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The Characterization Of Pb2+ Toxicity In Rat Neural Development: An Assessment Of Pb2+ Effects On The Gaba Shift In Neural Networks And Implications For Learning And Memory Disruption

Lorenz Simon Neuwirth
Graduate Center, City University of New York

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THE CHARACTERIZATION OF Pb2+ TOXICITY IN RAT NEURAL DEVELOPMENT: AN ASSESSMENT OF Pb2+ EFFECTS ON THE GABA SHIFT IN NEURAL NETWORKS AND IMPLICATIONS FOR LEARNING AND MEMORY DISRUPTION

By

LORENZ SIMON NEUWIRTH

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2014
This manuscript has been read and accepted for the Graduate Faculty in Biology subprogram neuroscience in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy

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THE CITY UNIVERSITY OF NEW YORK
Abstract

THE CHARACTERIZATION OF Pb2+ TOXICITY IN RAT NEURAL DEVELOPMENT: AN ASSESSMENT OF Pb2+ EFFECTS ON THE GABA SHIFT IN NEURAL NETWORKS AND IMPLICATIONS FOR LEARNING AND MEMORY DISRUPTION

By

LORENZ SIMON NEUWIRTH

Adviser: Professor Abdeslem El Idrissi, Ph.D.

The toxic effects of Pb$^{2+}$ on the developing rat nervous system has been investigated to assess early developmental GABAergic disruption and its implications with altering inhibitory learning and memory. This goal was achieved using a multi-systems approach: blood lead levels (clinical physiology), qRT-PCR (molecular genetics), brain and primary neuronal culture immunology (immunohistochemical and cellular approaches), physiological cellular components (synaptosomes and protein expression) and finally through learning and memory assessment with GABA mimetic drug manipulations in the intact animal (behavioral pharmacology). The influence of a 956ppm Pb$^{2+}$ gestational diet (i.e. from birth to sacrifice) resulted in pup mean blood lead levels (BLLs measured in μg/dL) (Range 28-47) and Dams (Range 33-51) respectively. In contrast, control pups and dams were Pb$^{2+}$ negative. These ages were selected to determine neurodevelopmental trajectories of the GABA-shift from excitation-to-inhibition postnatally in our model. qRT-PCR studies evinced a delay in mRNA expression regulating GAD 80, 65, CACAN β3, GABA$_{AR}$ and were differentially regulated cortex and hippocampus as a function of age in response to Pb$^{2+}$. Brain slice immunohistochemistry revealed an early shift of KCC2 expression in both cortex and hippocampus. Notably, these alterations were differentially regulated by age, brain region and subcellular circuitry within structures (i.e. DG vs. CA3). Neuronal cultures revealed that in response to Pb$^{2+}$ at low micro molar concentrations
induced VSCC-β3 nuclear translocation and GABA<sub>AR</sub> upregulation. KCC2 expression was inhibiting in cultures by Pb<sup>2+</sup>. Synaptosomal effects of Pb<sup>2+</sup> revealed altered glutamate accumulation and handling with increased spontaneous and decreased evoked release in significantly modulated by Pb exposure suggesting altered brain synaptogenesis. Pb<sup>2+</sup> exposure resulted in increased binding suggesting post synaptic modification in cortex and hippocampus increasing brain excitability. Behaviorally, Pb<sup>2+</sup> exposure resulted in increased anxiety, impulsivity, stress, and disrupted learning and memory regulated by inhibitory circuits that were recovered with taurine, a GABA<sub>AR</sub> agonist, administration. Specifically, Pb<sup>2+</sup> disrupted contextual and auditory associative learning. Taken together, these results suggest that Pb<sup>2+</sup> interferes with early VSCCs and GABA<sub>AR</sub> synergistic action that establishes GABAergic neural networks and in turn produces increased brain excitability and over reactivity as a consequence of reduced inhibition.
Dedication

I would like to dedicate this work to my beloved father, Lorenz J. Neuwirth. For without his love, support, teaching, moral and ethical values that were instilled in him since immigrating from Düsseldorf, Germany, I would have never further pursued my education. Unfortunately, my father was unable to witness the accomplishments I have made due to his passing on January 10th, 2012. He will forever be missed and never forgotten. May he continue to rest in peace and watch over my family; especially my mother. This work is also dedicated to my beloved mother. If it weren’t for her tireless sacrifices, unselfish acts, and abilities to adapt through some of the most trying times emigrating from Vieux Fort, St. Lucia, I may not have had as many fortunate opportunities throughout my youth and young adulthood establishing the person I am today. Wherever she is I pray that she remains in good health, stature, and continues to enjoy the simpler things that life has to offer. In addition, I would like to dedicate this dissertation to my sister, Renata, my nephew, Shaun, my brother, Michael, and to my dearly loved wife, Angela DiTomasso. If it weren’t for Angela I would have never been able to muster up the strength daily to continue with my Ph.D. Moreover, I am eternally grateful for the tolerance she had for me and the acceptance for the endless hours away from one another during this endeavor; especially as newlyweds.
Acknowledgements

I would like to thank the many individuals who have contributed invaluably to my doctoral education. Dr. Theodore I. Lidsky became a true inspiration to me during my undergraduate education and more importantly became a genuine friend. He introduced me into the world of lead toxicology, behavioral neuroscience and electrophysiology. I valued his ability to see past my educational marks and his nurturing of my critical thinking. He will forever be a dear friend of mine. I thank both Dr. Probal Banerjee and Dr. Ekkehart Trenkner for the many discussions on my early education and encouraging me to continue towards a Masters’ degree and pursue a doctorate. I would like to also acknowledge Dr. Joshua Wallman's contribution to my education with his enthusiastic teachings of cellular biophysics. I never found electrophysiology more interesting until I experienced his lectures and translation of the content. Dr. Joshua Wallman unfortunately passed away ~ two years ago. He will be dearly missed. I would like to acknowledge Dr. Edward Meehan, for his discussions and feedback regarding behavioral psychology and the principles of learning and behavior with respect to the methods I employed in my dissertation. I am grateful for Dr. Robert Freedland's many discussions on the statistics employed in my dissertation and the ongoing financial support through the Center for Developmental Neuroscience and The New York State Institute for Basic Research. I would like to formally thank and acknowledge The CUNY Graduate Center for their most generous Science Fellowship that offset the financial cost of my degree over the last five years, whilst providing me an invaluable opportunity in teaching undergraduate education at the professoriate level. This experience has offered me an invaluable insight into academia and research which I will pursue as a career beyond my doctorate.
Dr. Gail Smith contributed invaluably to my education by supporting minority students through The Alliances for Graduate Education and the Professoriate (AGEP) CUNY initiative. If it weren’t for Dr. Gail Smith's efforts in maintaining such a resourceful program I would not have secured two NSF summer research Grants supporting my dissertation work. Mrs. Evans Green I am especially grateful to for her support over the many years. I was an entry level minority student at The College of Staten Island’s Science and Technology Entry Program (STEP) in the 6th grade where I first met her. Years later, I was reunited with her through The College of Staten Island’s Collegiate Science and Technology Entry Program (C-STEP). Through STEP and C-STEP I was fortunate to become part of a wonderful networking opportunity and another form of financial assistance in supporting my doctoral research as a minority student. I would like to thank Dr. Claude Brathwaite for his ongoing support, encouragement, and being hard on me throughout the years to complete my Ph.D. If it weren’t for him and The Louis Stokes Alliance for Minority Participation (LSAMP) program I would not have been exposed to the many prospective career networks that I currently have today. In addition, the LSAMP funding contributed significantly to my dissertation work and permitted me access to many forums to present my research both local and abroad. I have continued to have a remarkable relationship with him over the years throughout my Ph.D.

As for my committee members, Dr. Andrzej Wieraszko became a very close mentor to me over the years. He encouraged me to strive more and approach questions from many angles in science. His impartial character and his focus on developing my methodological approaches in synaptosomal and hippocampal slice physiology were invaluable. Dr. William J. L’Amoreaux was a very influential person in my early career. He never stopped criticizing my work, thinking, research designs, and especially my writing. He always made me strive longer and
harder than any other doctoral student in approaching my dissertation. He truly taught me to fail, accept the situation, and problem solve in order to experience success. His discussions and feedback with regard to my immunological experimentation and microscopy has resulted in this body of work. I would like to thank both Dr. Jay S. Schneider and Dr. Theodore I. Lidsky for their inspiration to work in the field of lead toxicology and their invaluable commentary and expertise with aligning my dissertation and its relationships to clinical correlates of human lead toxicity. They both have afforded me tremendous opportunities that I am forever grateful. I would like to thank Dr. George C. Wagner for his invaluable insight into neurotoxicology and his suggestions to the many methods I employed in the behavioral pharmacology aspect of my dissertation. His commentaries on my behavioral neuropharmacological methods were critical in developing my thinking and research designs during my dissertation. I would like to thank Dr. Chang-Hui Shen for his willingness and invaluable opportunity to teach me qRT-PCR and the intricate concepts behind such a powerful technique in molecular biological science. I am very grateful to his open door policy within his lab and cherish the relationship we built over such a short time.

I cannot forget the many doctoral students that became close friends over the years: Dr. Jonathan Blaize, Dr. Janto Tachjadi, Dr. Sarah Guariglia, Dr. Susan Briffa-Mirabella, Dr. Ahmed Abouelela, Dr. Amit Mogha, Dr. Kelly Levano, Dr. Christopher Corbo, Dr. Latifa Boukarrou, Dr. Salim Bendaoud, Alex Marsillo, Daniel Kerr, Brian Iskra, Simon Ng, Nicholas Volpe, Evelyn Okeke, Navita Madan, Alyssa Ferraro, and my dearest of friends Francoise Sidime and Dr. Xin Yan. I am especially grateful for the assistance, support, and aid of the animal vivarium staff: Joanne, Ana, Alyssa, David, Matt, Lisa, Pia, and Sarah for taking excellent care of my animals during my dissertation. I am very grateful for Ms. Angela Cartmel for her IACUC
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Lastly, I am eternally grateful to my mentor and dear friend Dr. Abdeslem El Idrissi. As I look back and reflect over the years I would not be able to imagine myself where I am today if it were not for his genuine investment in my education and me. I am especially honored that he was so understanding of my families’ situations with my father’s passing and my mother’s hardships following this unfortunate circumstance. In many ways, he may not have realized it, but I looked up to him in many paternal ways throughout my master’s and doctoral education. Having come from a family of minimally educated relatives, it is very hard to find a genuine role model who will invest into you their lifelong love and passion of their career in science. I have never met a more inspirational man who would give nearly everything of himself for the betterment of others. He has not only inspired me, but further has been an immovable cornerstone to my education. His tireless commitment to push me to be better, wiser, concise, more technical, and especially independent has developed not only my academic character, but the young scientist that I am today. I cannot thank Dr. Abdeslem El Idrissi enough for everything he has done to aid me, keep me at times in check, and notably tolerate me as his graduate student over the years. I have truly learned an invaluable amount of science and life lessons from him, but also regarding collegial relationships and of myself through this doctoral
experience. He will remain as a close friend for the many years to come and a future research collaborator.

A final thank you is rightfully deserved for the many animals whose sacrifices resulted in this body of work that has contributed to the ongoing understanding of lead toxicity on neurodevelopment and GABAergic disruptions. I hope that their contributions results in a new area of focus within the field of lead toxicity with hopes of finding translational approaches to treat and/or ameliorate this environmentally induced neurodevelopmental disorder.
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ABBREVIATIONS

Pb$^{2+}$ lead
Ca$^{2+}$ calcium
Ca-ATPase calcium-adenosine triphosphate enzyme pump
IQ intelligent quotient
BLL’s blood lead levels
CNS central nervous system
USEPA United States Environmental Protection Agency
LTP long term potentiation
cAMP cyclic adenosine monophosphate
AC adenyl cyclase
CDC center for disease control and prevention
PNS peripheral nervous system
CNPase 2', 3'-cyclic-nucleotide 3'-phosphodiesterase
K$^+$ potassium
PKA protein kinase A
PKC protein kinase C
VSCCs voltage sensitize calcium channels
NMDA$_{Rs}$ N-methyl-D-aspartate receptors
Mg$^{2+}$ magnesium
Na$^+$ sodium
CREB cyclic AMP response element binding protein
MAPK mitogen activated protein kinase
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>CAMKII</td>
<td>Ca^{2+}/calmodulin-dependent kinase</td>
</tr>
<tr>
<td>CAMKIV</td>
<td>Ca^{2+}/calmodulin-dependent kinase-IV</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular-regulated kinases</td>
</tr>
<tr>
<td>NKCC1</td>
<td>Na+-K+-Cl-Cl-cotransporter</td>
</tr>
<tr>
<td>KCC2</td>
<td>K+-Cl-Cl-cotransporter</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>chloride</td>
</tr>
<tr>
<td>GDPs</td>
<td>Giant Depolarization Potentials</td>
</tr>
<tr>
<td>AMPARs</td>
<td>$\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
</tr>
<tr>
<td>Ach</td>
<td>acetyl choline</td>
</tr>
<tr>
<td>LTD</td>
<td>long term depression</td>
</tr>
<tr>
<td>CA</td>
<td>Cornu Ammonis</td>
</tr>
<tr>
<td>Efcab1</td>
<td>extracellular face hand calcium binding domain</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain derived neurotrophic factor</td>
</tr>
<tr>
<td>DREAM</td>
<td>downstream regulatory element antagonist modulator</td>
</tr>
<tr>
<td>CCAT</td>
<td>Ca^{2+} channel associated transcriptional regulator</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamate decarboxylase</td>
</tr>
<tr>
<td>LE</td>
<td>Long Evans Norwegian hooded rats</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>Lux</td>
<td>luminescence</td>
</tr>
<tr>
<td>OF</td>
<td>open field test</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>EPM</td>
<td>elevated plus maze</td>
</tr>
<tr>
<td>LD</td>
<td>light/dark test</td>
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<tr>
<td>HB</td>
<td>hole board test</td>
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<tr>
<td>CFC</td>
<td>context fear conditioning test</td>
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<tr>
<td>ACFC</td>
<td>auditory cued fear conditioning test</td>
</tr>
<tr>
<td>ITI</td>
<td>inter-trial-interval</td>
</tr>
<tr>
<td>Acq</td>
<td>acquisition learning</td>
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<tr>
<td>Retent</td>
<td>retention learning</td>
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<tr>
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<tr>
<td>ASR</td>
<td>acoustic startle response test</td>
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<tr>
<td>dB</td>
<td>decibel</td>
</tr>
<tr>
<td>ITI</td>
<td>inter-trial-interval</td>
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<tr>
<td>PPI</td>
<td>pre-pulse inhibition</td>
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The Characterization of Pb$^{2+}$ Toxicity in Rat Neural Development:
An Assessment of Pb$^{2+}$ effects on the GABA shift in neural networks and implications for learning and memory disruption.

1.0 - Background and Significance:

1.1-Blood lead levels and their relation to neurotoxicity

Lead (Pb$^{2+}$) is an antiquated poison with resourceful malleable metallic properties, yet its toxicity has been reported to result in various neurological disruptions, with particular sensitivity in the cortex, hippocampus, and cerebellum (Finkelstein et al., 1998). Goldstein (1992) first hypothesized that “Pb$^{2+}$ may negatively affect early synaptogenesis disrupting early brain plasticity during critical stages of development.” Children are most vulnerable to Pb$^{2+}$ neurotoxicity due to an increase in absorbed lead via respiration and/or ingestion. In contrast, dermal absorption is rare and toxicity benign. Sixty percent of absorbed Pb$^{2+}$ is cleared in urine via filtration in the kidneys (Chisolm et al., 1991). Absorbed Pb$^{2+}$ enters the blood stream, 95% is transported by erythrocytes and distributed throughout the bodily organs; whereas 5% remains in the plasma (Lidsky and Schneider, 2003). The half-life of Pb$^{2+}$ in blood is ~ 35 days, ~ 2 years in brain, and dependent upon the size and amount of calcium (Ca$^{2+}$) accumulation in bone, it can remain circulating for decades (Lidsky and Schneider, 2003). In addition, Pb$^{2+}$ is transported into the brain by calcium-adenosine triphosphate enzyme pumps (Ca$^{2+}$-ATPase) located on the endothelial cells that comprise the blood brain barrier (Kerper and Hinkle, 1997a). This route of brain lead deposition suggests an early susceptibility for children to be at increased risk for neurotoxicity during critical stages of brain development altering growth, myelination, synaptogenesis, and inhibitory regulation.

Chelation therapy is an invaluable preventative measure for lowering Pb$^{2+}$ toxicity. However, it fails to address the neurological sequelae associated with childhood Pb$^{2+}$ poisoning (Toscano and Guilarte, 2001) due to continuous need for invasive procedures that children find aversive. Moreover, it does not prevent mobilization of skeletal Pb$^{2+}$ stores that can persist years following exposure (e.g. the femur has a duration of Pb$^{2+}$ mobilization from bone over a course of ~30 years post initial exposure) (Gulson et al., 2003). Moreover, Pb$^{2+}$ exposed mothers
bearing children can produce increased toxicity to their children by \(\text{Pb}^{2+}\) ability to enter the placenta (Hu and Hernandez-Avila, 2002). Children chronically exposed to \(\text{Pb}^{2+}\) endure brain damage as evidenced by reductions of 0-5 intelligent quotient (IQ) scaled points for every increase in 10\(\mu\)g/dL of \(\text{Pb}^{2+}\) concentration in blood (Lidsky and Schneider, 2006; Bellinger, 1995). The inverse relationship between high blood lead levels (BLLs) and decreased IQ points is well documented. Emergent chelation therapy is advised when BLLs reach and/or exceed 39 \(\mu\)g/dL; which may have already caused irreparable IQ deficits ranging from 15-20 points (Lidsky and Schneider, 2006; Bellinger, 1995) depending on the initial \(\text{Pb}^{2+}\) concentration and duration of exposure. Moreover, reduced IQ scores induced by \(\text{Pb}^{2+}\) poisoning are correlated with learning disabilities, hyperactivity, aggression, antisocial behaviors, attention deficit disorders, autism, hearing and speech impediments, and seizure disorders (Bellinger, 1996; Lidsky and Schneider, 2006; Kovatsi et al, 2010; Abayzan et al, 2013). Notably, the higher the \(\text{Pb}^{2+}\) burden the more observable symptomatic conditions were observed.

The body of literature on \(\text{Pb}^{2+}\) toxicity research, in both clinical and animal models, historically focused on alterations in adult cognitive impairment and behavioral sequelae at high BLLs. Silbergeld (1992) initially proposed that although \(\text{Pb}^{2+}\) poisoning causes insults to neural development, biological compensation ensues through as yet described mechanisms, masking observable cognitive and behavioral symptoms throughout youth and adolescence. In addition, these symptoms are revealed later in life by inevitable age-dependent neurodegeneration (Figure 1.). However, given the advancement of technologies and more sensitive measurements for scrutinizing behavioral methods animals will low BLL's can evince behavioral differences indicating that Siblergeld's (1992) model is deemed inaccurate and unspecific when explaining all possibilities relating to \(\text{Pb}^{2+}\) effects on early brain development. Moreover, early brain developmental overgrowth may contribute to masking effects of observable symptoms prior to synaptogenic events, equivocally substantial damage to early brain development will not be masked simply to brain overgrowth as they compromised functional brain mechanisms contributing to behavioral outcomes will produce deficits in action.
Previously, clinical studies in children through employing neuropsychological testing (Lidsky and Schneider, 2006) and perturbations in animal models of postnatal development correlated Pb$^{2+}$-induced impairments in sensory cortex (Wilson et al., 2000) and hippocampal learning and memory (Gilbert et al., 1996; Murphy and Regan, 1998). These animal models are used as valid model systems to investigate the underlying neurobiological consequences of Pb$^{2+}$ and their relationship to IQ. Neurocognitive behaviors that regulate attention, visual-motor reasoning skills, social skills, mathematics, and reading skills are reported to be abnormal in children with BLLs lower than 10µg/dL (Canfield et al., 2003; Lanphear et al., 2000; Lanphear et al., 2005; Wasserman et al., 1997). Nevin (2007) used correlational models between individual’s preschool BLLs with concurrent rates of homicides, crimes, and tetra-ethyl lead gasoline exposure predicting that children chronically exposed to Pb$^{2+}$ would have reduced IQ, fail out of school, become delinquent, increased risk for imprisonment and ultimately result in an increased government financial burden due to leaded gasoline manufacturing.

