2 was as followed: striatum > hippocampus > cortex > midbrain > cerebellum > hindbrain. With NMDAR2 expression was increased in the cortex, and seemed to be decreased in the hippocampus and striatum and remained unchanged in the other regions. Following two-way ANOVAs, a significant main effect of Age was only reported in the cerebellum (p=0.004). Overall, treatment did not affect the level of expression of NMDAR2, except in the cortex of old mice. However, the main effect of Diet or interaction did not reach significance after a two-way ANOVA (all ps>0.081).

3.7.4 Determination of mGluR1 concentration

The effects age and diet on mGluR1 expression in homogenates from brain regions are shown in Figures 18 – 19. The expression pattern was as followed: cerebellum >
midbrain=striatum > hippocampus=hindbrain > cortex. Expression of mGluR1 was increased with age in the cerebellum, decreased in the midbrain and striatum and unchanged in the others. In old mice, treatment was associated with lower expression levels in the cerebellum, cortex, hindbrain and hippocampus. However, two-way ANOVAs yielded only significant main effects of Age for the midbrain and striatum (all ps<0.032).
4.0 DISCUSSION

Our study aimed at determining the effect of induced metabolic acidosis on the brain function of young and old mice, and to identify the mechanisms underlying the observed changes. The main effects of the supplementation with ammonium chloride were: (1) drastic weight decrease in old mice, (2) reversed age-related impairments in motor and cognitive function, (3) improved spatial learning and memory of young mice, and (4) no major alterations in expression of ASICs 1 and 2a, NMDAR1 and 2b, or mGluR.

For this study, we utilized the C57BL/6J mouse model of aging. This model has been has been successfully shown to approximate human aging, particularly with regard to cognitive function assays (49-51). In the current study, the young four-month old mice would be equivalent to a 25-year old human, and the older twenty-month old mice would be equivalent to a 60-year old human. Previous studies on this specific model have reported age-related declines in motor and cognitive function (49-52). Our study reports similar levels of declines with age on all tests performed and consistent with the literature.

Induction of metabolic acidosis was achieved through methods described by Nowick, et al., Tashima, et al., and Hafner et al., resulting in a mild decrease in urine pH (46-48). Hafner et al. noted in an analysis of serum from mice given ammonium chloride supplementation, pH decreased to 7.23±0.03 from a physiological normal of 7.35±0.02. This decrease in serum pH was associated with a decline in urine pH from physiological normal of 6.7±0.14 to 5.7±0.08. In the current study, we noted a decline in urine pH in both young and old mice consistent with that
noted by Hafner et al., indicating an induction of acidosis. However, after one month on the diet the old mice urine pH returned to control levels. It is also noteworthy that the urine pH of older mice is within the acidotic range as defined by Hafner et al. prior to any diet modification or behavioral testing, then subsequently declines further into the acidotic range in the treatment group before returning to previous levels. While the specific mechanism behind the return to basal urine pH in the old mice remains unknown, we speculate that adaptive mechanisms were activated in the younger treated mice to avoid any potential damaging effects of acidosis, yet the older treated mice were less able to adapt over time thereby urine pH increased due to declining kidney function causing an inability to excrete hydrogen ions. Further studies will be required to determine the underlying mechanisms for this observed change in urine pH.

A substantial decline in body weight was observed in the current study in both young and old treated groups with the older group experiencing a more dramatic change than the diet-matched younger mice. This was contrary to what had been observed by Tashima, et al. (47). The reason for this dramatic weight loss remains unclear, particularly when considering that food intake was not affected by the ammonium chloride diet. Further studies will be needed to understand the specific causes for such dramatic weight loss.