Estimates by the United States Environmental Protection Agency (USEPA) have calculated that a decline in a single point of a child's IQ results in an added financial burden of $8,346 per poisoned child annually (USEPA, 1997). Summative small IQ deficits can shift an
entire population distribution leftward further increasing government financial aid of a given country (Godwin, 2009) (Figure 2.).

Figure 2. Simulation of governmental financial burden as a function of Pb$^{2+}$ induced IQ reductions in children [Adapted from Godwin, 2009].

In the United States from 1976-1980 it was estimated that 88% of children between 1-5 years old had elevated BLLs $>$10µg/dL (Meyer et al., 2003). By 1997, the percentage of Pb$^{2+}$-exposed children dropped from 88% to 5% (Meyer et al., 2003), and 3% of Pb$^{2+}$-exposed children are concentrated in low income and socially disadvantaged urban areas (Lidsky and Schneider, 2003). Globally, the percent of children requiring similar supports is striking but must be examined with caution (Toscano and Guilarte, 2005) (See Figure 3.).
Interpretation of data from Pb\textsuperscript{2+} research during the last ~60 years is complicated by the use of varied clinical studies with high BLLs that are rare today. The Center for Disease Control and Prevention (CDC) has recommended 10µg/dL (i.e. 0.48µmol/L) as the “level of concern” actionable intervention criteria for pediatric screenings in children (CDC, 1991). Using this criteria, clinical studies reveal brain alterations at levels below 10µg/dL, suggesting even this supposed minimum concentration is not safe (Koller et al., 2004). To further elucidate the molecular mechanisms underlying cognitive disruption at BLLs below 10µg/dL, various animal models have been used (For Review see Silbergeld and Goldberg, 1980; Jason and Kellog, 1980). In response to convincing clinical and animal studies of low BLL’s inducing neurological impairment, the CDC’s Advisory Committee on Childhood Lead Poisoning Prevention (2012) redacted the term “level of concern”, and are now reliant on a “reference level” of 5µg/dL (i.e. 0.24 µmol/L) based on 97.5% of the US population of lead poisoned children in this range.

Inconsistencies from animal studies makes it difficult to establish the effects of Pb\textsuperscript{2+} on parameters used in human subjects. Among the inconsistencies are: a) age (Jet et al., 1997; Singh and Jiang, 1997; Hussain et al., 2000; Zaiser and Miletic, 2000), b) altered Pb\textsuperscript{2+} kinetics in long term potentiation (LTP) [a model of hippocampal learning and memory consolidation] (Gilbert et al., 1996; Gilbert et al., 1999; Hussain et al., 2000, Liu et al., 2000; Zaiser and Miletic, 2000), and c) observable behavior learning deficits (Regan and Keegan, 1990; Rashidy-Pour et al., 1995; Bourjeily and Suszkiw, 1997; Jett et al., 1997). Moreover, inconsistent dose-dependent concentrations of Pb\textsuperscript{2+} have resulted in unrefined animal models (Spence et al., 1985; Minnema and Michaelson, 1986; Strużyńska and Rafałowska, 1994; Gilbert et al., 1999). Effects of Pb\textsuperscript{2+} on animal tissues studied in vitro are also conflicting due to both poor detection methods in overcoming Pb\textsuperscript{2+} precipitation from test solutions (Drew et al., 1989) and gender differences between studies (Vahter et al., 2007). Vahter et al. (2007) reported that young males are more susceptible for Pb\textsuperscript{2+} neurotoxicity in early life, whereas young females are more susceptible to immunotoxic effects of Pb\textsuperscript{2+}. This suggests sex differences being differentially affected in response to Pb\textsuperscript{2+}. These findings elucidate that Pb\textsuperscript{2+} may disrupt specific pathways in the endocrine system with influence towards emotional learning, anxiety, and fear conditioning.
1.2-Pb$^{2+}$ effects on Central Nervous Tissues

To compensate for species differences in extrapolating lead toxicity effects that would most appropriately generalize to human clinical conditions. In vivo studies are advantageous as it permits researchers to identify the effects of Pb$^{2+}$ in a live behaving organism with assessment of the Pb$^{2+}$-induced underlying biological changes and its interaction with environment. Such an approach permits the researcher with the ability to discover drug to treat the induced condition and to subsequently identify the pharmacotherapeutic tolerance of the organism as a bridge to translational biomedicine. However, in vivo studies are limited in that experimental manipulations during real-time physiology are less precise than methods employed in vitro. In vitro methods offer more careful control of manipulating neurochemical and genetic interactions with the complex environment of a simulated intact organism. Notably, not all results produced by well controlled in vitro methods are accurately extrapolated back to a behaving organism.

Research shifted to predominantly animal in vitro approaches to isolate central nervous system (CNS) tissues to investigate direct Pb$^{2+}$ consequences on neuron populations. Oligodendrocytes are most vulnerable to Pb$^{2+}$ exposure (Tang et al., 1996) and their early progenitors far more susceptible to Pb$^{2+}$ disruptions than mature oligodendrocytes (Deng et al., 2001). Suggesting fetal neurons are more vulnerable to Pb$^{2+}$ toxicity than mature neurons. Pb$^{2+}$-induced delayed myelination, hypomyelination, and demyelination of neurons resulting in aberrant and/or absent CNS signaling (Coria et al., 1984). Within the first 2-3 years of postnatal development the brain undergoes massive myelination. Pb$^{2+}$ insults during this critical stage generates abnormalities in CNS myelin sheaths (Dabrowska-Brouta et al., 1999) and Schwann cells in peripheral nervous system (PNS) (Dyck et al., 1977). This malformation of the myelin sheath drastically affects brain structure and function. A major enzyme critical for myelin synthesis (2’, 3’-cyclic-nucleotide 3’-phosphodiesterase (CNPase) has reduced activity in response to Pb$^{2+}$ exposure (Dabrowska-Brouta et al., 2000).

Tiffany-Castiglioni et al., (1989) proposed a 'lead sink' hypothesis suggesting that Pb$^{2+}$ accumulates in astrocytes within the developing and mature brain, indirectly perturbing neurons. Astrocytic Pb$^{2+}$ accumulation is observed in non-mitochondrial sites as a protective mechanism against cellular respiratory processes and surrounding vulnerable neurons (Lindahl et al., 1999). Moreover, evidence from tissue cultures indicate that immature astrocytes are more sensitive to
Pb$^{2+}$ sequestration, yield higher concentrations and retain these levels far after initial exposure in contrast to mature astrocytes (Tiffany-Castiglioni et al., 1989; 2001; Lindahl et al., 1999). Notably, consistent with the 'lead sink' hypothesis, Pb$^{2+}$-storing astrocytes in early development may cause age-related leakage, either by increasing local Pb$^{2+}$ concentrations or inducing focal Pb$^{2+}$ lesions contributing to increased intracranial pressure, edema, and decreased brain volume (Cecil et al., 2008).

Pb$^{2+}$ has a unique function in neurons given its ability to be substituted for calcium (Ca$^{2+}$), producing widespread toxic effects (Bressler and Goldstein, 1999). Studies have characterized molecular pathways and established clear toxic dose dependent thresholds, in response to Pb$^{2+}$/ Ca$^{2+}$ competition (Kerper and Hinkle, 1997b). Taken together, these reports suggest that neurons are extremely sensitive to Pb$^{2+}$ exposure during development, and are more sensitive to lead than other divalent cations. This is consistent with the suggestion that there is no safe neurobiological concentration of Pb$^{2+}$ (Koller et al., 2004).

Pb$^{2+}$ at nanomolar concentrations competes with intracellular Ca$^{2+}$ levels, activating calmodulin and in turn, stimulates protein kinase A (PKA), cyclic adenosine monophosphate (cAMP), phosphodiesterases, and voltage gated potassium (K$^+$) channels (Bressler et al., 1999). Moreover, Pb$^{2+}$ at picomolar concentrations substitutes for Ca$^{2+}$ and increases calmodulin activity, whereas at high concentrations reduces calmodulin activity in neurons resulting in altered intracellular Ca$^{2+}$ homeostasis (Ferguson et al., 2000; Kern and Audesirk, 2000). PKA activation stimulates transcription factors that regulate gene expression. Pb$^{2+}$ may alter gene expression through PKA downstream of adenynyl cyclase (AC) pathways (whose downstream modulator is cAMP), quickly inactivating or prolonging activation through phosphodiesterases altering gene expression.

Moreover, picomolar concentrations of Pb$^{2+}$ in neurons activates protein kinase C (PKC), a major cellular regulator for neuronal proliferation and differentiation that is involved in neuroplasticity via long-term potentiation (LTP) (Bressler and Goldstein, 1991), which is more sensitive than nanomolar concentrations of Ca$^{2+}$ that typically activate PKC (Bressler et al., 1999). Markedly, hippocampal PKC expression has been shown to be reduced at 31.9µg/dL, consistent with the emergent chelation criteria for intervention at BLLs <39µg/dL (i.e. ~ 1.5-1.7µM brain tissue concentration), implicating a clear correlation with increased BLLs and decreases in hippocampal PKC expression that may result in learning and memory disruption.
(Sun et al., 1999; Mazzonlini et al., 2001; Hussain et al., 2000). Together these reports suggest Pb$^{2+}$ competition with Ca$^{2+}$ mishandling in neonatal and adult brains (Singh and Jiang, 1997; Silbergeld et al., 1980). However, with increased focus of Pb$^{2+}$ effects on early CNS developmental consequences, in vivo and in vitro, the precise molecular mechanisms occurring at BLLs below 10µg/dL resulting in neurological sequelae during early development remains to be elucidated.

1.3-L-type VSCCs: Encoding, functioning, and spatial distribution

Ca$^{2+}$ enters the cell via two distinct routes: the voltage sensitive calcium channels (VSCCs) or the glutamatergic N-methyl-D-aspartate receptors (NMDA$_R$s); which are both ligand and voltage-sensitive resulting in widespread changes in gene expression through NMDA$_R$ Ca$^{2+}$ influx. Of the Glutamatergic receptors the NMDA$_R$ have higher selective permeability to Na$^+$ and Ca$^{2+}$; which produce fast activation and slow inactivation channel kinetics. The activation of NMDA$_R$s require large membrane depolarizations (above `50mV), as well as, the binding of glutamate and its co-agonist glycine to remove the magnesium (Mg$^{2+}$) ion depolarization block which resides in the ion channel pore (Gallin and Greenberg, 1995). Once Mg$^{2+}$ is removed, sodium (Na$^+$) and Ca$^{2+}$ influx increase membrane excitability and Ca$^{2+}$-dependent second messenger signals.

In contrast to NMDA$_R$s, VSCCs have selectivity for only Ca$^{2+}$ ions, but vary in their distinct functional membrane excitability, which is regulated by the type of VSCC activated and their subunit configurations. For the purposes of this work we will remain focused on the L-type VSCC. Ca$^{2+}$ enters the VSCCs with very high permeability resulting in fast activation and slow inactivation rates producing very large and precise localization of influx, permitting rapid and efficient interaction with Ca$^{2+}$ specific second and third messenger systems.

Calcium channels are assembled through a variety of subunit configurations exceeding 20 different types in the human genome (Herlitze and Mark, 2005). L-Type VSCCs are comprised of functionally specific subunits $\alpha_1$, $\beta$, $\alpha_2$, $\delta$ and $\gamma$ (See Figure 4).
Figure 4. The L-Type VSCCs main α1 subunit and its β, δ and γ ancillary subunits. Note that the β subunit is the only intracellular target of this channel in which nuclear translocation of the C-terminus has been reported to augment and maintain Ca$^{2+}$ homeostasis in neurons [Adapted from Benarroch, (2010)].

From these arrangements, α1 is the main subunit responsible for Ca$^{2+}$ conductance and bridges the ancillary subunits together forming the channel pore; whereas the remaining ancillary subunits (i.e. β, α2, δ and γ) are responsible for intracellular and extracellular transmembrane VSCC modulation. VSCC subunits encoded by Ca$_{v1.1}$ are expressed in skeletal muscle and cardiomyocytes (Hase et al., 1994; Mejia-Alvarez et al., 1994; Klugbauer et al., 2002). The subunits Ca$_{v1.4}$ are expressed in retinal tissues and dorsal root ganglia (Morgans, 2001; Yusaf et al., 2001). Notably, two particular subunits encoded by Ca$_{v1.2}$ and Ca$_{v1.3}$ mRNA in brain tissue contributes to α1 subunits L-Type VSCC currents (Snutch et al., 1991; Williams et al., 1992).

Of the many brain regions, L-Type VSCCs in the cortex, hippocampus, and cerebellum (Snutch et al., 1991; Williams et al., 1992) linking integrative dendritic and somatic signals to excitation transcription coupling from the membrane to nucleus. An example of this excitation transcription coupling would be L-Type VSCCs and cyclic AMP response element binding protein (CREB) phosphorylation regulating mechanisms for learning and memory through the mitogen activated protein kinase (MAPK) pathway (Atkins et al., 1998, Dolmetsch et al., 2001; Riberio et al., 2005). Consistent with these findings, spatial distribution analyses have revealed that L-Type VSCCs are localized to basal dendrites (Westenbroek et al., 1990) with weaker immunoreactivity in distal arbors (Hell et al., 1993). Notably, gamma-aminobutyric acid
(GABAergic) mouse cortical neurons were observed to lack such spatial distribution patterns of Ca\textsubscript{v1.2} and Ca\textsubscript{v1.3} α1 subunits and instead both were localized to the proximal dendrites and soma. This spatial patterning of the precise location of L-Type VSCCs relative to the soma suggests a close interaction between the L-Type VSCC and GABAergic neurons regulating genes (See Figure 5.).

**Figure 5.** The L-Type VSCCs β3 subunit autoregulation of cell excitability in early immature neurons.

L-Type VSCCs are critical for early immature neurotransmitter release, facilitating specific developmental events that dictate the temporal and spatial expression patterns of Ca\textsubscript{v1.2} and Ca\textsubscript{v1.3} mRNA. This developmental Ca\textsubscript{v1.2} and Ca\textsubscript{v1.3} mRNA expression trajectory may be susceptible for altered expression of the VSCC auxiliary subunits in response to Pb\textsuperscript{2+}.

In studies using regenerated neuromuscular junctions, L-Type VSCC antagonists increase evoked and inhibit spontaneous neurotransmitter release, signifying early involvement of L-Type VSCCs in immature acetylcholine (Ach) neurotransmitter release (Sugiura and Ko, 1997). Furthermore, L-Type VSCCs are localized on early migrating axons along with their growth cones developmentally regulating acetyl choline (Ach) release from immature neurons (Sun and Po, 1987; Zakharenko et al., 1999). We hypothesize, that these findings suggest in early development, immature neurons in distinct brain regions may developmentally express more L-Type VSCCs as a compensatory mechanism when exposed to competitive antagonists, including
Pb\(^{2+}\). These Pb\(^{2+}\) induced perturbations of Ca\(^{2+}\) signaling may alter spontaneous and evoked neurotransmitter release resulting in aberrant synaptogenesis during critical periods of neural development (*For Review See* Rice and Barone Jr., 2000); consistent with Goldstein’s (1992) hypothesis of Pb\(^{2+}\) disrupting synaptogenesis and developmental plasticity.

### 1.4-Pb\(^{2+}\) competition with Ca\(^{2+}\) through L-type VSCCs: Inhibition of neurotransmission

Pb\(^{2+}\) has been shown to compete with Ca\(^{2+}\) influx altering signal processing. In particular the L-type VSCCs are critical for neurotransmission, gene induction, neuronal guidance in early development, synaptogenesis and synaptic plasticity. Pb\(^{2+}\) mechanisms of cellular action have been closely linked to mimetic properties of Ca\(^{2+}\) signal activation (Audesirk and Tjalkens, 2004). Thus, Pb\(^{2+}\) disrupts events that maintain Ca\(^{2+}\) cellular homeostasis. In particular Pb\(^{2+}\) has been shown to inhibit voltage-sensitive calcium channels (VSCCs) (Saliba et al., 2009), disrupting Ca\(^{2+}\) ionic gradient and electrochemical driving forces (i.e. typically 2mM:[Ca\(^{2+}\)]\(_e\) vs. 50-200nM:[Ca\(^{2+}\)]\(_i\)), and inducing aberrant cellular depolarization (Audesirk and Tjalkens, 2004).

Greenberg et al., (1986) discovered that neuronal excitability can regulate gene transcription via the binding of agonists to the *nicotinic acetylcholine receptor* (nAChR), which induced membrane depolarization and subsequent Ca\(^{2+}\) influx through VSCCs increasing *c-fos* proto-oncogene expression in neural cell lines. Interestingly, Pb\(^{2+}\) has been shown to reduce Ca\(^{2+}\) influx through L-type VSCCs via competitive action (Büsselberg et al., 1991; Evans et al., 1991; Tsien et al., 1988) Thus, L-type VSCCs competition with Ca\(^{2+}\) influx Pb\(^{2+}\) can reduce neurotransmission efficiency, alter membrane excitability, and essentially alter gene regulation.

In the cytosol, Pb\(^{2+}\) influx through VSCCs may alter the phosphorylation levels of second messenger-activated systems such as: Ca\(^{2+}/\)calmodulin-dependent kinase (CAMKII), Ca\(^{2+}/\)calmodulin-dependent kinase-IV (CAMKIV), PKC, PKA, MAPK, and extracellular-regulated kinases (ERK) (*For Review See* Toscano and Guilarte, 2005); therefore, indicating a complex Pb\(^{2+}\) disruption of second messenger systems causing early developmental learning and memory disruption. It is noteworthy to consider that if the expression and function of L-type VSCCs are disrupted by Pb\(^{2+}\), then subsequent altered down-stream signaling cascades during early brain development may result in premature or delayed developmental gene expression.

**1.5-Pb$^{2+}$ reduces GABA neurotransmission through inhibiting presynaptic VSCCs**

Of the many Pb$^{2+}$ studies pertaining to learning and memory impairments in animals, glutamate systems have been the major focus (*For Review See* Toscano and Guilarte, 2005) due to the NMDA$_R$s involvement in calcium-dependent LTP as a cellular form of learning and memory (Bliss and Collingridge, 1993). Squire (1992) initially observed that the hippocampus is a fundamental brain region that regulates learning and memory, which further encodes the cellular properties of LTP in its intrinsic circuits. Lasley and Gilbert (2000) suggested that Pb$^{2+}$-depressed LTP in hippocampus, suggesting a cellular correlation of cognitive learning impairments. Notably, these observations failed to report a clear mechanism within the hippocampal circuit responsible for explaining Pb$^{2+}$ disruption in learning and memory. Moreover, these studies solely focused on glutamatergic pathways, whereas GABAergic interneurons provide critical synchronization of distinct neuronal rhythms between the hippocampus and other integrative brain structures essential for learning and memory (Soltesz and Deschenes, 1993; Bragin et al, 1995; Buzaki and Chrobak, 1995). Moreover, NMDA$_R$s, GABA$_A$R$s, and VSCCs collectively drive early brain Ca$^{2+}$ oscillations through giant depolarizing potentials (GDPs) to synchronize brain activity across structures; whereby they shape the timing and tonic phases of excitation-inhibition balancing (Ben-Ari, 2002) prior to the onset of glutamatergic synapse formation in the brain (See *Figure 6.*).
When hippocampal GABAergic activity is desynchronized, the resultant effects may produce seizures due to improper GABA inhibition-to-glutamate excitation ratios. These critical neuronal discharges balances in the hippocampus are disrupted by high BLLs (For Review See Lidsky and Schneider, 2003). Pb$^{2+}$ exposure decreases the threshold for membrane depolarization, making neurons easily excitable, and increases hyper-excitability and potential seizure activity. In a case study by Healy et al., (1984) an infant with Pb$^{2+}$ neurotoxicity suffered seizures associated with their poisoning, in which valproic acid was prescribed to control convulsive activity successfully. Valproic acid enhances GABA synaptic transmission through inhibiting the enzyme responsible for breaking down GABA, GABA transaminase. Thus, valproic acid increased GABA neurotransmission reducing postsynaptic neuronal excitability and increasing seizure thresholds in this child.

Krishnamoorthy et al., (1993) elegantly showed in rats that Pb$^{2+}$ exposure increased seizure onset and induced more severe convulsions induced by selectively blocking the GABA$_{AR}$ with picrotoxin; thereby reducing GABA efficiency and increasing hyperexcitability. Taken together, these studies indicate that the effects of Pb$^{2+}$ exposure on the GABAergic system are

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**Figure 6.** Neurodevelopmental trajectory and GDP critical period illustrating GABAergic neurons preceding the onset of functional glutamatergic synapse formation [Adapted from Ben-Ari et al., (2007)].
equally important with respect to learning, memory, and seizure susceptibility as are the glutamatergic systems. However, the GABAergic system must be carefully considered due to its synapse formation arising prior to glutamatergic systems in early development.

To further support the claim that Pb$^{2+}$ inhibits GABA neurotransmission, nerve terminal preparation studies, otherwise known as synaptosomes, have been widely used to focus solely on transynaptic communication. Ca$^{2+}$ influx through VSCC regulates the liberation of neurotransmitters from presynaptic nerve terminals (Turner et al., 1993). In synaptosomal preparations, Pb$^{2+}$ enters isolated nerve terminals and is further sequestered in mitochondria (Silbergeld et al., 1977). Previously, Regunathan and Sundaresan, (1985) conducted synaptosomal studies under Pb$^{2+}$ treatments and observed reduced postsynaptic glutamate binding and presynaptic uptake from rat cortex, cerebellum, and brainstem.

Jablonska et al., (1994) showed that chronically Pb$^{2+}$-treated rats have decreased GABA uptake, increased dopamine uptake that were independent of Ca$^{2+}$ concentrations, with morphological changes in the synaptic vesicles and mitochondria. Pb$^{2+}$ has been shown to induce biphasic properties where it inhibits depolarization-evoked release (i.e. increasing the neurons threshold to fire specific chemical signals) and increases spontaneous release (i.e. increasing non-specific neurotrophic factors and neurotransmitters) from rat hippocampal synaptosomes (Minnema et al., 1988). These biphasic properties have shown that low concentrations of Pb$^{2+}$ increases spontaneous neurotransmitter release and block depolarization dependent neurotransmitter release across transmitter systems (Goldstein, 1980).

To further elucidate Pb$^{2+}$ toxicity and the relationship between glutamate/GABA-glutamine cycles with respect to neuronal-astrocyte excitability, synaptosomal studies investigated GABA binding release and uptake. Neurons cannot synthesize glutamate or GABA from glutamate decarboxylase (GAD) and must rely on astrocyte uptake through transport proteins to convert these neurotransmitters into glutamine via catabolism of GABA transaminase and succinate-semialdehyde dehydrogenase. Strużyńska and Sulkowski, (2004) showed diminished GABA transport, decreased uptake and depolarization evoked release, lower expression of GAD, the GABA synthesizing enzyme, and over expression of the GABA transport protein GAT-1. Their work implicates another supporting argument for Tiffany-Castiglioni’s (1989) 'lead sink' hypothesis, that Pb$^{2+}$ may disrupt both glutamate and GABA transporters activity and altered neuronal-astrocyte glutamine interactions, reducing GAD levels.
Astroglia are critical for glutamate/GABA-glutamine metabolism and Pb\textsuperscript{2+} disruption of this pathway as may result in cognitive impairments and seizure susceptibility. Importantly, Strużyńska and Sulkowski, (2004) used only rat forebrain preparations, which may not generalize towards region specific differences, but should be interpreted with caution. Spence et al., (1985) showed that acute Pb\textsuperscript{2+} exposure reduced KCl dose-dependent GABA release from spinal cord synaptosomal preparations. Pb\textsuperscript{2+} inhibition of GABA release from synaptosomes (Minnema and Michaelson, 1986; Strużyńska and Rafalowska, 1994) and GABAergic hippocampal neurons in culture (Braga et al., 1999) were also reported. In chronic Pb\textsuperscript{2+} treated synaptosomes, GABA release was altered as a function of dose and duration (Minnema and Michaelson, 1986; Strużyńska and Sulkowski, 2004; Lasley et al., 1999; Lasley and Gilbert, 2002).

Borisova et al., (2011) proposed that presynaptic disturbances induced by Pb\textsuperscript{2+} may cause partial dissipation of the synaptic vesicle proton gradient resulting in decreased VSCC dependent neurotransmitter release, incomplete vesicle filling with transmitter substances, and inhibition of the Na\textsuperscript{+}-dependent glutamate transporter. These results may also explain the increase in spontaneous neurotransmitter release under Pb\textsuperscript{2+} exposure. VSCC inhibition has been well documented as the best reported mechanism for presynaptic inhibition (Giustizieri et al., 2005). Xiao et al., (2006) has shown that Pb\textsuperscript{2+} induces direct inhibition of VSCC in developing hippocampal slices attenuating action potential dependent GABA release as a more precise mechanism for Pb\textsuperscript{2+} neurotoxic cognitive impairments in early development.

1.6-The GABA shift is developmentally regulated by the Ca\textsuperscript{2+}-dependent gene induction of the KCC2-cotransporter

The GABA shift is defined as the developmentally regulated switch between early immature neurons responding to GABA neurotransmission with excitation-to-mature neurons responding to GABA neurotransmission with inhibition (Ben-Ari, 2002). There are two Ca\textsuperscript{2+}-dependent inducible Slc genes (i.e. solute carries that transport molecules across the cell membrane) critical for developmentally regulating the GABA shift: (1) the gene that encodes for the Na\textsuperscript{+}-K\textsuperscript{+}-Cl\textsuperscript{−} cotransporter (NKCC1) is Slc12a2 and (2) Slc12a5 for the K\textsuperscript{+}-Cl\textsuperscript{−} cotransporter (KCC2). These genes arise from a family of electroneutral cation-chloride cotransporters that were originally characterized in Drosophila melanogaster (Sun et al., 2010).
This gene family is responsible for maintaining cell volume regulation, epithelial transport, and GABAergic circuitry (Kahle et al., 2010). The latter sets the stage for appropriate GABAergic and glutamatergic regulation of neural development in organisms.

In the mature brain the two major neurotransmitters, GABA and glutamate, mediate a balance between excitation and inhibition of neuronal networks, respectively. However, in the immature brain the GABAergic system is initially excitatory and precedes the subsequent functional excitatory activity of the glutamatergic system due to a large chloride $[\text{Cl}^-]$, when compared to the $[\text{Cl}^-]_e$ (Ben-Ari, 2002; Ben-Ari et al., 2007) (See Figure 7.).

![Diagram of GABAergic and glutamatergic systems](image)

**Figure 7.** The $\text{Cl}^-$ concentration and electrochemical gradients that maintain early GABAergic excitation and mature inhibition based on the NKCC1 and KCC2 $\text{Cl}^-$ transport proteins [Adapted from Ben-Ari et al., (2007)].

Therefore, presynaptic GABA secreting neurons can excite immature post synaptic neurons expressing $\text{GABA}_{AR}$'s, as long as the threshold is below $E_{\text{Cl}}$ and the driving force of $\text{Cl}^-$ remains at higher intracellular concentrations. This critical developmental process is regulated by synergistic activity between VSCC and $\text{GABA}_{AR}$'s. $\text{Ca}^{2+}$ influx through VSCCs depolarize neurons, activate GABA receptor permitting the efflux of $\text{Cl}^-$ inducing further depolarization.
evidencing synergistic activity between VSCCs and GABA<sub>AR</sub>s. Following VSCC and GABA<sub>AR</sub> synergistic action, GABA neurotransmission excites post synaptic cells, in turn, activating NMDA<sub>R</sub>s. NMDA<sub>R</sub>s facilitate synchronized activity between the Ca<sup>2+</sup> wave oscillations and GDPs; which have been identified as the hallmark principle for developing neuronal networks (Ben-Ari, 2002). The major regulators of the GABA excitation-to-inhibition shift are the KCC2 cotransporters. The shift is a Ca<sup>2+</sup>-mediated process in which KCC2 cotransporters are expressed as a result of VSCC activation secondary to GABA-mediated depolarizations (Ben-Ari, 2002). Thus, it is prudent to evaluate Pb<sup>2+</sup> effects on KCC2 expression during the GABA shift in early development.

The developmental shift in GABA from excitation in immature neurons to inhibition when neurons mature, are developmentally regulated by the expression levels of NKCC1 and KCC2 (Sun et al., 2010) and their subsequent functional activity. In early development NKCC1 cotransporters are highly expressed and very active, while the KCC2 cotransporters have a relatively low expression level and are primarily inactive (Gamba, 2005). As the immature neurons are releasing spontaneous GABA to remain viable and functional they also maintain their cued guidance via neurotrophic factors in conjunction with long-range secretions by their target cells. Once these immature neurons are fully guided to their fate destination they begin to signal GDPs which allow higher conductances of Ca<sup>2+</sup> influx due to the cooperativity from the initially inactivated α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA<sub>R</sub>s) and NMDA<sub>R</sub>s.

To further evidence the KCC2 cotransporters regulating the GABA shift, studies in culture evaluated excitatory GABAergic cells that were injected with KCC2 cotransporter mRNA. The results indicated a shift from GABA excitation-to-inhibition in response to the increase in KCC2 expression. The shift was determined to be a GABA mediated process in which KCC2 cotransporter responded with feedback to the GABA<sub>AR</sub>s (Ben-Ari, 2002). Interestingly, when immature neurons were exposed to GABA<sub>AR</sub> antagonists the GABA shift was prevented, but resulted in a peculiar effect in that KCC2 cotransporters were not affected (Ben-Ari, 2002). Therefore, the KCC2 cotransporter activity and its expression in relation to brain maturation remain to be elucidated. This indicates that the KCC2 cotransporter activity may be regulated by PKA (Gomez-Ospina et al., 2006) through VSCCs (Ben-Ari, 2002) that can
stimulate by Pb$^{2+}$ at nanomolar concentrations (Bressler et al., 1999) prematurely establishing inappropriate neural network signals altering GABA excitation-to-inhibition.

1.7-Pb$^{2+}$ alters immediate-early gene induction via the L-type VSCC

VSCCs maintain their precise spatio-temporal expression and signal functions through principally conserved biophysical properties: (a) they are highly voltage dependent allowing for rapid activation and inactivation of the channel pore which permits Ca$^{2+}$ influx in response to changes in membrane potential, (b) they are highly permeable to Ca$^{2+}$ in contrast to other potential ions, (c) VSCCs must be localized in areas on neurons where they can easily access relevant regulatory targets of Ca$^{2+}$-dependent signaling cascades; thus allowing for rapid localized increases in [Ca$^{2+}$]$_i$, (d) lastly, they must undergo modulation and diverse conformations to permit selective regulation of different classes of VSCCs (Tsien and Barrett, 2005).

Moreover, Pb$^{2+}$ can influx into the cytosol in bovine adrenal medullary cells through L-type VSCCs (Sun and Suszkiw, 1995; Simons and Pocok, 1987). Once Pb$^{2+}$ invades the intracellular cytosol it is able to perturb L-type VSCCs kinetics by increasing I$_{\text{Ca}}$ through attenuation of the calcium-dependent steady state inactivation of the L-type VSCCs (Sun and Suszkiw, 1995). In response to cytosolic Pb$^{2+}$ increasing Ca$^{2+}$ conductance, the subsequent increase in [Ca$^{2+}$]$_i$ results in rapid and prolonged second messenger system activation, and ultimately third messenger gene induction affecting an array of regulatory protein transcription factors such as CREB (Toscano et al., 2003; Toscano et al., 2002), NR1 (Toscano and Guilarte, 2003; Nihei and Guilarte, 1999; Guilarte and McGlothan, 1998), c-fos and jun (Toscano and Guilarte, 2005; Finkelstein, Markowitz, Rosen, 1998), and brain derived neurotrophic factor (BDNF) (Schneider et al., 2001); all of which resulted in altered mRNA expression levels for each of these target genes in response to Pb$^{2+}$ exposure. In addition, internal endoplasmic reticulum stored of Pb$^{2+}$ may be released into the cytosol activating second messenger systems altering gene activity; otherwise, cytosolic Pb$^{2+}$ can activate hormonally regulated G-proteins that stimulate VSCC activity (Nussey and Whitehead, 2001).

More recently, Schneider et al., (2011) investigated the effects of a 30-day postnatal Pb$^{2+}$ diet in the rat and changes in hippocampal gene expression via microarray analyses. They found that Pb$^{2+}$ may differentially affect gender; one gene in male rats, the extracellular face hand
calcium binding domain (Efcab1), had a significant 10-fold increase in response to Pb$^{2+}$ treatment, whereas females exhibited a significant 0.6-fold reduction in hippocampal gene expression (Schneider et al., 2011). Ca$^{2+}$ signaling can be either indirectly activated by Ca$^{2+}$-dependent kinases and phosphatases regulating transactivating properties of transcription factors (Dolmetsch et al., 2001 West et al., 2001; Kornhauser et al., 2002; Spotts et al., 2002) or directly via nuclear Ca$^{2+}$ sensors (Leclerc et al., 2012).

Currently, a Ca$^{2+}$-dependent DNA regulating transcription factor is the downstream regulatory element antagonist modulator (DREAM); derived from the recoverin 4-EF hand subfamily, involved in transcription repression (Carrion et al., 1999; Mellstrom and Naranjo, 2001). Moreover, a unique concept for VSCCs arose, whereby the Ca$^{2+}$ domains may act in a functional way as a self regulating transcription factor, such as the Cav$_{1.2}$ C-terminal fragments; otherwise known as the Ca$^{2+}$-channel associated transcriptional regulator (CCAT) (Gomez-Ospina et al., 2006). The CCAT has been shown to translocate to the nucleus, regulate gene expression, and increase dendritic arborization once activated (Gomez-Ospina et al., 2006).

Schneider et al., (2011) identified 175 genes that were differentially regulated in response to Pb$^{2+}$ and gender further altered expression patterns, which may have resulted from Pb$^{2+}$ alterations in CCAT regulation. NMDA$_{R2A}$ mRNA in the developing hippocampus of gestational Pb$^{2+}$ treated rats (Nihei and Guilarte, 1999), and mRNA NR1 splice variants were reported to variably alter hippocampal expression in all cornu ammonis (CA) regions when compared to controls in response to gestation and developmental Pb$^{2+}$ exposure evidencing altered synaptogenesis (Averil et al., 1980; McCauley et al., 1982; Kawamoto et al., 1984; ) and negatively effecting learning in these rats (Guilarte et al., 2000; Guilarte and McGlothan, 2003).

In primary neuronal cultures, treatment with Pb$^{2+}$ (10µM-100µM) inhibits NMDA$_R$ activity during the critical stage of synaptogenesis resulting in altered glutamatergic connectivity later in life (Neal et al., 2011). These data may indicate that alteration in the glutamatergic system induced by lead may result in aberrant connectivity in the brain. Despite the importance of Ca$^{2+}$ channels being localized near NMDA$_R$ in cells and their synergistic actions in learning and memory, NMDA$_R$s during early development are present but inactive leaving only GABA$_A$s and VSCCs as mediators of excitation. Not until the GABA shift is completed in early development do the NMDA$_R$s take on a sequential role in regulating Ca$^{2+}$ activity to
synchronize GDPs as the molecular substrate for initially hard wiring the brain and subsequently establishing the networks for experience dependent learning and memory.

Given this information it is prudent to evaluate the potential for Pb\textsuperscript{2+} to alter the expression of genes critical in early GABA development prior to the activation of the NMDA\textsubscript{Rs} and the establishment of functional neuronal circuits. Here, we hypothesize that in response to gestational Pb\textsuperscript{2+} exposure in the rat the Slc12a family genes that regulate the timed expression of the NKCC1- and KCC2-transporters are disrupted. These transporters regulate the developmental changes in GABAergic excitation-to-inhibition. Therefore, alteration in the expression pattern would interfere with critical periods of brain development. The potential for aberrant synaptic connections prior to the NMDA\textsubscript{R} system being activated would suggest that not only are glutamatergic neurons susceptible to Pb\textsuperscript{2+} insult, but GABAergic neurons may be equally affected prior to glutamatergic neurons. However, given the importance of the GABA system in early neural development, it may be more informative in elucidating the molecular mechanisms of Pb\textsuperscript{2+} action on the GABAergic neural development.

Studies by Murphy et al., (1991) and Deisseroth et al., (1996) showed that in the presence of pharmacological agents that block L-type VSCCs, inhibition of immediate-early gene inducers occurs. Therefore, the influx of Ca\textsuperscript{2+} is responsible for immediate-early gene induction, indicating that any disruption of this signaling pathway, such as those produced by Pb\textsuperscript{2+}, may result in altered immediate-early gene induction consistent with published data (Schneider et al., 2011).

The findings from the aforementioned studies in accordance to our hypothesis suggest the following signaling cascade of Pb\textsuperscript{2+} resulting in altered gene induction during early development (See Figure 8.):
Figure 8. Hypothesis Model Pb$^{2+}$ Competition Through VSSCs Reduces β3 Subunit Autoregulation of Cell Excitability Reducing Gene Regulation Resulting in Developmental Delays.

1) Pb$^{2+}$ may compete with Ca$^{2+}$ influx through the L-type VSCCs; 2) intracellular Pb$^{2+}$ may increase the I$_{Ca}$ through VSCCs by delaying its ability to inactivate the channel; 3) increased [Ca$^{2+}$]$_i$ in response to Pb$^{2+}$ may alter the activity of gene induction pathways through a) sustained Ca$^{2+}$-dependent activity with second messengers or alternatively b) Pb$^{2+}$ may directly activate third messengers altering DNA; and 4) Pb$^{2+}$ may alter transcription factors of the VSCCs as a self-regulating mechanism in response to Pb$^{2+}$ exposure. The VSCCs-β3 subunit (CCAT) upon neuronal excitation, translocates from the membrane to the nucleus and transcribes mRNA coding from the VSCCs gene CACNB β3 (Bros et al., 2011; Satin, et al., 2011).

Thus, this signaling serves as an autoregulatory process in neurons through a distinct C-terminal encoding of a VSCCs-β3 subunit transcription factor in response neuronal excitability (Gomez-Ospina et al., 2006). Moreover, VSCCs-β3 activity increases as a function of post natal development and is highly expressed in GABAAergic neurons in early development (Gomez-Ospina et al., 2006). Thus, Pb$^{2+}$ may disrupt this VSCCs-β3 autoregulation, resulting in altered gene transcription, decreased Ca$^{2+}$ signaling, and altering neurite formations and extensions during the critical stages of early development.
1.8-Postulated molecular mechanism of Pb\textsuperscript{2+} perturbation of GABAergic immediate-early gene induction via the L-type VSCC and reduced GABA release during synaptogenesis.

The goal of this study was to investigate how chronic gestational Pb\textsuperscript{2+} exposure leads to a neurodevelopmental delay in the rat model through a multi level system approach using molecular, cellular, physiological and behavior analyses described as follows.