Due to the decline in body weight in the older treatment group, consideration of calorie restriction (CR)-like effects on behavior are warranted in the context of this study. Dubey, et al., noted that old CR C57BL/6J mice were 35% more active than their age-matched controls (53). In the current study, distance travelled was 24% higher in the supplemented mice compared to the old controls. Furthermore, Dubey et al. also reported an improvement in the active avoidance performance of the CR mice compared to the old controls. Similarly, in our study, the supplemented mice had better cognitive flexibility than the old controls. It is noteworthy that the
The effect of ammonium chloride supplementation was only observed on cognitive flexibility and not initial learning, suggesting different underlying mechanisms of the two aspects of cognition. Additionally, we speculated that our observed improvement in treading and balancing abilities might be due to the significant weight loss, an ANCOVA was performed and revealed that when weights of the mice are taken into consideration the beneficial effects of the diet disappear. Overall, reversal of age-related declines in motor and cognitive functions might be attributable to an unexpected effect of CR, however more studies will be needed to determine whether the signaling pathways often associated with CR effects are indeed activated under this paradigm.

As previously mentioned, the overall lower urine pH seen in older controls suggests a chronic acidotic condition is already present, therefore, due to this existing condition, we must consider the effects with regard to ASIC desensitization kinetics. Activation of ASICs occur with a rapid decline in extracellular pH, usually at around pH of 5.8-6.2 (38), however studies by Babini et al., Bässler et al., Benson et al., and Gründer et al. observed desensitization without prior activation can occur within a pH range of 7.2-7.3 (22, 25, 32, 33). Additionally, Wemmie et al. observed deficits in cue and conditioned fear response, and spatial learning of ASIC-null mice, indicating that ASIC activation is required for synaptic plasticity in normal fear behaviors (28, 29). Further research by Wemmie et al. showed that ASIC+/− hippocampal neurons had impaired LTP when compared to ASIC wild-type neurons (28). Therefore, within the context of the current study, the learning deficits observed in the older mice with chronic mild acidosis could potentially be due in part to desensitization of ASIC channels causing learning deficits similar to that which was observed in ASIC-null mice. However, if this is the case, then the observed trend toward reversal in older treated mice in some our behavioral assays might not be
due to increased ASIC activation, therefore further research is necessary to elucidate the specific mechanism causing this effect.

The effect of long-term chronic acidosis on neuronal structure such as that which was observed in older mice in this study should also be considered. Acidotic conditions caused by traumatic brain injury, stroke, or seizure activity have been shown to cause neuronal cell death (1, 9, 10). It has been shown that a decrease in ASIC1a expression in hippocampal slices causes a reduction in dendritic spine density, while an increase in ASIC1 causes an increase in spine density through Ca\(^+\)/calmodulin-dependent protein kinase II (CaMKII) signaling (40). Therefore, within the context of this study, we can speculate that the chronic mild acidosis found in old control mice could potentially cause desensitization leading to channel inactivation, which in turn cause a decrease in dendritic spine density in hippocampal neurons through CaMKII pathways.

A study by Coryell, et al. noted that ASIC1a channel activation is necessary for normal fear behavior, and Ziemann et al. found that fear behavior relating to the amygdala depended on pH reduction in the synaptic cleft (54, 55). In our tests of cognitive flexibility and learning, we observed an overall trend toward aversive stimuli avoidance among treatment groups indicating a higher fear response, therefore, we can speculate that the induced acidosis in the treatment groups sufficiently lowered pH in the synaptic cleft for ASIC1a channel activation. These results are also consistent with the previously mentioned studies by Wemmie, et al. regarding ASIC activation and synaptic plasticity in fear conditioning (28, 29, 38, 56, 57). Additionally, in a study of protons as neurotransmitters, Du et al. noted that ASICs as a postsynaptic receptor were critical for learning and memory associated with the amygdala (58). Warden et al. also observed an increase in active behavior of mice in a forced swim test, however no difference was observed
in wild-type mice in behavioral tests with less-aversive stimuli (59). Further speculation by Warden regarding these observed behaviors propose that extreme aversive stimuli cause a more dramatic decline in pH in the synaptic cleft by release of acidic vesicles. These studies would suggest that an increase in extracellular protons by ammonium chloride supplementation might activate those ASICs with higher pH activation kinetics such as ASIC1 homomers, and the aversive stimuli used for spatial learning and active avoidance in the current study additionally activate other ASIC channels with lower pH activation kinetics such as ASIC2 homomers and heteromers (Table 1). According to Wemmie et al., activation of post-synaptic ASICs increase the likelihood of long-term potentiation (LTP) through increased activation of N-methyl-D-aspartate receptors (NMDAR) (28, 29). Our observation that older treated mice were better able to learn the task than their age-matched peers is consistent with both Warden and Wemmie, however if chronic mild acidosis causes a steady-state desensitization/inactivation of ASIC in older mice, then an additional increase in proton concentration would not effectively activate these channels and other means of learning must be considered. However, if the chronic mild acidosis does not effectively cause desensitization, then we could speculate that the observed learning behavior in older treated mice was due in part to ASIC activation.

In contrast to the previously mentioned findings of Wemmie et al., Wu et al. observed that mice lacking ASIC1a had normal performance in hippocampal dependent spatial memory, therefore indicating that ASIC activation is not required for LTP (41). He also noted that post-synaptic ASIC activation does not play a role in NMDA receptor activation and would therefore not involved in reversal of learning deficits observed in the older treatment group. However, Chiang et al. found that baso-lateral amygdala (BLA) glutamatergic synapses required ASIC activation for formation of fear memories (60). Therefore, when considering the results
observed in the current study with regard to spatial learning in the younger treatment group, an increase in ASIC activation may or may not lead to an activation of glutamatergic receptors that contribute to LTP. If ASIC activation leading to an increase in LTP does not occur in the ammonium treated groups, then other mechanisms must be considered. This would also explain our findings of a trend toward a reversal of behavior in older treated mice. If, in fact, desensitization leading to inactivation of ASICs has occurred, alternative pathways leading to LTP would be necessary to explain the observed reversal of learning behavior.

Current immunohistochemical studies have found that ASIC1 and 2 have overlapping expression which is inconsistent among brain regions (28). Higher concentrations of both ASIC1 and 2 homomers can be found in areas of the brain associated with synaptic plasticity such as the hippocampus, as well as in areas with high excitatory synaptic activity such as the amygdala (27-29). In the current study, we found that expression of ASIC1 seemed highest in hindbrain, followed by cortex, and cerebellum. Expression in the hippocampus, striatum, and midbrain was the lowest of all brain regions, which is in contrast to what had previously been reported. For ASIC2a, we observed a higher expression in the cerebellum, an almost equal expression in the striatum, followed by hindbrain, cortex, and hippocampus, which is again inconsistent with previously published studies. Older treated mice were found to have lower expression of ASIC1 in the hippocampus when compared to age-matched controls, therefore our previous speculation with regard to a reversal of learning behavior potentially being due to activation of alternate learning pathways rather than ASIC’s would be substantiated. A trend toward greater expression of both ASIC1 and 2a was observed in the cerebellar region of treated mice, as well as a decrease in cortical tissue. This would be consistent with our findings of decreased latency in alley turning and an increase in latency to tread through increased activation of ASIC causing an
increase in reflex activity but not learning behaviors. Whether these results represent a true cell surface expression of functional trimeric units is unknown. The methods utilized in the current study were not able to differentiate between functional channels and internalized monomers because of whole-cell tissue preparation techniques, therefore additional study with techniques specific to cell surface proteins is warranted.

Acidification has also been found to inhibit activation of NMDA receptors (NMDARs), and deletion of ASIC’s reduces the ratio of AMPA:NMDA in in vitro hippocampal cultures (61). Additionally, activation of ASIC1a could alleviate the voltage-dependent Mg\(^{2+}\) blockade and therefore activate the NMDA receptor to facilitate LTP (42, 43). With that in mind, we analyzed these receptors in the same tissues as ASIC1 and 2a to determine what effects, if any, chronic acidosis might have. NMDA expression in the cerebellum increased in older mice compared to diet-matched younger mice. This is inconsistent with our observations of an age-effect in the behavioral assays. However, we also noted a significant reversal in expression of NMDAR1 in older treated mice when compared to age-matched controls which is consistent with the previously mentioned studies of the effects of acidification and inhibition of NMDARs, but does not explain our observations of decreased reflex latencies and increased balancing latencies in the older treated mice. In the cortex, we observed a decrease in NMDA expression in the older mice compared to younger mice, with the older treatment group being more significantly declined when compared to age-matched controls. This is once again inconsistent with our behavioral observations with regard to reflex, orienting, and executive function, however it is consistent with the previously mentioned literature. In the hippocampal region, there was a trend toward a decrease in NMDA expression among the treatment groups, and an additional decrease in NMDAR2b expression in older mice when compared to younger mice. The overall decline in
expression in treatment groups once again is consistent with previous literature, however is inconsistent with our findings especially with regard to the more steep learning observed in the young treatment group in path length during session one of the Morris Water Maze.