- We hypothesized that Pb\textsuperscript{2+}, through competition with initial Ca\textsuperscript{2+} influx and inhibition of Ca\textsuperscript{2+}-dependent processes, leads to premature increased expression of key regulatory proteins directing the GABA shift resulting in premature wiring of neuronal circuits. To evaluate this primary cerebellar neuronal cultures from harvested from postnatal day 6 rats were used as an in vitro model system. Chronic gestational Pb\textsuperscript{2+} exposure may significantly alter the Ca\textsuperscript{2+}-dependent activity and autoregulatory transcription and translation of the VSCCs β-3 subunit expression resulting in perturbations in GABAergic signaling and GDP-induced neuronal network miss-wiring and de-synchronization with altered synaptogenesis in early development. We sought to evaluate the presences of VSCC β-3 subunit nuclear translocation and relative GABA\textsubscript{AR} expression in cultured cerebellar neurons to test our hypothesis. We hypothesize that Pb\textsuperscript{2+} attenuation of VSCC's in early development produces an upregulation in CACNB β3 mRNA expression channelopathies and a down regulation of GABA\textsubscript{AR} β-3 chain mRNA expression, which is associated with neurodevelopmental disorders such as schizophrenia and autism (Hyde et al., 2011; Khale et al., 2008). In order to assess these changes we sought to characterize the altered mRNA expression of the KCC\textsubscript{2} transporter, which is responsible for shifting from GABA excitation-to-inhibition, mRNA for GAD isoforms GAD80, GAD86 (i.e. as markers for early excitatory GABAergic events) GAD65, and GAD67 (i.e. as markers for mature inhibitory GABAergic events), NKCC1 (i.e. a marker for early Cl\textsuperscript{−} importers regulating GABAergic excitation), KCC2 (i.e. a marker for late Cl\textsuperscript{−} exporters regulating GABAergic inhibition), GABA\textsubscript{AR} β-3 (i.e. a GABAergic marker for inhibitory regulation of fear learning and memory and a susceptibility marker for autism neuropathology), CACNB β3 (i.e. a marker for L-Type VSCCs β-3 subunits to assess nuclear transcription), MECP2 (i.e. a marker for presynaptic neurotransmitter release),
and FMR1 (i.e. a marker for postsynaptic plasticity through mGlurs) in response to Pb\textsuperscript{2+} exposure.

- We predict that the effects of Pb\textsuperscript{2+} on these genes are age- and brain region-dependent. We will investigate the Pb\textsuperscript{2+}-induced changes in the whole frontal cortex and hippocampus to assess the spatial and temporal distribution of CACNB β3 and GABA\textsubscript{AR} β-3 across development.

- We postulate that the aforementioned altered molecular mechanisms of action in response to Pb\textsuperscript{2+} neurotoxicity may result in enhanced brain excitability resulting in increased spontaneous neurotransmitter release during early development, attenuation of evoked neurotransmitter release, disrupted synaptic activity with respect to presynaptic vesicle liberation and post synaptic modification impairing early synaptogenesis. We hypothesize that gestational Pb\textsuperscript{2+} exposure will produce alterations in spontaneous and evoked neurotransmitter release, transmitter uptake, and postsynaptic binding of transmitter substances.

- Consistent with these disruptions in age-dependent frontal cortex and hippocampal brain plasticity induced by gestational Pb\textsuperscript{2+} exposure aberrations in experience dependent learning and memory, negative effects on emotionality and disruption in reversal and/or inhibitory learning (i.e. either by prefrontal cortical disinhibition or over excitation) (Burtkowski, 1964) behavioral consequences are expected. In addition, we will assess the potential recovery on Pb\textsuperscript{2+} induced disruptions by GABAergic agonists as a potential pharmacotherapeutic intervention for Pb\textsuperscript{2+} toxicity. Together the aforementioned effects may help explain the initial stages of Pb\textsuperscript{2+} induced perturbations of early GABAergic development prior to the onset of the AMPA\textsubscript{RS} and NMDA\textsubscript{RS} role in learning and memory.

The delicate interplay between both the GABA\textsubscript{AR}\textsubscript{S} and VSCCs when challenged by chronic gestational low level Pb\textsuperscript{2+} exposure do not result in organism death. Thus, biochemical compensations exist in response to this Pb\textsuperscript{2+} neurotoxicity that, in turn, produces altered brain wiring and synaptogenesis underlying the aforementioned behavioral outcomes produced in our rat model.
2.0-Chronic Gestational Pb\(^{2+}\) exposure produces a high BLL that remains throughout development

2.1-Methods

2.1.1-Subjects

Long-Evans Norwegian hooded male and female rats were paired. Control rats were fed a regular Purina rat chow (Dyets Inc. # 61212) [containing 970gm/Kg Purina RMH 1000 chow, 30gm/Kg maltose dextrin], while the pairings for Pb\(^{2+}\) rats were fed a diet containing 1.5g/Kg lead acetate (Dyets Inc. # 612113) [containing 968.4gm/Kg Purina RMH 1000 chow, 30gm/Kg maltose dextrin, 1.5gm/Kg lead acetate, and 0.1gm/Kg yellow dye] (956ppm) ad libitum from pairing throughout gestation and continued through parturition and weaning at 21 days of age.

At postnatal day (PND) 2, 7, 14, and 22 rats (\(N = 5\)) were anesthetized with 50mg/kg Nembutal® i.p., transcardial blood samples were collected, then rats were sacrificed and whole brains were taken in accordance with The College of Staten Island (CUNY) IACUC approval. Dams of the pups (\(n = 3\)) were also sacrificed at the same time to track whether or not BLL concentrations changed as a function of age and/or their relationship to the mother’s level of Pb\(^{2+}\) toxicity. Additionally, rat brains were harvested, Cryoprotected (or homogenized) and stored at \(-80^\circ\)C for further processing of proteins.

2.1.2-Blood lead level analyses

At the point of animal sacrifice transcardial blood samples were collected in ethylenediaminetetraacetic acid (EDTA) coated S-Monovette® syringes (Sarstedt, Germany) mixed and immediately frozen on dry ice. Samples were then sent out for BLL determination to Magellan Diagnostics (North Bellirica, MA). BLLs were determined using atomic absorption spectrophotometry (AAS) with a sensitivity detection level of 1µg/dL.
2.1.3.-Statistical analyses

All data were analyzed in Statistica V. 6.1 (Statsoft, Inc. Tulsa, OK). An Age X Subject and an Age X Treatment ANOVA were used to identify factor and interaction effects. Significant differences were determined by an equal N Tukey’s HSD post hoc comparison test. Significance levels were set at $p < 0.05$ with a SEM of 95%. Data are presented as mean ± SEM.

2.2.-Results

![Blood Pb2+ Concentration (ug/dL) vs Post Natal Day graph](image)

**Figure 9.** Dams and pups BLL subject comparisons as a function of age. Pups at PND 2 had increased BLLs when compared to their dams. Following PND 2 a reversal of BLL profiles were observed and remained until PND 22 where both subjects leveled off.
Figure 10. BLL treatment comparisons as a function of age during development. Control rats were Pb\textsuperscript{2+} negative throughout development. There was a significant difference in Pb\textsuperscript{2+} treated rats in all ages when compared to controls (p<0.001***). Pb\textsuperscript{2+} treated rats showed significant differences at PND 7 (p<0.001*** ) and 14 (p<0.001*** ) when compared to PND 2 rats. In addition, the Pb\textsuperscript{2+} rats showed an inverted curvilinear relationship in elevated BLLs at PND 2 and 22, suggesting a difference in absorption from early lactation feeding, relative rat blood volume and ability to independently feed from the food cages at PND 22.

The influence of a 956ppm Pb\textsuperscript{2+} gestational diet (i.e. from birth to sacrifice) resulted in pup mean blood lead levels (BLLs measured in μg/dL) at PND 2= 41.83 SEM± 14.17, PND 7= 34 SEM± 10.4, PND 14= 30.17 SEM± 7.77 and PND 22= 38.67 SEM±10.67. The dams at corresponding Pb\textsuperscript{2+} exposure times evinced mean BLLs at PND 2= 37 SEM± 9, PND 7= 41.33 SEM± 21.33, PND 14= 39.33 SEM± 60.33 and PND 22= 43.6 SEM± 22.3 respectively.

In contrast, control pups and dams were Pb\textsuperscript{2+} negative. BLL samples revealed an Age effect (DF=3, MS=66.81, F=4.128, p<0.01**) and Subject effect (i.e. Dam or pup) (DF=1, MS=147.62, F=9.121, p<0.01**) (See Figure. 9). Moreover, an Age X Subject interaction was observed (DF=3, MS=77.88, F=4.8, p<0.01**) (See Figure. 9). Figure 10. shows the differences in BLLs based on treatment conditions having an Age effect (DF=3, MS=79.06, F=14.708, p<0.001*** ) a Treatment effect (DF=1, MS=14,840.33, F=2,760.99, p<0.001*** ) and an Age X Treatment interaction (DF=3, MS=779.06, F=14.71, p<0.001*** ).
2.3.-Discussion

*Figure 9.* shows that at PND 2 pups had an elevated BLL (~ 42μg/dL) in contrast to their dams (~ 37μg/dL) and at PND 7 these observations reversed in that the dam had elevated BLLs (~42μg/dL) when compared to their pups, whose BLL dropped remarkably (~34μg/dL). These reversed BLLs/age ratios persisted throughout postnatal development (PND 7-14), whereas at weaning (PND 22) pups BLLs were quite similar (~ 39μg/dL) to that of their respective dams (~43μg/dL). These results suggest that rat pups were vulnerable to Pb\(^{2+}\) exposure and accumulated more Pb\(^{2+}\) at PND 2 during critical stages in early development. Notably, at PND 7 there was a decrease in pup BLLs, which may be due to either increased excretion, but was more likely due to brain/body deposition. If early brain Pb\(^{2+}\) deposition was highest at PND 7 it would suggest that this age in postnatal development may contribute considerably to altering the critical periods in early brain development and further disrupt fine tuning and wiring during synaptogenesis at PND 14. Lastly, the decrease in BLLs from PND 2 through 14 and increase in BLLs from PND 14 to 22 could be explained by the rats’ respective weight to blood volume ratio as a function of age and the pups independence of eating leaded food (See *Figure. 9.*). In addition, at PND 2 the Dams milk is more concentrated and may contain more Pb\(^{2+}\) that is readily absorbed when compared to later postnatal ages. *Figure 10.* illustrates that there were no BLL concentrations detected above or below the 1μg/dL range in control rats irrespective of age. Notably, the rats exposed to Pb\(^{2+}\) showed significant body burdens with highest levels at PND 2 and 22 evinced by an inverted curvilinear relationship as a function of age.
3.0-Pb$^{2+}$ exposure alters Ca$^{2+}$-dependent GABA shift: Novel mechanism for early Pb$^{2+}$ induced neuropathology via altered GABA brain networks:

3.1-Methods

3.1.1-Subjects

Experimentally naïve male Long Evans Norwegian Hooded rats (Taconic, N.J.) (Control $n = 12$; Pb$^{2+} n = 12$; $n = 3$ for PND 2, 7, 14, and 22) were maintained under controlled temperature ($24 \pm 1^\circ$C) and humidity ($55 \pm 5\%$), on a 12-hr light (17:00–7:00 hr):12-hr dark (7:00–17:00 hr) cycle. Lead was administered as described in section 2.1.1. Age-matched controls were used as a reference group.

3.1.2-Sample preparation

At PNDs 2, 7, 14, and 22 rats were selected from each treatment condition (i.e. $N = 3$) for brain extraction of both frontal cortices and hippocampi respectively. Briefly, rats were anesthetized with Nembutal® (i.p. injection 50mg/Kg) and sacrificed in accordance with The College of Staten Island’s IACUC approval procedures. Their frontal cortices and hippocampi were homogenized and phenol: chloroform extracted in less than 2 minutes and frozen on at $-80^\circ$C until use.

3.1.3-RNA preparation

RNA was prepared from brain tissue samples in accordance with manufactures’ protocol (TRIzol Reagent; Invitrogen 15596-026). Briefly, 50-60mg of wet brain tissues were weighed and homogenized in 1ml of TRIzol regent per 100mg of tissue using a glass-Teflon for 1 hr. Post centrifugation, RNA was subjected to a chloroform extraction and subsequently precipitated with isopropyl alcohol. RNA samples were then resuspended in 100μl of DEPC-treated H$_2$O.
3.1.4-Preparation of cDNA and real-time PCR analysis

Identical amounts of total RNA (i.e. 10µg) were treated with RNase-free DNase (Qiagen cat. #79254) at 37°C for 1 hr and purified by phenol/chloroform extraction (i.e. 3:1) followed by an ethanol precipitation. From the purified RNA, 1µg was used in the SYBR GreenER Two-Step qRT-PCR kit (Invitrogen cat. #11765-100) for the first strand cDNA synthesis and in the real-time PCR reaction preparation according to the manufactures protocol. The real-time PCR primers are noted in Table 1. All experiments were repeated three times. In addition, within each experiment PCR reactions were done in triplicates and analyses conducted through a 7500 sequence detection system (Applied Biosystems).

| Table 1. Oligonucleotides used in the real-time qRT-PCR reactions. |
|---------------------------------|-----------------|
| GAPDH ORF                       |                 |
| Forward primer                  | 5’-ACAGGGTGGTGAGCCTCATG-3’ |
| Reverse primer                  | 5’-GTGGGGATAGGACCTCTCTTG-3’ |
| GAD 80 ORF                      |                 |
| Forward primer                  | 5’-AGTGTGGCCTCCAGAGGTTC-3’ |
| Reverse primer                  | 5’-TGATATGGCTCCCCAGGAG-3’ |
| GAD 86 ORF                      |                 |
| Forward primer                  | 5’-TGCCCTCCAGAGGTATG -3’ |
| Reverse primer                  | 5’-TGATATGGCTCCCCAGGAG-3’ |
| GAD 65 ORF                      |                 |
| Forward primer                  | 5’-GGCTCTGGCTTTTGTCCTTC -3’ |
| Reverse primer                  | 5’-TGCCAATCCAATTACTCTTGAA-3’ |
| GAD 67 ORF                      |                 |
| Forward primer                  | 5’-GCCTGGAAGGCTGAAAA-3’ |
| Reverse primer                  | 5’-AATATCCCATCATCTTTATTTGACC -3’ |
| GABA_Aβ3 ORF                    |                 |
| Forward primer                  | 5’-CCACGGAGTGACATGGAAGTTTA-3’ |
| Reverse primer                  | 5’-CATGCTGCTGCTGATGAT-3’ |
| CACNB β3 ORF                    |                 |
| Forward primer                  | 5’-TGATCGGGAGGCTAGTGA-3’ |
Reverse primer 5’-CACGCTGCTCGTAGTGAT-3’

**NKCC$_1$ ORF**
- Forward primer 5’-ATGAGTCTTCCAGTTGCCCCG-3’
- Reverse primer 5’-GCAACGTGTCATGTGCTTTT-3’

**KCC$_2$ ORF**
- Forward primer 5’-GGACCCCCGCATAAAGGA-3’
- Reverse primer 5’-CCTCCAGACCTGTGCGAC-3’

**MECP2 ORF**
- Forward primer 5’-CAGGTCATGGTGATCAAGC-3’
- Reverse primer 5’-CCACACTCCAGCTTTCTA-3’

**FMR1 ORF**
- Forward primer 5’-GTGAGATTCCACCACCTGT-3’
- Reverse primer 5’-CCAACAGCAAGGCTTCTTT-3’

### 3.1.5-Target DNA sequence estimations

Target DNA sequence quantities were estimated using the procedures described by Andrew et al., (2012); Ford et al., (2007); Wimalarathna et al., (2011), and Zhang et al., (2009). Briefly, the target DNA sequence quantities were estimated from the threshold amplification cycle number ($C_T$) using Sequence Detection System Software (Applied Biosystems). A $\Delta C_T$ value was obtained by subtracting the respective primer $C_T$ value from the $C_T$ value from the corresponding *glyceraldehyde 3-phosphate dehydrogenase* (GAPDH) value in order to normalize the differences in cDNA aliquots as a house keeping gene. Therefore, the relative mRNA levels were expressed as $2^{(-\Delta C_T)} \times 100\%$ of GAPDH and data transformed to a Log10 value for fold change comparisons.
3.1.6-Statistical analyses

All data were transformed from the relative mRNA $2^{(-\Delta CT)} \times 100\%$ of GAPDH to a Log10 value to easily compare differences in fold expression. Data were then further analyzed in Statistica V. 6.1 (Statsoft, Inc. Tulsa, OK). An Age X Treatment X Gene ANOVA was used to identify factor and interaction effects. Significant differences were determined by either an equal or unequal $N$ Tukey’s HSD post hoc comparisons test. Significance levels were set at $p < 0.05$ with a SEM of 95%. Data are presented as mean ± SEM.
3.2-Results

Figure 11. Chronic gestational Pb$^{2+}$ exposure down regulated Caβ3 mRNA expression in cortex and hippocampus during early brain development. Cortical Caβ3 mRNA was down regulated from PND 7 through PND 22 (p<0.001***) (A). Hippocampal Caβ3 mRNA was down regulated affected at PND 7 and 22 (p<0.001***), but recovered at PND 14 (B). GABA β3 mRNA was down regulated at PND 22 in cortex (A) and hippocampus (B) in response to Pb$^{2+}$ (p<0.01**).
**Figure 11.** GABA<sub>AR</sub>'s showed that in cortex GABA<sub>AR</sub>-β3 and Ca β3 mRNA expression changes induced by Pb<sup>2+</sup> exposure caused a **Gene** effect (DF=1, MS=2.31, F=82.69, p<0.001***), a **Age** effect (DF=3, MS=1.76, F=63.15, p<0.001***), a **Gene X Age** Interaction (DF=3, MS=0.36, F=12.93, p<0.001***), an **Age X Treatment** interaction (DF=3, MS=0.769, F=27.55, p<0.001***), a **Gene X Age X Treatment** interaction (DF=3, MS=0.21127, F=7.565, p<0.001***) (A).

**Figure 11.** showed that in hippocampus GABA<sub>AR</sub>-β3 and Ca β3 mRNA expression changes induced by Pb<sup>2+</sup> exposure caused an **Age** effect (DF=3, MS=0.837, F=5.56, p<0.01**), a **Treatment** effect (DF=1, MS=1.129, F=7.50, p<0.01**) a **Gene** effect (DF=3, MS=29.00, F=192.58, p<0.001***), an **Age X Treatment** interaction (DF=3, MS=0.96, F=6.35, p<0.001***), an **Age X Gene** interaction (DF=9, MS=0.47, F=3.13, p<0.001***), a **Treatment X Gene** interaction (DF=3, MS=0.68, F=4.53, p<0.006166) and an **Age X Treatment X Gene** interaction (DF=9, MS=0.78, F=5.17, p<0.001***) (B).
Figure 12. Chronic gestational Pb$^{2+}$ exposure decreases KCC2 and increases NKCC1 mRNA in cortex and hippocampus during early brain development. Cortical KCC2 mRNA is down regulated from PND 7 (p<0.05*), through PND 14 (p<0.001***), whereas NKCC1 mRNA is up regulated at PND 2 and 7 (A). Interestingly, in hippocampus the KCC2 mRNA is down regulated at PND 2 through 7 (p<0.001***), and up regulated at PND 14 (p<0.05*), whereas NKCC1 mRNA is up regulated at PND 2 (B).
Figure 12 showed that in cortex NKCC1 and KCC2 mRNA expression changes were altered by Pb\(^{2+}\) exposure resulting in an Age effect (DF=3, MS=1.76, F=63.15, p<0.001***), a Gene effect (DF=1, MS=2.31, F=82.69, p<0.001***), an Age X Treatment interaction (DF=3, MS=0.77, F=27.55, p<0.001***), an Age X Gene interaction (DF=3, MS=0.36, F=12.93, p<0.001***), and an Age X Treatment X Gene interaction (DF=3, MS=0.21, F=7.57, p<0.001***) (A).

Figure 12 showed that in hippocampus NKCC1 and KCC2 mRNA expression changes were altered by Pb\(^{2+}\) exposure resulting in an Age effect (DF=3, MS=1.43, F=17.86, p<0.001***), a Treatment effect (DF=1, MS=4.22, F=52.68, p<0.001***), a Gene effect (DF=1, MS=0.92, F=11.46, p<0.001***), and an Age X Treatment interaction (DF=3, MS=0.36, F=4.48, p<0.01**) (B).
Figure 13. Chronic gestational Pb$^{2+}$ altered early and late GAD mRNA expression levels. Cortical Pb$^{2+}$ exposure disrupted early GAD 80 and 86 and late GAD 67 mRNA expression at PND 2 (p<0.01**) and altered late GAD 65 and 67 mRNA at PND 22 (p<0.001***) (A). Hippocampal, early GAD 86 mRNA expression was up regulated at PND 2 (p<0.01**) and late GAD 67 mRNA expression was down regulated (p<0.001***) (B).
Figure 13. showed that in cortex early GAD 80 and 86 and late GAD 65 and 67 were perturbed by Pb\textsuperscript{2+} resulting in an Age effect (DF=3, MS=3.11, F=34.66, p<0.001***), a Gene effect (DF=3, MS=29.61, F=330.35, p<0.001***), an Age X Treatment interaction (DF=3, MS=1.34, F=14.95, p<0.001***), an Age X Gene interaction (DF=9, MS=0.79, F=6.58, p<0.001***), a Treatment X Gene interaction (DF=3, MS=0.59, F=6.58, p<0.001***), an Age X Treatment X Gene interaction (DF=9, MS=0.50, F=5.56, p<0.001***) (A).

Figure 13. showed that in hippocampus early GAD 80 and 86 and late GAD 65 and 67 were perturbed by Pb\textsuperscript{2+} resulting in an Age Effect (DF=3, MS=0.84, F=5.56, p<0.01**), a Treatment effect (DF=1, MS=1.13, F=7.50, p<0.01**), a Gene effect (DF=3, MS=29.00, F=192.57, p<0.001***), an Age X Treatment interaction (DF=3, MS=0.96, F=6.35, p<0.001***), an Age X Gene interaction (DF=9, MS=0.47, F=3.13, p<0.001***), a Treatment X Gene interaction (DF=3, MS=0.68, F=4.53, p<0.01**) and an Age X Treatment X Gene interaction (DF=9, MS=0.78, F=5.17, p<0.001***) (B).
Figure 14. Chronic gestational Pb$^{2+}$ effects on synaptic vesicle release MECP2 and synaptic plasticity FMR1 mRNA expression levels. Pb$^{2+}$ up regulated FMR1 mRNA expression levels at PND 2 through 7 ($p<0.001^{***}$) and down regulated expression levels at PND 14 ($p<0.05^*$) in cortex (G). Interestingly, FMR1 mRNA expression of MECP2 mRNA expression reversed at PND 14 when comparing Pb$^{2+}$ rats to controls (A). Pb$^{2+}$ exposure down regulated FMR1 and MECP2 mRNA expression at PND 7 and MECP2 mRNA expression at PND 22 (B).
Figure 14 showed that in cortex FMR1 and MECP2 mRNA expression levels were disrupted resulting in an Age effect (DF=3, MS=1.21, F=50.98, p<0.001***), a Treatment effect (DF=1, MS=0.15, F=6.49, p<0.02*), a Gene effect (DF=1, MS=0.63, F=26.61, p<0.02*), an Age X Treatment interaction (DF=3, MS=0.093, F=3.95, F=0.02*) and an Age X Gene interaction (DF=1, MS=0.08, F=3.19, p<0.04*) (A).

Figure 14 showed that in hippocampus FMR1 and MECP2 mRNA expression levels were disrupted resulting in an Age effect (DF=3, MS=2.48, F=32.73, p<0.001***), a Treatment effect (DF=1, MS=0.47, F=6.2, p<0.02*), a Gene effect (DF=1, MS=0.63, F=8.36, p<0.01**) and an Age X Treatment interaction (DF=3, MS=0.23, F=3.00, p<0.05*) (B).

3.3-Discussion

We examined Pb²⁺ effects on gene expression during early development in cortex and hippocampus. The qRT-PCR data revealed that Pb²⁺ decreases VSCC-β3 mRNA expression at PND 7, 14, and 22 in cortex and PND 7 and 22 in hippocampus when compared to control rats (Figure 11-A & B.). Pb²⁺ effected these genes differentially resulting in the VSCCs mRNA down regulation in expression, whereas the GABA<sub>AR</sub>-β3 mRNA expression was observed to increase in cortex at PND 2, 7, and 14 then decreased at PND 22 from control rats (Figure 11-A.). Interestingly, in hippocampus the GABA<sub>AR</sub>-β3 mRNA expression was decreased at PND 7 and 22 under Pb²⁺ treatment (Figure 11-B.). These findings suggest that GABA<sub>AR</sub>-β3 mRNA increases expression when VSCC-β3 mRNA decreases in response to Pb²⁺ insult.

With respect to the chloride transport proteins that regulate the GABA-shift, we observed increased NKCC1 mRNA expression at PND 2 and 7 in cortex (Figure 12-A.), whereas in hippocampus an increase at PND 2 was noted and then a decrease at PND 22 when compared to control rats (Figure 12-B.). The KCC2 mRNA was shown to decrease at PND 2 and increase at PND 7 in both cortex (Figure 12-A.) and hippocampus (Figure 12-B.) when compared to control rats. Notably, in cortex at PND 14 KCC2 mRNA expression was decreased (Figure 12-A.), whereas in hippocampus it was increased under Pb²⁺ treatment (Figure 12-B.). These findings suggest brain regions specific differences in the proteins required for the onset of the GABA-shift were observed in cortex at PND 22 and in the hippocampus at PND 14 in response
to Pb\textsuperscript{2+} exposure. However, the ratio of the NKCC1 and KCC2 transport proteins and their functional activation through phosphorylation remain to be elucidated under Pb\textsuperscript{2+} exposure.

To determine how Pb\textsuperscript{2+} may affect early GABAergic neurons we investigated how early GAD 80 and 86, as well as, late GAD 65 and 67 mRNA expression altered during post natal development. We observed that in cortex GAD 80 and 86 mRNA expression increased at PND 2 and 14 when compared to control rats (\textit{Figure 13-A.}). In hippocampus, GAD 80 mRNA expression decreased at PND 2, whereas GAD 86 increased at PND 2 in response to Pb\textsuperscript{2+} treatment (\textit{Figure 13-B.}). At PND 14 both GAD 80 and 86 increased in hippocampus when compared to control rats (\textit{Figure 13-B.}).

With respect to late GAD 65 we observed in cortex that mRNA expression increased from PND 2 through 14 and decreased at PND 22 under Pb\textsuperscript{2+} exposure (\textit{Figure 13-A.}). In hippocampus, GAD 65 showed decreased mRNA expression at PND 2 and 7 in response to Pb\textsuperscript{2+} (\textit{Figure 13-B.}). In cortex GAD 67 mRNA expression was observed to increase at PND 7 and decrease at PND 22 (\textit{Figure 13-A.}), whereas in hippocampus a decrease in GAD 67 mRNA expression was observed at PND 7 and PND 22 (\textit{Figure 13-B.}). These findings suggest that Pb\textsuperscript{2+} interferes with early GAD 80 and 86 that prolong early GABAergic excitation during development. Together, these findings show a direct effect of Pb\textsuperscript{2+} insult unto GABAergic neurotransmission during critical stages of development with the temporal programming of GABAergic networks at the transcriptional level.

In order to assess how Pb\textsuperscript{2+} would affect presynaptic neurotransmitter vesicular release and post synaptic plasticity we evaluated the changes in mRNA expression for MECP2 and FMR1 respectively during development. In the cortex, Pb\textsuperscript{2+} exposure caused increased MECP2 mRNA expression at PND 14 when compared to controls (\textit{Figure 14-A.}). In hippocampus Pb\textsuperscript{2+} decreased mRNA expression in PND 7 and 22 (\textit{Figure 14-B.}). In cortex FMR1 mRNA expression was observed to increase in PND 2 and 7 followed by a decrease at PND 14 (\textit{Figure 14-A.}), whereas in hippocampus decreased mRNA expression was shown at PND 7 in response to Pb\textsuperscript{2+} (\textit{Figure 14-B.}). These findings indicate that in cortex at PND 14 Pb\textsuperscript{2+} perturbs vesicular release and post synaptic plasticity. In hippocampus at PND 22 Pb\textsuperscript{2+} disrupts vesicular release.
4.0- Chronic gestational Pb\textsuperscript{2+} exposure reduces \textit{GABA}_{AR} expression developmentally and causes nuclear translocation of the L-Type VSCCs β-3 subunit in cortex and hippocampus

4.1-Methods

4.1.1-Subjects

Experimentally naïve male Long Evans Norwegian Hooded rats (Taconic, N.J.) (Control \( n = 12; \) Pb\textsuperscript{2+} \( n = 12; \) \( n = 3 \) for PND 2, 7, 14, and 22). Long Evans male and female rats were paired. Rats were fed as described in section 2.1.1.

4.1.2- Brain slice immunohistochemistry

At postnatal day (PND) 2, 7, 14, and 22 rats were sacrificed and perfused 15 minutes with PBS followed by 10 minutes 4% PFA. The following 24 hours, brains were transferred into 30% sucrose in PBS. Brains were aligned to cut mid-sagittal sections on a cryostat with O.C.T. TissueTek\textregistered{} compound at 26°C. Brain sections were cut in 30µm thick slices and transferred to a 12 well plate with cryoprotectant 30% glycerol, 30% ethylene glycol, and 40% PBS for floating immunohistochemistry (IHC). Repeated serial sections were collected in each well to increase reliability of staining between slices. Slices were blocked with 2% NFDM-PBS, 10% NGS, and 0.02% Triton X100, washed for 30 minutes and blocked in same solution without Triton X100 with primary antibodies MS x \textit{GABA}_{AR} beta chain (MAB341 Chemicon International), MS x KCC2 (MyNeuroAb) and Rbt x Calcium β3 (AB5230-200Ulb Chemicon International) then incubated for 24 hours at 4°C. The following secondary antibodies were used: goat anti-Ms FITC (ab\textsuperscript{'} 2 (Santa Cruz Biotechnology) and Cy5 goat anti-Rbt (Santa Cruz Biotechnology). For confocal data, the FITC fluorophore was excited at 488 nm; the Cy5 at 633 nm. Emission for these dyes are in the green and red range, respectively. Slices were transferred onto gelatin-coated slides manually and mounted with Slow Fade Gold anti-fade reagent with DAPI nuclear counter stain (Invitrogen), then cover slipped using a number 1 ½ coverglass.
4.1.3-Primary neuronal granule cell cultures

Twelve postnatal day 6 male rats were sacrificed and cerebellums were dissected away from the rest of the brain under sterile conditions. Cerebellums were washed 3 xs with calcium-magnesium free phosphate buffered saline (CMF-PBS). Samples were subjected to trypsinisation and placed on a manual rotor for 5 minutes. Samples were washed again 3x with CMF-PBS then treated with DNase and triturated sequentially 10xs with 3 pasture pipettes of decreasing size to dissociate cerebellar granule cells. Samples were left to sit on ice and 2 minutes later the supernatant was collected and transferred to a new tube. Samples were then centrifuged at 4°C for 1,000 RPM for 5 minutes. DNase was then removed from the sample leaving only the pellet, which was then resuspended in 1mL of Minimum Essential Medium (MEM) Eagles Serum. Samples were triturated again in the same manner as before. A 1:10 dilution of sample into MEM was used to count cells using a hemocytometer. Desired cell suspensions were calculated using a dilution factor to yield 1.5x10^6 cells per poly-D-lysine coated cover slip in a 24 well plate. The next day MEM was replaced with fresh MEM containing N₂ supplement and cells were treated with dose responses of PbCl⁻ (i.e. 0.05μM, 0.1 μM, 0.5 μM, 1 μM, and 1.5 μM). The following day (i.e. 3rd day in vitro) cells were fixed and subjected to IHC same as the procedures above.

4.1.4- Confocal microscopy and Imaris image processing

Tissue were visualized under a Leica SP2 AOLS confocal microscope with a 40x oil objective for brain slices and a 63x oil objective for primary neuronal granule cell cultures. Z-stacks were taken of the sections and regions of interest. Images were then quantified using the Imaris software (Bitplane, South Windsor CT) digital intensity mean analysis and reconstruction of the sections. All settings were equivalent within comparative brain/granule cell culture regions and standardized across samples and treatments.

4.1.5-Statistics

All data were analyzed in Statistica V. 6.1 (Statsoft, Inc. Tulsa, OK). An Age X
Treatment X Protein ANOVA was used to identify factor and interaction effects. Significant differences were determined by either an equal or unequal *Tukey’s HSD* post hoc comparisons test. Significance levels were set at *p* < 0.05 with a SEM of 95%. Data are presented as mean ± SEM.

### 4.2-Results

![Figure 15. Pb$^{2+}$ effects on KCC2 expression (Green) patterns in cortex as a function of age.](image)

![Figure 16. Pb$^{2+}$ effects on KCC2 IHC mean intensity expression in cortex as a function of age.](image)
Figures 15-16 showed an Age effect (DF=3, MS=146,787, F=150.895, p<0.001**) and an Age X Treatment interaction (DF=3, MS=78,563, F=80.762, p<0.001**).
Figures 17-18 showed an Age effect (DF=3, MS=175,754, F=167.295, p<0.001***), a Treatment effect (DF=1, MS=71,122, F=67.6999, p<0.001***), and an Age X Treatment interaction (DF=3, MS=51,263, F=48.796, p<0.001***).

Figure 19. Pb\(^{2+}\) effects on KCC2 expression (Green) patterns in CA3 as a function of age.

Figure 20. Pb\(^{2+}\) effects on KCC2 IHC mean intensity expression in CA3 as a function of age.

Figures 19-20 showed an Age effect (DF=3, MS=29,660, F=22,621, p<0.001***), a
Treatment effect (DF=1, MS=72,474, F=55,274, p<0.001***), and an Age X Treatment interaction (DF=3, MS=37,357, F=28,491, p<0.001***).

**Figure 21.** PbCl\(^-\) alters GABA\(_{AR}\) expression (Green) in culture and VSCC-β3 nuclear translocation (white arrow pointing to Red) in cerebellar granule cell cultured neurons. (uppercase letter shows field and lower case letter shows magnified cell). Images were taken with a 40x oil objective.
Figure 2. PbCl⁻ induces a curvilinear effect on VSCC-β3 nuclear expression in cerebellar granule cell cultured neurons.

Figure 2. shows a curvilinear relationship between increased dose responses of PbCl⁻ on increasing VSCC-β3 nuclear expression at lower and decreased nuclear expression at higher doses. PbCl⁻ has a treatment and Dose effects as independent factors in a one way ANOVA (DF=5, MS=413.913, F=105.44, p<0.001*** with an unequal Tukey post hoc analysis.
Figure 23. PbCl\textsuperscript{2-} induces a curvilinear effect on GABA\textsubscript{AR} expression in cerebellar granule cell cultured neurons.

Figure 23. shows a curvilinear relationship between increased dose responses of PbCl\textsuperscript{2-} on GABA\textsubscript{AR} expression in cerebellar granule cell cultured neurons. PbCl\textsuperscript{2-} has a treatment and Dose effects using a one way ANOVA (DF=5, MS=880.243, F=598.4, p<0.001*** ) with an unequal Tukey post hoc analysis.
Figure 24. PbCl\textsuperscript{-} decreases KCC2 expression (Green) in cerebellar granule cell cultured neurons regardless of dose (uppercase letter shows field and lower case letter shows magnified cell). Images were taken with a 40x oil objective.
Figure 25. PbCl\textsuperscript{-} reduces KCC2 expression in cerebellar granule cell cultured neurons regardless of dose.

Figure 25. shows a decreased KCC2 expression in cerebellar granule cell cultured neurons in response to PbCl\textsuperscript{-} regardless of dose. PbCl\textsuperscript{-} has a Treatment and Dose effects using a one way ANOVA (DF=5, MS=108,607, F=2,400.4 p<0.001***) with an unequal Tukey post hoc analysis.

4.3-Discussion

Our data showed that in the gestational rat model Pb\textsuperscript{2+} toxicity prematurely shifted KCC2 protein expression for PND 7 to PND 2 in cortex, DG, and CA3 regions of the hippocampus evidenced by immunohistochemistry (Figures 15-20.). In addition, the normal trajectory of KCC2 peak expression was observed at PND 14 in cortex, DG, and CA3 in control rats (Figures 15-20.). Interestingly, in the cortex Pb\textsuperscript{2+} exposure reduced KCC2 immunoreactivity at PND14 and PND 22 when compared to controls (Figures 15-18.). In the DG Pb\textsuperscript{2+} decreased KCC2 immunoreactivity at PND 14 and remained elevated in contrast to controls at PND 22 (Figures 19-20.). These findings implicate that gestation Pb\textsuperscript{2+} exposure may
prematurely increase expression of KCC2 resulting in a premature GABA-shift in early development resulting in altered excitation-to-inhibition balancing and an inappropriately wired brain.

In order to assess the exact effects on the VSCC-β3 and GABA<sub>AR</sub> expression as a function of increased Pb<sup>2+</sup> exposure we examined the dose response effects of PbCl<sup>-</sup> in primary cerebellar granule cell cultured neurons 3 days in vitro (3 DIV). Our results showed that PbCl<sup>-</sup> at low concentrations increases VSCC-β3 and at high concentrations reduced immunoreactivity with peak expression at PbCl<sup>-</sup> 0.1µM in contrast to control conditions (Figures 21-23.). These findings describe an atypical dose response curve where higher doses of PbCl<sup>-</sup> may appear to be less detrimental, but result in irreparable neurotoxicity. Essentially, other neurobiological factors, such as astroglia cells, may help compensate with mediating high doses of PbCl<sup>-</sup>, whereas at low doses such compensation may not be possible.

Interestingly, the cerebellar granule cells GABA<sub>AR</sub> immunoreactivity showed similar effects as did the VSCC-β3 (Figures 21-23.); however, peak expression of GABA<sub>AR</sub> was observed at PbCl<sup>-</sup> 0.5µM. These findings showed that VSCC-β3 is more sensitive to PbCl<sup>-</sup> insult and the early GABAergic system is altered suggesting interference with increased VSCC-β3 and GABA<sub>AR</sub> synergistic activity at low and reduced synergistic activity with high doses of PbCl<sup>-</sup>.

When we applied the same dose response of PbCl<sup>-</sup> to the cerebellar granule cells we observed a drastic decrease in KCC2 immunoreactivity (i.e. ~40-50%) irrespective of dose; indicating that PbCl<sup>-</sup> produced delays in the GABA shift in isolated cultured neurons when compared to control conditions (Figures 24-25.). These findings indicate that Pb<sup>2+</sup> can interrupt early GABAergic development through perturbing the precise interplay between the VSCC-β3 and GABA<sub>AR</sub>, thereby secondarily affecting KCC2 expression resulting altered excitation-to-inhibition signals in the brain with altered neural networks.
5.0 - Chronic gestational lead exposure enhances spontaneous and inhibits stimulation-evoked release of glutamate analog, D-2, 3-^3^H-aspartic acid from synaptosomes in early cortical and post natal hippocampal development:

5.1-Methods

5.1.1-Subjects

Experimentally naïve male Long Evans Hooded rats (Taconic, N.J.) *Rattus norvegicus* (Control *n* = 96; Pb^2+* n* = 96) were tested in accordance with The College of Staten Island (CUNY) IACUC approval procedures. Long Evans male and female rats were paired. Rats were fed as described in section 2.1.1. Age matched controls were used as a reference group. At postnatal days (PND) 2, 7, 14 and 22 rats were selected from each treatment condition (i.e. *n* = 8) for frontal cortex and hippocampal synaptosomal preparations to assess neurotransmitter release properties and (i.e. *n* = 4) for both neurotransmitter uptake and binding studies respectively. To assess chronic gestation Pb^2+ effects as a function of brain injury and plasticity during early development we selected the following time points to map a developmental trajectory: PND 2 during astrocyte proliferation, dendritic arborization, and gonadal hormones of immature neurons prior to the critical period, PND 7 during the critical period including synapse formation and spine growth, PND 14 post critical periods, and PND 22 as mature neurons (*See Review Kolb and Gibb, 2001*).