The metabotropic glutamate receptor 1 (mGluR1) is a G-protein coupled and a major excitatory receptor involved in learning and memory behaviors, therefore we also analyzed what effects acidification might have on its expression. We found a decrease in expression in the midbrain of older mice versus younger mice. This would be consistent with the observed decline in spatial learning and memory in older mice versus diet-matched younger mice. A trend toward reversal of expression was also observed in older treated mice is also consistent with the observed results with regard to a decrease in path length and a corresponding reversal in learning index when compared to age-matched controls. Additionally, a decrease in expression was also noted in the striatum of older mice which also corresponds to our finding of age-effects in our behavioral assays.

Based on the observations in the current study, we speculate that the decrease in hydrogen proton excretion in the urine of older untreated mice corresponds to a decrease in serum pH that desensitize ASIC’s and inhibit NMDA activity thus reducing cognitive function as compared to young untreated mice. The mild decline in NMDA expression seen in the older treated mice is inconsistent with the reversal of behavior observed, however we speculate that the increase in serum protons may have increased activity of ASIC channels that require a lower pH for activation, such as heteromeric ASIC1a/2, thus further activating the remaining voltage-dependent NMDA receptors that are blocked by Mg⁺ at higher pH. If this is speculation is true, ASIC activation would be beneficial for improvement in motor and cognitive function in older treated mice. However, the observed ASIC expression in the current study did not support this
theory, and future studies with additional immunohistochemical techniques would be better suited to investigate ASIC expression in specific brain regions which might further clarify these results.

In conclusion, we found that induction of mild chronic acidosis by ammonium chloride supplementation drastically decreased weight in old mice, reversed age-related impairments in motor and cognitive function, and improved spatial learning and memory of young mice. However, we observed no major alterations in expression of ASIC1 or 2a, NMDAR1 or 2b, or mGluR. Whether ASIC activity contributed to these cognitive and learning behavior effects are unclear, therefore future studies are warranted to elucidate the mechanisms causing these effects.
5.0 REFERENCES

1. Espay AJ. Neurologic complications of electrolyte disturbances and acid-base balance [Internet]; 2014 [cited 18 May 2015].


37. Tikhonova TB, Nagaeva EI, Barygin OI, Potapieva NN, Bolshakov KV, Tikhonov DB. Monoamine NMDA receptor channel blockers inhibit and potentiate native and recombinant proton-gated ion channels. Neuropharmacology. 2015;89:1-10.


40. Zha X-, Wemmie JA, Green SH, Welsh MJ. Acid-sensing ion channel 1a is a postsynaptic proton receptor that affects the density of dendritic spines. Proc Natl Acad Sci U S A. 2006;103(44):16556-61.


57. Wemmie JA, Zha X-, Welsh MJ. Acid-sensing ion channels (ASICs) and pH in synapse physiology. Structural and Functional Organization of the Synapse. 2008:661-81.


60. Chiang P-, Chien T-, Chen C-, Yanagawa Y, Lien C-. ASIC-dependent LTP at multiple glutamatergic synapses in amygdala network is required for fear memory. Scientific Reports. 2015;5.