5.1.2-Synaptosomal sample preparation

Long Evans Hooded rats at PND 2, 7, 14, and 22 were anesthetized with Nembutal® (i.p. injection 50mg/Kg) and sacrificed in accordance with The College of Staten Island’s (CUNY) IACUC policies and procedures. In two sets of experiments either frontal cortices or hippocampi were dissected, extracted, weighed, and homogenized in a 1:10 dilution (w/v) buffer containing 0.32M sucrose, 1mM MgSO₄, 20mM HEPES and pH adjusted to 7.4 with NaOH (Sigma/Aldrich, MO). Samples were pestled in a glass homogenizer with 18 strokes, and 1ml of the sample was collected into an eppendorf tube. The 1ml sample was then centrifuged at 5,000
rpm x 10 minutes at 4°C (Beckman TL-100 Ultra Centrifuge, MD). The supernatant from the sucrose gradient was collected and re-centrifugated at 19,000 rpm x 20 minutes at 4°C, while the pellet was discarded. The subsequent pellet containing synaptosomes was re-suspended in 800µl of oxygenated (O₂ 95%/CO₂ 5%) artificial cerebrospinal fluid (aCSF) solution containing 124mM NaCl, 2mM CaCl₂, 4.5mM KCl, 1mM MgCl₂, 26mM NaHCO₃, 1.2mM NaH₂PO₄, and 10mM C₆H₁₂O₆ with pH 7.4. (Sigma/Aldrich, MO) then centrifugated for 2 minutes at room temperature (Beckman # 348750 Microfuge, CA). This step was repeated twice. The synaptosomal pellet was then dissolved by pipette in 1ml oxygenated aCSF solution. Lastly, 200µl of the synaptosomes were used in the experiments that follow.

### 5.1.3-Synaptosomal spontaneous and evoked neurotransmitter release experiments

Synaptosomal neurotransmitter release samples (i.e. 200µl) were loaded into an electrically inducible chamber (Brandel SF-12 Suprafusion System, MD) along with 3µl of 1mCi/mL D-2,3,5-H-aspartic acid (ARC, Inc., MO); a non-metabolized glutamate analogue to assess Glutamatergic synaptosomal excitability. Samples were incubated for 1 hour while being temperature regulated at 37-39°C. Perfusion flow rates were adjusted to 90% pumping rate (2.25ml/min). After the incubation period, a 30 minute pre-collection period was done to wash out excess background radioactivity that was not accumulated into the synaptosomes. The experimental samples were collected every 2 minutes in individual scintillation vials. The first 9 scintillation vials were averaged and taken as a baseline measure of spontaneous release. Sample 10 was then electrically stimulated with a 10Hz, 10mA stimulus for 20 seconds (Brandel 12 channel electric stimulation unit, MD) and another 10 scintillation vials were collected post stimulation. These remaining samples were taken as measurements of evoked release. Post experiment, 3.4ml of ready safe scintillation cocktail liquid (Beckman Coulter™, CA) were added to all collection vials capped and vortexed for 10 seconds. All scintillation vials were set in trays for 24 hours. The following day scintillation counts per minute (CPM) were collected for 10 minutes per vial (Beckman Coulter™ LS 6500 Multipurpose Scintillation Counter, CA).

### 5.1.4-Synaptosomal neurotransmitter uptake experiments

Watman circle filter papers 24mm (GF/B Circles: Cat: 1821-024) were soaked for 1 hr in a 0.1% poly ethyleneimine solution to create an adherent substrate to the filter for the
synaptosomes used in both uptake and binding experiments. Filters were then left to air dry. Once dry the filters were placed on the Hoefer Scientific Model # FH224V/FH225V 10 PLC FLTR HLDR w/valves 25mm (San Francisco, CA) followed by installing the valve chambers atop the filters. Each chamber was filled with 500µl of aCSF. A radioactive glutamate solution was prepared with 2µl of stock D-2, 3,3H-aspartic acid (ARC, Inc., MO) and adjusted to 22ml using the same aCSF solution as in the release studies. From this solution 1ml was added to each chamber. Synaptosomal samples were prepared identical to the release studies except that the synaptosomal pellet was dissolved with a 200µl pipette in 1ml of oxygenated aCSF solution. Then 200µl of sample was added to each chamber (i.e. total volume 1.7ml) and left to incubate for 2 minutes. After 2 minutes, each sample was vacuumed out and rinsed with 1mL of aCSF then vacuumed 3 times. Samples were then left to air dry for 2 minutes and filter papers were collected into scintillation vials and prepared identically for CPM determination as the release samples.

5.1.5-Synaptosomal neurotransmitter binding experiments

Whatman circle filters were prepared identically to the uptake studies. Brain homogenates from cortex and hippocampus were centrifuged at 3,400g X 20 minutes and replaced with fresh binding buffer 4 times to wash out endogenous glutamate (i.e. 1-3µM) to create glutamate free synaptosomal samples. Binding buffer comprised of: 30mM Tris-HCl, 2.5mM CaCl₂, and pH 7.3 (Sigma-Aldrich, MO). From the synaptosomes two equal amounts of 200µl were placed into separate Eppendorf tubes (i.e. one for hot D-2, 3,3H-aspartic acid (ARC, Inc., MO) and the other for cold glutamate) to assess total amount of binding hot glutamate vs. hot + cold glutamate binding was determined via a subtraction method divided by the amount of protein in the synaptosomal fraction. A 100mM glutamate (Sigma-Aldrich, MO) was made and diluted to a 1mM glutamate solution and then a final concentration of 100µM glutamate. The radioactive (i.e. hot) D-2, 3,3H-aspartic acid solution was made by taking 5µl of D-2, 3,3H-aspartic acid and adding it to 5µl of 1mM cold glutamate. The cold glutamate sample contained 200µl of synaptosomes, 200µl of D-2, 3,3H-aspartic acid solution, 300µl of binding buffer and 80µl of 1mM cold glutamate. The hot glutamate sample contained 200µl of synaptosomes, 200µl of D-2, 3,3H-aspartic acid solution, 380µl of binding buffer. These samples were incubated for 45 minutes at 37°C. Following incubation samples were immediately filtered and
washed 3 times with binding buffer. Samples were then left to air dry for 2 minutes and filter papers were collected into scintillation vials and prepared identically for CPM determination as the release samples.

5.1.6-Synaptosomal protein determination procedures

The sample tubes from each of the experiments that were prepared for the synaptosomal, uptake, and binding experiments were used to quantify the protein concentration (µg/µl) per 1:10 dilution of sample (mg/ml). Proteins were subjected to a Bradford assay using bovine serum as the reference protein with a Bio-Rad DC Protein colorimetric assay kit (BIO-RAD 500-0116, CA) according to manufacture guidelines. Briefly, standards of bovine serum were freshly made using the following protein concentrations 0.0, 0.0125, 0.250, 0.5, 0.75, 1.0, 1.25, 1.5, and 2µg/µl respectively. Standards were run in triplicates simultaneously with unknown proteins from the synaptosomal experiments. Using a 96 well plate, 5µl of standards and unknown protein samples were added into each well, followed by 25µl of working reagent A+S, and finally 200µl of reagent B were added (i.e. total volume 230µl/well). The plates were gently agitated to mix the reagents and were stored in Napco Model 6300 (Surplus Solutions, LLC., MA) CO2 incubator for 30 minutes prior to being read at 750nm absorbance spectrum on a Spectra Max 340PC microplate reader (Molecular Devices, CA). Protein absorbencies were read and data computed using SoftMax Pro® data analysis software. A standard protein curve was calculated using an average of the triplicate samples verses the unknown protein concentrations (R² > 0.9) as a function of protein concentration (µg/µl) against optical density absorbed from the protein when read at 750nm.

5.1.7-Normalization of synaptosomal protein against CPM

Counts per minute (CPM) of D-2,3,3H-aspartic acid were recorded over a 10 minute period per sample using a Beckman Coulter LS6500 Multipurpose Scintillation Counter (Fullerton, CA). Obtained values for D-2,3,3H-aspartic acid CPM data were taken and divided by the amount of protein from each respective 1ml sample. The resultant CPM/mg protein was graphed as amount of basal uptake and spontaneous release rates of 3H-D-Aspartate prior to and post electrical stimulation. Results from the release studies were normalized against the first 9 spontaneous baseline scintillation vials prior to electrical stimulation to determine the relative
difference in D-2,3,3H-aspartic acid release from synaptosomes. In both cases Pb\textsuperscript{2+} exposed samples were normalized against controls.

In the release studies the CPM/mg protein were collected from the 2 minute incubated samples and Pb\textsuperscript{2+} exposed samples were normalized against controls. For the binding studies the CPM/mg protein were collected from both the hot (i.e. D-2,3,3H-aspartic acid only) and cold glutamate (i.e. D-2,3,3H-aspartic acid +100μM glutamate) samples to determine total binding by subtracting the difference from a competitive binding from non-competitive binding.

5.1.8-Statistical analyses

All data were analyzed in Statistica V. 6.1 (Statsoft, Inc. Tulsa, OK). For the spontaneous and evoked release, uptake, binding, and Ca\textsuperscript{45} accumulation studies factorial ANOVAs were used to assess Age, Treatment, and Age X Treatment interaction effects. For both uptake and binding studies a factorial ANOVA was used to assess Age, Treatment, and Age X Treatment interaction effects. Significance levels were set at α= 0.05% with a 95% ± SEM. Significant differences were determined by equal and unequal Tukey’s HSD post hoc comparisons test.
5.2-Results

Figure 26. Effects of chronic gestational Pb\(^{2+}\) exposure on spontaneous \(^{3}H\)-D-Asparate release in cortex (A) and hippocampus (B) as a function of age during early post natal development. Pb\(^{2+}\) increases spontaneous release at PND 2, 7, and 22 in cortex (A) and PND 14 in hippocampus (B).
**Figure 26.** In the cortex the spontaneous neurotransmitter release studies revealed an *Age* effect (DF=3, F=40.906, p<0.001***), a *Treatment* effect (DF=1, F=32.74, p<0.001***), and an *Age X Treatment* interaction (DF=3, F=17.36, p<0.001***). (A). **Figure 26.** In the hippocampus the spontaneous neurotransmitter release studies showed an *Age* effect (DF=3, F=25.99, p<0.001***), and an *Age X Treatment* interaction (DF=3, F=6.63, p<0.001***). (B).
Figure 27. Effects of chronic gestational Pb$^{2+}$ exposure on evoked $^3$H-D-Asparate release in cortex (A) and hippocampus (B) as a function of age during early post natal development. Pb$^{2+}$ inhibits evoked neurotransmitter release at PND 2 and 7 in cortex (A) and PND 2-22 in hippocampus (B).
Figure 27. in the cortex the evoked release studies showed an Age effect (DF=3, MS=11,415, F=6.62, p<0.001**), a Treatment effect (DF=1, MS=10,658, F=6.18, p<0.02**) and an Age X Treatment interaction (DF=3, MS=9,239, F=5.36, p<0.001**) (A). Figure 27. in the hippocampus the evoked release studies showed an Age effect (DF=3, MS=4,180, F=6.1, p<0.001***) and a Treatment effect (DF= 1, MS=, F=5.46, p<0.02*) (B).
Figure 28. Effects of chronic gestational exposure of Pb\textsuperscript{2+} on \textsuperscript{3}H-D-Asparate uptake and binding in cortex (A & B) and hippocampus (C & D) as a function of age during early post natal development. Pb\textsuperscript{2+} decreases neurotransmitter uptake at PND2 and increases uptake at PND 7 in cortex (A). In hippocampus uptake is increased at PND 22 (B). In cortex Pb\textsuperscript{2+} increases...
neurotransmitter binding at PND 2, 14, and 22, but reduces it at PND 7 (C). In hippocampus Pb²⁺ follows the same trajectory, but with a less pronounced effect at PND 7 and with enhanced effects at PND 14 and 22 (D).

Figure 28. in the cortex the uptake studies showed an Age effect (DF=3, MS=1,419.91, F=40.58, p<0.001***), a Treatment effect (DF=1, MS=189.69, F=5.42, p<0.05**) and an Age X Treatment interactions (DF=3, MS=603.74, F=17.25, p<0.001***) (A). Figure 28. in the hippocampus the uptake studies revealed an Age effect (DF=3, MS=13,319.4, F=21.41, p<0.001***), a Treatment effects (DF=1, MS=2,086.28, F=33.85, p<0.001*** and an Age X Treatment interaction (DF=3, MS=1,630, F=26.45, p<0.001***) (B).

Figure 28. in the cortex the binding studies showed an Age Effect (DF=3, MS= 748,317, F=29.48, p<0.001*** and an Age X Treatment interaction (DF=3, MS=192,600, F=7.59, p<0.001***)(C). Figure 28. in the hippocampus the binding revealed an Age effect (DF=3, MS=1,658,008, F=73.66, p<0.001***), a Treatment effect (DF=1, MS=398,405, F=17.72, p<0.001*** and an Age X Treatment interaction (DF=3, MS=122,675, F=5.46, p<0.01**) (D).

5.3-Discussion

In the spontaneous vs. evoked ³H-D-Asparate experiment, chronic gestational Pb²⁺ exposure induced early developmental changes in synaptosomal physiology at select age points between the cortex and hippocampus (Figure 26-A&B.). In the cortex, Pb²⁺ enhanced spontaneous release of ³H-D-Asparate at PND 2 and 22 when compared with controls (Figure 26-A.). In addition, Pb²⁺ exhibited a significant increase in spontaneous neurotransmitter release at PND 2 followed by a delayed and gradual recovery with elevations at PND 22 (Figure 26-A.). Peak spontaneous neurotransmitter release in the cortex was observed in PND 14 in controls, whereas it was observed at PND 2 in Pb²⁺ treated rats (Figure 26-A.). Interestingly, in the hippocampus, Pb²⁺ treatment only exhibited a reduction in spontaneous release at PND 14 (Figure 26-B.). The peak ages of hippocampal spontaneous release was observed at PND 2 and 14 in controls, with Pb²⁺ treatment showing a consistent pattern of early elevations in spontaneous neurotransmitter release followed by a delayed and gradual recovery by PND 22 (Figure 26-B.). In contrast to spontaneous neurotransmitter release, in the cortex, Pb²⁺ reduced evoked neurotransmitter release at PND 2 and 7 followed by benign effects at PND 14 and 22 (Figure 27-A.). In hippocampus Pb²⁺ reduced evoked neurotransmitter release consistently at
PND 7, 14, and 22 as a function of age (Figure 27-B). This indicates that the cortex is most vulnerable to Pb\(^{2+}\) disruption in neurotransmission at PND 2 and 7, whereas the hippocampus is vulnerable at later developmental ages (i.e. PND 7, 14, and 22) (Figure 27-A&B).

In the cortex Pb\(^{2+}\) reduced uptake at PND 2 and increased uptake at PND 7 when compared to controls (Figure 28-A). Peak cortical uptake in controls was observed at PND 7 and was significantly greater in Pb\(^{2+}\) treated rats at this age. In contrast, in hippocampus Pb\(^{2+}\) treatment resulted in reduced uptake at PND 7 and increased uptake at PND 14 and 22 when compared to controls (Figure 28-B). Peak uptake hippocampal uptake in controls was observed at PND 14 and was significantly greater at PND 22 in Pb\(^{2+}\) treated rats.

In cortex peak binding was observed at PND 7 in both treatment groups. Notably, Pb\(^{2+}\) treatment increased binding at PND 2, 14, and 22, while at PND 7 resulted in a decrease in binding (Figure 28-C). Pb\(^{2+}\) significantly reduced PND 7 binding and increased PND14 binding evidencing a shift in post synaptic communication consistent with developmental delays. The same binding effects in response to Pb\(^{2+}\) were observed in hippocampi, with the exception that at PND 7 no Pb\(^{2+}\) effects were noted (Figure 28-D).

We evaluated gestational Pb\(^{2+}\) effects on cortical and hippocampal synaptosomal spontaneous and evoked release, uptake, and binding of glutamate analog, D-2,3,\(^{3}\)H-aspartic acid which we considered as indicative of changes at Glutamatergic synapses. D-2,3,\(^{3}\)H-aspartic acid was selected as a reliable determinant of neurotransmitter properties given that it is not metabolized through the glutamate/glutamine/GABA-cycle. In addition, changes observed in neurotransmitter properties were due to altered gene and protein expression as a result of chronic Pb\(^{2+}\) insult, rather than an acute in-vitro test solution application. Interestingly, these lifelong changes in neurotransmitter properties by Pb\(^{2+}\) produced results consistent with the biphasic properties reported in the literature in-vitro.

These experiments evidence the three major aspects of neurotransmission: 1) release, 2) uptake, and 3) binding of postsynaptic targets. Pb\(^{2+}\) disrupts the efficiency on synaptosomal vesicular accumulation of glutamate resulting in increased extracellular glutamate with the potential for excitotoxicity, increased glial cells clearing away excess transmitters from the synaptic cleft and increased transporter activity to maintain appropriate signaling. Since Pb\(^{2+}\) causes synaptic vesicles to accumulate less neurotransmitter at PND 2 and more at PND 7 in cortex it will result in reduced glutamate recycling and subsequent release. Pb\(^{2+}\) increases
spontaneous release of neurotransmitters suggesting altered non-specific transmitter signals producing outcomes consistent with altered synaptogenesis in early development. Pb2+ also showed an increase in binding at PND 2, 14, and 22 indicating increased glutamate post synaptic receptors and sensitivity to glutamate which may be a compensatory mechanism for a reduced number of post synaptic contacts if Pb^{2+} resulted in reduced neuronal survival during development. Increasing glutamate post synaptic receptor expression would increase the probability of neuron survival in response to Pb^{2+} insult developmentally. In addition, between PND 7-14 there is much brain plasticity to compensate for injuries and enduring insults prior to or after this critical window will reduce long term outcomes associated with this neurotoxicant injury.

Pb^{2+} enhanced spontaneous release at PND 2 in the cortex, whereas in hippocampus at PND 14 release was inhibited. Evoked release was inhibited at PND 2 and 7 in cortex and trends of inhibition were evident at PND 7, 14, and 22 in hippocampi. These data show Pb^{2+}-induced cortical aberrations at PND 7 and at PND 14 in hippocampi. Pb^{2+} reduced cortical uptake at PND 2 and increased at PND 7. In contrast, uptake was enhanced at PND 22 in hippocampi. At PND 7 there was reduced binding in cortex, whereas at PND 14 binding was increased in hippocampi. We suggest that gestational Pb^{2+} exposure causes imprecision of cortical and hippocampal signal-mediated pruning post natally which may obscure relevant signals while increasing non-specific background activity resulting in improper network connections throughout development.
6.0-Neurobehavioral effects of chronic gestational Pb$^{2+}$ exposure: Taurine as a neuroprotective agent to recover these GABAergic learning deficits

6.1-Methods

6.1.1-Subjects

Long Evans male and female rats were paired. Rats were fed as described in section 2.1.1 ad libitum from pairing, throughout gestation, and continued through parturition and weaning. At postnatal day (PND) one set of 22 rats were assigned to behavioral assays as follows: Open Field (OF), Elevated Plus Maze (EPM), Light/Dark Test (LD). Also, independent sets of rats were assigned to the Hole Board Test (HB), Context Fear Conditioning Test (CFC) and Auditory Cued Fear Conditioning Test (ACFC), and the Acoustic Startle Response (ASR) with pre-pulse inhibition (PPI) test to control for carryover effects and to ensure novelty. Rats were administered taurine, a GABA$_{AR}$ agonist, 43mg/Kg i.p. injection 15 minutes prior to behavioral testing to assess taurine's influences on GABAergic behavioral regulation in reducing anxiety, irritability, stress and increasing inhibitory learning and memory.

6.1.2-Open field

At PND 22 rats were examined during 10 minutes of locomotor exploration in the Open Field test (OF) (376mm H x 914mm W x 615mm L) in an illuminated room 300 Lux. This test was used to evaluate if there were any locomotor problems and or anxiety issues in the rats behaviors. Locomotor variables including time mobile (s) in zone and latency for first exit from zone (s) were recorded and post analyzed using the Anymaze® video tracking software transmitted via a ceiling mounted SONY-Handy Camera ran to a standard HP 1.6GHz computer.

6.1.3-Elevated plus maze

At PND 23 rats were examined during 10 minutes of anxiety testing in the Elevated Plus Maze (EPM) (159mm H x 70mm W x 730mm L) in an illuminated room 300 Lux. This tests evaluates anxiety phenotypes. Locomotor variables including time mobile (s) in zone, number of
zone entries, time head dipping over open arm ledge, and number of head dips over open arm ledge were recorded and post analyzed using the Anymaze® video tracking software transmitted via a ceiling mounted SONY-Handy Camera ran to a standard HP 1.6GHz computer.

6.1.4-Light/dark test

At PND 24 rats were examined during 10 minutes of anxiety testing in the Light/Dark test (LD) (50mm H x 150mm W x 300mm L) in an illuminated room 300 Lux. This test uses intrinsic motivation (dark escape) as an anxiety test. Locomotor variables including time in zone and time immobile (s) in zone were recorded and post analyzed using the Anymaze® video tracking software transmitted via a ceiling mounted SONY-Handy Camera ran to a standard HP 1.6GHz computer.

6.1.5-Hole board test

At PND 30-40 rats were examined during 10 minutes of anxiety testing in the Hole Board test (HB) (610mm H x 610mm W x 610mm L) in an illuminated room 300 Lux on day 1. This test is a neurotoxicity test that evaluates fear and cognitive behaviors. On day two rats were tested in the same chamber and lighting, but 4 olfactory gradients (i.e. vanilla, orange, lemon, and almond) were placed in petri dishes below the four corners of the HB test to promote exploratory movement. Locomotor variables including time freezing (s) time exploring (s)in zone, number of head pokes, latency to first head poke, and time head poking were recorded and post analyzed using the Anymaze® video tracking software transmitted via a ceiling mounted SONY-Handy Camera ran to a standard HP 1.6GHz computer.

6.1.6-Contextual fear conditioning

At PND 24-30 behaviorally naive rats were transferred to the testing room and remained in their home cage for 60 minutes in order to acclimate to the test room in the dark with red ambient light. After the acclimation to the testing room rats were gently picked up and placed into the context fear conditioning chamber (Med Associates) (215.9mm H x 260.35mm W x 254mm L). This test evaluates fear induced learning and memory. The testing paradigm was as
follows: (a) Day 1 acquisition phase: 120s acclimation, 10s later sound was emitted for 30s duration, after 10s of the sound presented a light was illuminated for 10s and during the last 2s of the sound a 0.5mA shock was given for 5s in duration as the conditioned aversive stimulus. Following the delivery of the shock the rats latency to break 3 infrared beams were measured every 10s for 60s followed by a 70s inter-trial-interval. Three trials were presented during day 1 which was considered the learning acquisition phase. (b) Day 2 retention phase: The exact same testing procedures were administered as in day 1 except that there was no shock delivered.

6.1.7-Auditory cued conditioning

At PND 24-30 behaviorally naive rats were transferred to the testing room and remained in their home cage for 60 minutes in order to acclimate to the test room in the dark with red ambient light. After the acclimation to the testing room rats were gently picked up and placed into the context fear conditioning chamber (Med Associates). This test used sound stimuli paired with environmental context to evaluate learning and memory. The testing paradigm was the same as the CFC test except that on Day 3 rats were place in an altered context. The chamber was the same size, but was divided diagonally with a black plexiglass (349.25mm) and the floor coated with a black rubber mat. On the opposite of the plexiglass where the rats could not access a Petri dish was filled with a vanilla extract to stimulate movement in the altered context chamber. Motion was recorded for a single trial with a baseline measure for 180 seconds followed by the onset of the same auditory cue used in the CFC training during the prior two days. However, the tone lasted 180 seconds.

6.1.8-Acoustic startle response habituation

At PND 30-46 behaviorally naive rats were transferred to the testing room and remained in their home cage for 60 minutes in order to acclimate to the test room in the dark with red ambient light. After the acclimation to the testing room rats were gently picked up and placed into the acoustic startle response chamber MED-ASR-Pro1 (Med Associates Inc., VA) for the length of the testing session (i.e. 30 minutes). This test evaluates habituation learning to repeated exposure to auditory stimuli. All subjects were administered a one block design consisting of 20 trials with 115 decibels (dB) with inter-trial-interval (ITI) of 15 ms to assess startle habituation.
6.1.9-Acoustic startle response and pre-pulse inhibition

At PND 30-46 behaviorally naive rats were transferred to the testing room and remained in their home cage for 60 minutes in order to acclimate to the test room in the dark with red ambient light. After the acclimation to the testing room rats were gently picked up and placed into the acoustic startle response chamber MED-ASR-Pro1 (Med Associates Inc., VA) for the length of the testing session (i.e. 30 minutes). This test evaluates the rats ability to reduce a startling reflex in response to a lower sound preceding a loud sound. All subjects were administered a three block design to assess pre-pulse inhibition learning. Block one consisted of 4 trials with a startle stimuli of 115 dB. Block two contained a random matrix of 16 trials with unpredictable prepulses of 75dB, 85dB, 95dB, and 105dB with an ITI of 15 ms. Block three was identical to block one. Data were driven via the motion sensor transducer platform and transmitted to a standard desktop computer to analyze the data with the supplied Med Associated Software.

6.1.10-Statistical analyses

All data were analyzed in Statistica V. 6.1 (Statsoft, Inc. Tulsa, OK). Factorial ANOVAs were used to assess Age, Treatment, and Age X Treatment interaction effects. Significance levels were set at $\alpha=0.05\%$ with a 95% ± SEM. Significant differences were determined by equal and unequal Tukey’s HSD post hoc comparisons test.
6.2-Results

Figure 29. Pb$^{2+}$ increased anxiety measures in OF and taurine reduced anxiety through GABAergic modulation in latency to exit center zone (A) and time mobile in center zone (B).

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Figure 29. Pb$^{2+}$ increased anxiety measures in OF and taurine reduced anxiety through GABAergic modulation in latency to exit center zone (A) and time mobile in center zone (B).
**Figure 29.** The OF test for center zone exploration latency revealed a *Tau Treatment* effect (DF=1, MS=14,459.5, F=8.97, p<0.004**) (A). **Figure 29.** In the OF the time mobile showed Pb$^{2+}$ effect (DF=1, MS=895.92, F=4.08, p<0.05*) and a *Tau Treatment* effect (DF=1, MS=2,348.56, F=10.7, p<0.001***) (B).
**Figure 30.** Pb$^{2+}$ showed no difference on EPM anxiety measures, but taurine decreased anxiety in control rats and increased anxiety in Pb$^{2+}$ treated rats (A&B).
**Figure 30.** The EPM test for time mobile in zone showed a *Taurine* effect (DF=1, MS=7,946.6, F=6.32, p<0.01 **), a *Zone* effect (DF=2, MS=49,216.2, F=39.12, p<0.001***), and a *Pb*²⁺ *X Taurine* interaction (DF=1, MS=6,158.3, F=4.9, p<0.03*) (A). **Figure 30.** The EPM test for number of zone entries revealed a *Pb*²⁺ effect (DF=1, MS=642.14, F=6.86, p<0.01**), a *Taurine* effect (DF=1, MS=1,138.62, F=12.16, p<0.001***), a *Zone* effect (DF=2, MS=2,389.47, F=25.51, p<0.001***), and a *Pb*²⁺ *X Taurine* interaction (DF=1, MS=571.03, F=6.09, 0.01**) (B).
Figure 31. Taurine treatment increased anxiety in Pb$^{2+}$ rats evidenced by reduced time head dipping over the open arm ledge (A) but showed no difference in total number of head dips in the EPM (B). Figure 31. The EPM time head dipping over the open arm ledge showed a Pb$^{2+}$ effect (DF=1, MS=116.21, F=4.93, p<0.03*) (A). Figure 31. Revealed no significant differences (B).
Figure 32. Taurine treatment increased anxiety in Pb\textsuperscript{2+} rats evidenced by increased acclimation time in light chamber during the first minutes in Pb\textsuperscript{2+} rats and for first 5 minutes in Pb\textsuperscript{2+} & Taurine rats in the LD test (A). However, this time spent in the light chamber was time spent freezing rather than exploring the chamber (B).
Figure 32. The LD test time in light chamber showed a $Pb^{2+}$ effect (DF=1, MS=4,196.16, F=25.80, p<0.001***) and a Taurine effect (DF=1, MS=3,538.16, F=21.75, p<0.001) and a $Pb^{2+}$ X Taurine interaction (DF=1, MS=3,866.57, F=23.77, p<0.001***) (A). Figure 32. The LD test time in light chamber immobile revealed a $Pb^{2+}$ effect (DF=1, MS=2,349.13, F=19.73, p<0.001***) and a Taurine effect (DF=1, MS=1,812.86, F=15.23, p<0.001**) and a $Pb^{2+}$ X Taurine interaction (DF=1, MS=2,419.16, F=20.32, p<0.001***) (B).
Figure 33. Frequency comparisons between the traditional HB test for anxiety escape behaviors (A) vs. a novel exploration test (B). The HB test showed a zone effect in which Pb\(^{2+}\) rats had reduced head pokes in the center zone, but this trait improved with taurine treatment (A). However, the day two’s testing procedures were more sensitive to assess neurotoxic behavioral signatures, evidencing reduced number of head pokes in both zones in Pb\(^{2+}\) rats and with recovery under taurine treatment (B).
Figure 33. We used a traditional escape response HB test along with a novel exploration HB test to tease apart the anxiety with the cognitive searching responses associated with total number of head pokes in this test. The escape response HB test (A) evidenced a Zone effect (DF=1, MS=1,392.56, F=13.1, p<0.001***). In contrast, the novel exploration HB test (B) revealed a Zone effect (DF=1, MS=925.25, F=23.22, p<0.001***), a $Pb^{2+}$ effect (DF=1, MS=3,346.05, F=83.97, p<0.001***), a Zone $X Pb^{2+}$ interaction (DF=1, MS=206.17, F=5.17, p<0.03*) and a $Pb^{2+} X Taurine$ interaction (DF=1, MS=188.93, F=4.74, p<0.03*).
Figure 34. Latency to first head poke comparisons between the traditional HB test for anxiety escape behaviors (A) vs. a novel exploration test (B). The HB test showed a zone and Pb\(^{2+}\) effect in the escape condition, with no taurine differences noted (A). However, the day two’s testing procedures were again more sensitive to assess neurotoxic behavioral signatures, evidencing increased latency to first head poke by Pb\(^{2+}\) rats in the center zone and with recovery under taurine treatment (B).
Figure 34. We assessed the differences in latency for first head poke between these two test conditions. The escape HB test (A) showed a Zone effect (DF=1, MS=10,976.8, F=11.53, p<0.001***), and a Pb$^{2+}$ effect (DF=1, MS=6,935.9, F=7.2873, p<0.009084**). The exploration HB test (B) revealed a Zone effect (DF=1, MS=135,108.2, F=32.02, p<0.001***), a Pb$^{2+}$ effect (DF=1, MS=41,485.2, F=9.83, p<0.003**), a Zone X Taurine interaction (DF=1, MS=22,882.2, F=5.42, p<0.02*) and a Zone X Pb$^{2+}$ X Taurine interaction (DF=1, MS=18, 395.4, F=4.36, p<0.04*).
Figure 35. Time comparisons between freezing and exploring behaviors (A) and total time engaged in hole poking behaviors during these test conditions (B). The HB test showed a Pb$^{2+}$ and a taurine effect in which Pb$^{2+}$ rats had increased time freezing in the escape condition and decreased time freezing in the exploration condition (A); notably, taurine recovered the behavior in the exploration condition (A). Interestingly, when comparing the time spent hole poking no Pb$^{2+}$ or taurine differences were noted in the escape conditions (B). Interestingly, Pb$^{2+}$ rats
showed a significant reduction in the exploration condition and taurine improved these behaviors treatment (B).

**Figure 35.** We evaluated the differences in time freezing vs. exploring and total time spent hold poking in each HB test condition. The behavioral engagement time comparisons between tests (A) showed a *Condition* effect (DF=1, MS=1,061,194, F=221.88, p<0.001***), a *Pb*²⁺ effect (DF=1, MS=29,813, F=6.23, p<0.01**), a *Condition X Pb*²⁺ interaction (DF=1, MS=213,911, F=44.73, p<0.001***), a *Condition X Taurine* interaction (DF=1, MS=51,794, F=10.83, p<0.001***), and a *Condition X Pb*²⁺ X *Taurine* interaction (DF=1, MS=59,831, F=12.51, p<0.001***). The comparisons for time spent head poking between tests (B) revealed a *Condition* effect (DF=1, MS=11,918.4, F=26.78, p<0.001***), a *Pb*²⁺ effect (DF=1, MS=14,878.9, F=33.44, p<0.001***), and a *Condition X Pb*²⁺ interaction (DF=1, MS=2,241.7, F=5.04, p<0.03*).
Figure 36. Differences in Context Fear acquisition and extinction learning with respect to context (A) and aversive stimuli (B). Pb\textsuperscript{2+} treated rats show no differences in fear learning, but have reduced memory consolidation of the prior days learning as evidenced by extinction trials in both context (A) and aversive (B) conditions. In addition, Pb\textsuperscript{2+} treated rats were more sensitive to aversive conditioning than controls, which may have attributed to poor working memory consolidation during training trials (B).
**Figure 36.** In the CFC context learning revealed in the extinction condition a $Pb^{2+}$ effect (DF=1, $F=21.74$, $p<0.001^{***}$) (A). **Figure 34.** In the CFC aversive learning showed in the acquisition trials a *Trial X Pb* $^{2+}$ interaction (DF=3, $F=5.14$, $p<0.001^{**}$), and in the extinction trials a $Pb^{2+}$ effect (DF=1, $F=48.89$, $p<0.001^{***}$) (B).
Figure 37. Differences in ACFC acquisition and retention learning with respect to context (A) and aversive stimuli (B). Pb\(^{2+}\) treated rats showed reduced context learning (A), and initial sensitization to aversive fear learning indicating stressful conditions that may impeded memory consolidation of the prior days learning as evidenced by retention and altered context trials in both context (A) and aversive (B) conditions.
Figure 37. In the ACFC test during the context condition showed a $Pb^{2+}$ effect ($DF=1$, $F=29.03$, $p<0.001^{***}$), a Trial effect ($DF=3$, $F=173.794$, $p<0.001^{***}$) and a $Pb^{2+} \times$ Trial interaction ($DF=3$, $F=10.09$, $p<0.001^{***}$) (A). In the AFCF aversive stimulus condition revealed a $Pb^{2+} \times$ Trial interaction ($DF=5$, $F=2.73$, $p<0.05^{*}$) and a Trial effect ($DF=5$, $F=75.718$, $p<0.001^{***}$) (B).

Figure 38. Differences in Auditory Cued Context Conditioning and generalization into alternate contexts. $Pb^{2+}$ treated rats showed reduced freezing in an altered context when compared to controls. However, once the tone previously paired with aversive foot shock was presented both control and $Pb^{2+}$ rats froze equivocally. This suggests that $Pb^{2+}$ rats may be hyper excitable and respond to new environments with increased stress.

Figure 38. In the ACFC test revealed a Condition effect ($DF=1$, $MS=18,644,039$, $F=335.41$, $p<0.001^{***}$) and a $Pb^{2}$ Effect ($DF=1$, $MS=1,265,333$, $F=22.76$, $p<0.001^{***}$).
Figure 39. Effects of Pb$^{2+}$ on non-associative startle habituation (A) and the effects of Pb$^{2+}$ treated rats PPI learning through a pre-post startle 115dB intervention and the use of taurine (B). The graph illustrates that Pb$^{2+}$ rats are hypersensitive to auditory stimuli and they have delays in habituation to non-associative stimuli (A). In addition, Pb$^{2+}$ rats have disruptions in PPI learning ability, but recover with taurine treatment. This suggests that taurine may recover GABAergic learning and memory impairments induced by Pb$^{2+}$ neurotoxicity during development.
**Figure 39.** The startle non-associative habituation test showed a *Trial* effects (DF=19, MS=424,655, F=2.47, p<0.001*** and a *Pb*²⁺ effect (DF=1, MS=11,108,541, F=64.52, p<0.001***) (A). **Figure 39.** The startle PPI pre and post test comparisons revealed a *Condition* effect (DF=1, MS=125,668, F=130.39, p<0.001***) , a *Taurine* effect (DF=1, MS=22,432, F=23.28, p<0.001***), a *Condition X Taurine* interaction (DF=1, MS=5,777, F=6.0, p<0.01**), a *Pb*²⁺ X *Taurine* interaction (DF=1, MS=8,193, F=8.50, p<0.01**) and a *Condition X Pb*²⁺ X *Taurine* interaction (DF=1, MS=10,982, F=11.4, p<0.001***) (B).

**Figure 40.** Effects of *Pb*²⁺ treated rats PPI learning through a pre-post startle 115dB intervention and the use of Baclofen.

**Figure 40.** There was no *Pb*²⁺ or Baclofen treatment differences observed. However, there was only a *Condition Effect* (DF=1, MS=148,161, F=121.72, p<0.001***).
6.3-Discussion

The toxic effects of Pb$^{2+}$ on the developing rat nervous system has been investigated to assess early developmental GABAergic disruption and its implications with altering inhibitory learning and memory. This goal was achieved using a multi-systems approach: blood lead levels (clinical physiology), qRT-PCR (molecular genetics), brain and primary neuronal culture immunology (immunohistochemical and cellular approaches), physiological cellular components (synaptosomes and protein expression) and finally through learning and memory assessment with GABA mimetic drug manipulations in the intact animal (behavioral pharmacology).

The influence of a 956ppm Pb$^{2+}$ gestational diet (i.e. from birth to sacrifice) resulted in pup mean blood lead levels (BLLs measured in μg/dL) at PND 2= 41.83 SEM± 14.17, PND 7= 34 SEM± 10.4, PND 14= 30.17 SEM± 7.77 and PND 22= 38.67 SEM±10.67. The dams at corresponding Pb$^{2+}$ exposure times evinced mean BLLs at PND 2= 37 SEM± 9, PND 7= 41.33 SEM± 21.33, PND 14= 39.33 SEM± 60.33 and PND 22= 43.6 SEM± 22.3 respectively. In contrast, control pups and dams were Pb$^{2+}$ negative. These ages were selected to determine neurodevelopmental trajectories of the GABA-shift from excitation-to-inhibition postnatally in our model.

We assessed the changes in GABAergic developmental regulatory mRNA gene expression of Caβ3, GABA$\_AR$-β3, NKCC1, KCC2, GAD 80, 86, 65, and 67 in response to Pb$^{2+}$ in the cortex and hippocampus at the aforementioned ages. Our results indicate a Pb$^{2+}$ induced up regulation of Caβ3 mRNA at PND 7 followed by a steady down regulation at PND 14 and 22. In hippocampus, Caβ3 mRNA was down regulated at PND 7 and 22. This suggests Pb$^{2+}$ inhibits Caβ3 gene regulation in both brain regions beginning at PND 7.
Notably, the GABA\(_{AR-\beta3}\) mRNA expression was slightly down regulated in cortex at PND 22, but was significantly down regulated in hippocampus at PND 7 and 22. This implicates that the hippocampus is more vulnerable to Pb\(^{2+}\) alterations in GABAergic gene expression suggesting interruption of inhibitory regulated learning and memory.

In cortex, NKCC1 mRNA was slightly up regulated at PND 2 and 7 in cortex, whereas in hippocampus it was up regulated at PND 2 and down regulated at PND 7 as a consequence of Pb\(^{2+}\). This suggests a Pb\(^{2+}\) specific divergent effect in cortex and hippocampus at PND 7 with early Cl\(^{-}\) importers increasing hyper polarization in both cortex and hippocampus at PND 2. Notably, at PND 7 Pb\(^{2+}\) increased hyper polarization would occur due to increased NKCC1 expression whereas, in hippocampus decreased NKCC1 mRNA expression would result in increased depolarization producing premature early brain excitability.

In contrast, KCC2 mRNA was up regulated at PND 2 and down regulated at PND 7 and 14 in cortex; whereas in hippocampus it was only down regulated at PND 2 and 7 then up regulated at PND 14 in response to Pb\(^{2+}\). This suggests that in cortex Pb\(^{2+}\) prematurely switches earlier than expected at PND 2 and is delayed until PND 22. In hippocampus KCC2 mRNA is reduced at PND 2-7 and over expression is observed as a compensatory consequence for early delay in this key GABAergic regulatory protein; resulting in increased Cl\(^{-}\) extrusion from the transporters and subsequently increasing depolarization. These premature delays and abnormal early expression of cotransporters indicate a disruption in the expression of the GABAergic networks in response to gestational Pb\(^{2+}\) exposure producing an organism with decreased inhibitory regulation and increased susceptibility to brain excitability.