Figure 1 – Effect of age and diet on body weight (A) and on urine pH (B) throughout the study in male C57BL/6J mice. Each value represents the mean ± SEM (n=8).
Figure 2 - Effects of age and diet on spontaneous activity as measured by total distance traveled (A), rearing activity (B) and time spent in the center of the box (C) of young and old male C57BL/6J mice. Each value represents the mean ± SEM (n=7-8). * p<0.05 compared to diet-matched young.
Figure 3 – Effects of age and diet on musculoskeletal reflexes measured as latency to initiate walking (A), to turn in a dead-end alley (B), and to turn 90 degrees (C) in young and old male C57BL/6J mice. Each value represents the mean ± SEM (n=7-8). * p<0.05 compared to diet-matched young.
Figure 4 – Effects of age and diet on musculoskeletal reflexes measured as ability to tread on a wire (A), and latency to fall (B) in young and old male C57BL/6J mice. Each value represents the mean ± SEM (n=7-8). * p<0.05 compared to diet-matched young. # p<0.05 compared to age-matched control.
Figure 5 – Effects of age and diet on bridge walking performance measured as latency to fall in male C57BL/6J mice. Each value represents the mean ± SEM (n=8). * p<0.05 compared to diet-matched young. # p<0.05 compared to age-matched control.
Figure 6 – Effects of acidic diet on learning (Session 1) and cognitive flexibility (Sessions 2 & 3) of young and old male C57BL/6J mice. Each value represents the mean ± SEM (n=7-8). * p<0.05 compared to diet-matched young. # p<0.05 compared to age-matched control.
Figure 7- Effects of age and diet on spatial learning and memory as measured by path length taken to reach a hidden platform (A) and speed (B) in male C57BL/6J mice. Each value represents the mean ± SEM (n=7-8).
Figure 8 – Effects of age and diet on the learning index as a measure of path length average performance for sessions 2, 3, and 4 in Morris Water Maze tests in young and old male C57BL/6J mice. Each value represents the mean ± SEM (n=8). * p<0.05 compared to diet-matched young. # p<0.05 compared to age-matched control.
Figure 9– Effects of age and diet on the performance of male C57BL/6J mice during the first session. Each value represents the mean ± SEM (n=7-8).
Figure 10 – Effects of age and diet on spatial bias during probe trials as measured by the percentage of time spent in the 40-cm perimeter around the hidden platform in male C57BL/6J mice. Each value represents the mean ± SEM (n=8).
Figure 11 - Effects of age and diet on ASIC1 expression in central nervous system tissue of male C57BL/6J mice. Representative columns (A) young control, (B) young treatment, (C) old control, (D) old treatment at ~ 60 kDa.
Figure 12 – Effects of age and diet on ASIC1 expression in central nervous system tissue of male C57BL/6J mice. Each value represents the mean ± SEM (n=4-6). # p<0.05 compared to age-matched control.
Figure 13 - Effects of age and diet on ASIC2a expression in central nervous system tissue of male C57BL/6J mice. Each value represents the mean ± SEM (n=4-6). Representative columns (A) young control, (B) young treatment, (C) old control, (D) old treatment at ~ 65 kDa.
Figure 14 – Effects of age and diet on ASIC2a expression in central nervous system tissue of male C57BL/6J mice. Each value represents the mean ± SEM (n=4-6).
Figure 15 – Effects of age and diet on NMDAR1 expression in central nervous system tissue of male C57BL/6J mice. Each value represents the mean ± SEM (n=4-6). Representative columns (A) young control, (B) young treatment, (C) old control, (D) old treatment at ~ 120 kDa.
**Figure 16** – Effects of age and diet on NMDAR1 expression in central nervous system tissue of male C57BL/6J mice. Each value represents the mean ± SEM (n=4-6). * p<0.05 compared to diet-matched young.
Figure 17 – Effects of age and diet on NMDAR2b expression in central nervous system tissue of male C57BL/6J mice. Each value represents the mean ± SEM (n=4-6). Representative columns (A) young control, (B) young treatment, (C) old control, (D) old treatment at ~ 190 kDa.
Figure 18 – Effects of age and diet on NMDAR2b expression in central nervous system tissue of male C57BL/6J mice. Each value represents the mean ± SEM (n=4-6). * p<0.05 compared to diet-matched young. # p<0.05 compared to age-matched control.
Figure 19 – Effects of age and diet on mGluR1 expression in central nervous system tissue of young and aged male C57BL/6J mice. Each value represents the mean ± SEM (n=8).

Representative columns (A) young control, (B) young treatment, (C) old control, (D) old treatment at ~ 145 kDa.
**Figure 20** – Effects of age and diet on mGluR1 expression in central nervous system tissue of young and aged male C57BL/6J mice. Each value represents the mean ± SEM (n=8). * p<0.05 compared to diet-matched young.