To determine more closely how and when these GABAergic networks are most susceptible to Pb\(^{2+}\) perturbations we examined the expression of GAD enzymes as they are
precursors for synthesizing early excitatory GABA (i.e. GAD 80 and 86) and late inhibitory GABA (GAD 65 and 67). In cortex, early excitatory GAD80 and 86 mRNA were both up regulated at PND 2 and 14 in response to Pb\(^{2+}\) indicating lasting expression of gestational profiles that should not be present at the observed levels in postnatal life; thus, indicating an altered GABAergic system with persistent excitation. The same observation was noted in hippocampus, however GAD 80 was significantly down regulated at PND 2 as a consequence of Pb\(^{2+}\).

Moreover, late inhibitory GAD 65 was slightly up regulated in cortex (i.e. increased inhibition) and down regulated in hippocampus (i.e. decreased inhibition) at PND 7; whereas GAD 67 was significantly down regulated at PND 22 in both cortex and hippocampus (i.e. decreased inhibition) suggesting an altered influence in regulating GABA-dependent learning and memory and increased susceptibility to brain excitability.

We also assessed MECP2 and FMR1 mRNA to determine whether or not we could indicate select alteration in presynaptic vesicular release and postsynaptic plasticity based on our previous findings suggesting altered GABAergic networks. In cortex MECP2 mRNA was up regulated at PND 14 (i.e. increasing neurotransmitter release); whereas in hippocampus it was down regulated at PND 22 (i.e. decreasing neurotransmitter release) in response to Pb\(^{2+}\). Interestingly, we observed FRM1 mRNA down regulation (i.e. reduced postsynaptic plasticity) at PND 14. Together these results indicate that Pb\(^{2+}\) induces brain region specific effects with different trajectories and some delays in brain wiring and/or maturation that may require further evaluation at a physiological level to elucidate how Pb\(^{2+}\) alters signaling based on these molecular changes.
Following these qRT-PCR findings we evaluated whether Pb\(^{2+}\) delays the spatial-temporal distribution (i.e. the pattern and age of onset) of developmentally regulated KCC2 expression in the cortex and hippocampus as a function of age. We observed that in cortex Pb\(^{2+}\) increases KCC2 expression at PND 2 and decreases its expression at PND 14 and 22. This suggests an early inhibition of GABA in the early postnatal cortex in response to Pb\(^{2+}\) and a decreased inhibition of GABA in adolescence and adult rat brains; consistent with the late brain being susceptible to excitability.

In hippocampus DG region KCC2 expression was increased at PND 2, 7 and 22; whereas it was decreased at PND 14. Moreover, in hippocampus CA3 region KCC2 expression was increased at PND 2, 7 and 14 then decreased at PND 22 in response to Pb\(^{2+}\). This suggests that the DG is more susceptible to brain excitability at PND 14; whereas, the CA3 region is more susceptible to brain excitability at PND 22. Thus, further suggesting that Pb\(^{2+}\) induces brain region specific alterations between cortex and hippocampus and inter region changes within the hippocampus in response to Pb\(^{2+}\).

We next evaluated in primary neuronal cerebellar cultures harvested from PND 5-7 rats and treated 3 days in-vitro (3-DIV) with dose responses of PbCl\(_2\), whether or not Pb\(^{2+}\) can induce VSCC-β3 nuclear translocation consistent with our hypothesis indicating the mechanism by which Pb\(^{2+}\) would alter GABAergic synergistic signaling via the GABA\(_{\text{AR}}\) and the VSCCs. Results show that cultured neurons treated with PbCl\(_2\) at 0.05μM, 0.1μM and 0.5μM produced an increase in immunodetection of VSCC-β3 nuclear expression; whereas at exposures exceeding these levels produced negligible nuclear expression when compared to control conditions. Moreover, these data suggest that cultured neurons are more sensitive to low dose Pb\(^{2+}\) exposure and as these levels increase neurons may appear to be insensitive, but more practically
mechanisms involved in clearing/removing Pb$^{2+}$ may have been saturated becoming essentially inactivated at higher concentrations of exposure. This may produce more latent effects that can further produce neuronal excitability and surrounding glial cell death if exposures remain chronic resulting in irreparable neurotoxicity.

When cultured neurons were exposed to increasing dose responses of PbCl$^{-}$ GABA$_{AR}$-β3 expression was increased at 0.05μM, 0.1μM, 0.5μM and 1μM; whereas at 1.5μM this expression was decreased. This suggests that Pb$^{2+}$ influences the increased expression of GABA$_{AR}$S to most likely compensate for the increased neuron excitability produced by Pb$^{2+}$. In addition, this increased excitability produces synergistic effects with VSCCs having increased sensitivity to low doses of Pb$^{2+}$, which may act together as a mechanism perturbing early GABAergic networks in the developing brain consistent with our hypothesis.

We also assessed the effects of PbCl$^{-}$ on KCC2 expression in cultured neurons. Results show that KCC2 expression in cultured neurons are inhibited nearly 50% from control conditions irrespective of PbCl$^{-}$ dose. Interestingly, this data is in opposition to what we have shown from brain slice immunohistochemistry indicating that there may be some undetermined compensatory mechanism preventing the intact brain from exhibiting conditions shown in culture due to isolated in-vitro conditions.

We return our discussion to whether Pb$^{2+}$ interferes with physiological functions at these respective ages. To address this question we evaluated the spontaneous and evoked release of glutamate analog, $^{3}$H-D-Asparate, its binding and uptake in synaptosomal tissue fractions from cortex and hippocampus to assess neuronal communication in response to Pb$^{2+}$. Results showed that in cortex Pb$^{2+}$ increased $^{3}$H-D-Asparate spontaneous release at PND 2, 7 and 22 and was reduced at PND 14. In contrast, in hippocampus $^{3}$H-D-Asparate spontaneous release was slightly
increased at PND 7 and decreased at PND 14. Comparatively, \(^3\text{H}\)-D-Asparate evoked release in the cortex diminished at PND 2 and 7; whereas in hippocampus showed a trend of decreased \(^3\text{H}\)-D-Asparate evoked release from PND 7, 14 and 22.

In addition, in cortex uptake was reduced at PND 2 and increased at PND 7; whereas in hippocampus \(^3\text{H}\)-D-Asparate uptake was reduced at PND 7 and increased at PND 14 and 22. Lastly, in both cortex and hippocampus \(^3\text{H}\)-D-Asparate binding was increased at PND 2, 14 and 22, but decreased at PND 7. Taken together these data suggest that the synaptic turnover of glutamate investigated on the synaptosomal fraction has been significantly modulated by \(\text{Pb}^{2+}\) exposure.

The spontaneous release was increased at early time points with brain region selective differences and evoked release was in opposite directions of these findings; evidencing a biphasic response of \(\text{Pb}^{2+}\) having altered the synaptic proteins involved in neurotransmission from development rather than being added \textit{in-vitro} to the synaptosomal fraction preparations. Additionally, in agreement with the previous observations of brain excitability the intensity of glutamate uptake was observed to change in the following way: 1) due to increased spontaneous release by \(\text{Pb}^{2+}\), synaptosomal fractions had difficulty accumulating \(\text{Pb}^{2+}\), 2) this diminished accumulation resulted in decreased evoked release, 3) there was a significant selectively decrease in uptake at PND 2 and increased uptake at PND 7 in cortex; whereas at PND 14 and 22 increased uptake was observed in hippocampus. 4) there was increased postsynaptic glutamate binding at PND 2, 14 and 22 in cortex. Moreover, there was reduced binding at PND 7 in cortex. These findings suggest that the cortex and hippocampus are negatively affected by \(\text{Pb}^{2+}\) producing increased brain excitability with the cortex alterations proceeding those of the hippocampus.
These changes show an increase in spontaneous release of glutamate (i.e. non-specific signals); which we presume would be the same for any other neurotransmitter; specifically GABA. These non-specific signals may inappropriately communicate with incorrect targets or over stimulate to correct targets, thereby altering brain excitability during development in response to Pb$^{2+}$. In addition, these Pb$^{2+}$ induced effects may cause developmental delays brought forth by imprecise neuronal communication efficiency. The impact of such a perturbation in early development may manifest in behaviors associated with GABAergic regulation, impulsivity control, anxiety, habituation, attentional mechanisms and learning and memory processes.

In order to determine whether or not Pb$^{2+}$ gestational exposure had caused alterations in GABAergic mediated behaviors, we subjected animals to a battery of tests for anxiety (i.e. open field, elevated plus maze, light: dark and hole board), Pavlovian fear learning extinction and altered context and cued conditioning, and acoustic startle non-associative learning and with pre-pulse conditioning. The responsivity of the rats were then further evaluated through the controlled effects of taurine, a GABAergic agonist, given as an i.p. injections of 43mg/Kg 15 minutes before each test with the exception of the context fear (i.e. as taurine has been reported to reduce nociception in the anterolateral pathway and may attenuate pain sensory inputs which are used as unconditioned stimuli in classical Pavlovian conditioning).

In the open field, Pb$^{2+}$ induced fear as evidenced by rats having shorter latencies to remain in the center zone and engaging in thigmotaxis (i.e. staying by the walls and not moving into the center area). Interestingly, taurine had reduced anxiety in both controls and Pb$^{2+}$ rats by increasing their latency to move from the wall to remain in the center area longer, while freezing less in the center.
In the elevated plus maze, taurine increased locomotor activity in control rats in all zones and made them more reactive in the open arm; thus reducing their anxiety in this test. In contrast, Pb\(^{2+}\) rats were not affected by taurine, with the exception of head dips. Taurine reduced Pb\(^{2+}\) rats time and number of head dips suggesting an anxiogenic, rather than anxiolytic effect.

In the light: dark test, control and Pb\(^{2+}\) rats did not differ from one another in anxiety profiles. In contrast, taurine increased initial time freezing in the light chamber for both controls and Pb\(^{2+}\) rats. Notably, the Pb\(^{2+}\) treated rats given taurine froze substantially in the light chamber for the majority of the test session indicating a anxiogenic effect.

This first set of rats showed preliminary inferences of taurine effects on anxiety based behaviors. Since the open field showed more positive effects on taurine reducing anxiety, we decided to use another set of rats in a hold board test which contained a similar arena, with the exception of 16 1" holes in the center, to assess whether or not we could evaluate Pb\(^{2+}\) induced effects on freezing behavior absent of any painful aversive stimuli and if we could further augment the Pb\(^{2+}\) rats behavior with contextual cues (i.e. odorants) to motivate them to move and explore the test environment. The hole board test showed an ability to assess the effects of zone within this test (i.e. center vs. outer) similar to the open field, but with greater sensitivity and abilities to parse taurine effects on rat freezing and exploration behaviors across two days of testing.

Pb\(^{2+}\) rats showed increased freezing and prolonged latency to make first head poke as a fear escape response when compared to controls on day 1. Taurine had no effects on escape latency to first head poke. Interestingly, taurine recovered Pb\(^{2+}\) rats exploration latency on day 2 when compared to Pb\(^{2+}\) rats and were equivocal to controls. Taurine did not produce an effect on control rats. Pb\(^{2+}\) rats administered taurine also exhibited less time freezing on day 1 and more
time exploring on day 2 when compared to Pb$^{2+}$, control and control and taurine rats. The same was observed for time spent hole poking. There were no taurine effects on control rats. These data suggest that Pb$^{2+}$ rats may respond to differently to contextual environmental cues. The state of anxiety or distress that the rat undergoes in novel testing situations may be over active in Pb$^{2+}$ rats which is why taurine recovers behaviors in the hold board test. In contrast, control rats experience the same contextual environmental cues, but are unaffected by taurine administration; suggesting that the Pb$^{2+}$ rats may have an altered GABAergic system which is more sensitive than the control rats.

In order to further assess whether Pb$^{2+}$ rats respond differently to contextual environmental cues we subjected another set of rats to a classical Pavlovian conditioning paradigm. This test revealed that Pb$^{2+}$ rats learning abilities are not different from controls, but Pb$^{2+}$ rats are more sensitive to aversive stimuli (i.e. Pb$^{2+}$ makes them more reactive to stressors) and they are less inclined to pick up the contextual environmental cues due to this elevated stress response. The consequence of these Pb$^{2+}$ induced effects produce diminished memory consolidation during testing and poor retention during extinction trials 24 hrs later.

In order to better understand context learning and its contribution to fear acquisition and extinction we tested another set of rats and subjected them to an auditory cued and altered context test paradigm. These Pb$^{2+}$ rats exhibited similar problems to the previous groups where reduced contextual learning was observed and poor retention; whereas, Pb$^{2+}$ rats exhibited increased sensitivity to aversive conditions, were more reactive and still produced poor retention. Notably, Pb$^{2+}$ rats were able to evince memory from the initial training sessions in an altered context 48 hrs later when they were presented with the conditioned sound that was paired with the aversive foot shock. This indicated that the rats were able to recall the sound and its
associative penalty in another context; therefore auditory processing was intact. However, we were not certain as to whether or not these animals were hypersensitive to auditory stimuli as high reactives, similar to the aversive foot shock. This was peculiar since in the initial testing contextual environment these rats produced poor retention, but in an altered context they froze more due to the increased reactivity to the sound duration rather than to context memory.

To address this last aspect of auditory sensory contribution/impairment to learning and memory we subjected rats to the acoustic startle response with pre-pulse inhibition. This test permits us to evaluate the contribution of the rats auditory sensory pathway and its subsequent sensori-motor behavior with taurine to determine whether Pb$^{2+}$ decreased inhibition can be recovered pharmacologically as a potential therapy for Pb$^{2+}$ exposure.

We first exposed naive rats to 20 continuous trials of 115dB with varied ITIs and assessed their ability to habituate without any other associated cues (i.e. non-associative learning). Results showed that Pb$^{2+}$ rats were more reactive to the sound stimulus and took longer to habituate than controls. This confirmed our observation of Pb$^{2+}$ rats being high reactives to stressors and this sensitivity could, by virtue alone, reduce learning based on poor attentional, increased impulsivity due to high reactivity, and neuronal processes due to a compromised/diminished GABAergic system (i.e. reduced GABAergic inhibition results in hypersensitivity and increased brain excitation).

We then tested another set of naive rats under a startle pre-post test followed by a pre-pulse paradigm (i.e. 75dB, 85dB, 95dB, and 105dB presented randomly prior to the startle 115dB) to evaluate inhibitory learning of the startle pulse. The results showed that Pb$^{2+}$ rats had minimal inhibition in response to the startle pulse following pre-pulse inhibition. Interestingly,
taurine had no effect on control rats, but taurine significantly increased inhibition in Pb$^{2+}$ treated rats; even more so than controls.

Lastly, we wanted to assess whether or not the GABA$_{AR}$ or the GABA$_{BR}$ was the contributing target of GABA inhibition or lack thereof in response to Pb$^{2+}$ treatment. To answer this question we ran another group of rats and tested them identically, however they were administered either 3mg/Kg, 6mg/Kg, or 10mg/Kg of Baclofen, a GABA$_{BR}$ agonist, i.p. 15 minutes before testing. The GABA$_{BR}$ showed no differences as an effect of dose in both controls or Pb$^{2+}$ treated rats. Moreover, there were no control (+) Baclofen effects when compared to Pb$^{2+}$ (+) Baclofen indicating that either the GABA$_{BR}$ system was not involved in regulating pre-pulse inhibition mediated by this behavioral assay and/or the GABA$_{AR}$ plays more of a critical role in pre-pulse inhibition then its possible ratio of GABA$_{BR}$s. In addition, GABA$_{BR}$s can also activate glycine receptors on the spinal cord and essentially equally reduce all motor activity in this test thereby making it indiscernible to parse between GABA$_{BR}$s influence in a sensori-motor task. The selective effects of taurine on recovering the inhibitory learning through the pre-pulse test is quite convincing that Pb$^{2+}$ induces stress and hyperactivity due to a reduced GABAergic system, which can be ameliorated by taurine.

This suggests that taken together taurine, or another GABA$_{AR}$ agonist, may be a potential exogenous pharmacotherapeutic agent for compensating for Pb$^{2+}$ induced neurodevelopmental perturbations and subsequent lifelong effects regarding the following: 1) reduced efficiency of the GABAergic system, 2) increased reactivity and hypersensitivity to sensory and aversive stimuli, 3) reduced attentional based mechanisms due to elevated stress responding, 4) difficulty in habituating to novel environments, 5) and impaired cognition and memory retention negatively impacting behavior.
In conclusions this body of work has identified the following:

- Gestational \( \text{Pb}^{2+} \) interferes with immature neuronal VSCCs and GABA\textsubscript{AR} mediated gene regulation in early development which has never been reported in this framework we present here.

- Specifically, gestational \( \text{Pb}^{2+} \) alters the expression pattern of the genes regulating the GABA-shift in cortex and hippocampus. Brain regions are differentially affected.

- Gestational \( \text{Pb}^{2+} \) induces developmental delays through altered expression of KCC2 differentially affecting cortex and hippocampus excitation-to-inhibition signaling.

- Gestation \( \text{Pb}^{2+} \) induces altered neurotransmitter signaling resulting in increased brain excitability post synaptically.

- Gestational \( \text{Pb}^{2+} \) disrupts GABAergic regulation of emotional and cognitive behaviors producing deficits in inhibitory learning and memory which can be recovered by taurine pharmacotherapy.
Future studies based on what we report here can be designed in the following ways to further this body of work:

- Investigate the effects of gestational taurine and Pb\(^{2+}\) exposure in potentially ameliorating Pb\(^{2+}\) induced gene dysfunction in early development.
- Assess in primary neuronal cultures taurine and Pb\(^{2+}\) dose responses and their effects on VSCC-β3, GABA\(_{AR}β3\), and KCC2 expression.
- Characterize the GDP profiles in hippocampus at PND 2, 7, 14, and 22. Then assess dose dependently how taurine may play a neuroprotective role in regulating Ca\(^{2+}\) homeostasis under Pb\(^{2+}\) altered gene expression.
- Assess seizure susceptibility as a result of increased brain excitability induced by Pb\(^{2+}\) under GABAergic and Glutamatergic pharmacology.
- Assess more advanced cognitive behavioral tests to understand Pb\(^{2+}\) induced impulsivity and reduced inhibitory control and taurine intervention.
7.0-Conclusions

Here we evaluated through qRT-PCR, immunohistochemistry of brain slices and primary cerebellar granule cell neuronal cultures, synaptosomal physiology, and behavioral pharmacological assays the effects of Pb$^{2+}$ on GABAergic neural development in the rat model. Our data suggest that Pb$^{2+}$ causes disruption of the GABA shift prior to glutamatergic activation. These disruptions in early brain wiring result in altered brain connections, disrupted synaptogenesis, and diminished GABAergic inhibition-to-excitation balancing resulting in hyper excitable, stress sensitive and learning compromised rats. In addition, GABA$_{AR}$ drugs may be of benefit to investigate as potential neuroprotective target therapies in this experimental model of Pb$^{2+}$ toxicity. Notably, other rodent strains and gender differences may not share the same outcomes identified in our studies. In addition, the timing at which and exposure of Pb$^{2+}$ may produce varied effects; however our model was restricted to a 956ppm Pb$^{2+}$ exposure level that began gestationally and proceeded throughout life. When such a pharmacotherapeutic intervention of GABA$_{AR}$ drugs should begin is unclear at this time, but evidence from this body of work suggest it may be of value. Early intervention would appear best. However, too much GABAergic activation may present with the same resultant issues identified here as increased brain excitability throughout life. We would suggest that the best time to intervene may be best suited during the postnatal day 7-14 plastic 'critical period' (i.e. defined as the period when astrocyte proliferation, dendrite growth, synapse formation, and spine growth are maximal). This ‘window’ would permit therapeutic action and possible increase the survival of many necessary neurons during synaptogenesis increasing the ratio between plasticity-to-pruning. We suggest that gestational Pb$^{2+}$ exposure causes a reduced accuracy in the precision of cortical and hippocampal neuronal pruning which may obscure the early developmental transmitting signals
while increasing non-specific background activity resulting in inappropriate or improper network connections that persist throughout the lifespan consistent with cognitive impairments purported in the vast literature.
8.0-References


