

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

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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 Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ 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 Pb²⁺. 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 Pb²⁺ at low micro molar concentrations

induced VS⁺CC-β3 nuclear translocation and GABA_{AR} upregulation. KCC2 expression was inhibiting in cultures by Pb²⁺. Synaptosomal effects of Pb²⁺ revealed altered glutamate accumulation and handling with increased spontaneous and decreased evoked release in significantly modulated by Pb exposure suggesting altered brain synaptogenesis. Pb²⁺ exposure resulted in increased binding suggesting post synaptic modification in cortex and hippocampus increasing brain excitability. Behaviorally, Pb²⁺ exposure resulted in increased anxiety, impulsivity, stress, and disrupted learning and memory regulated by inhibitory circuits that were recovered with taurine, a GABA_{AR} agonist, administration. Specifically, Pb²⁺ disrupted contextual and auditory associative learning. Taken together, these results suggest that Pb²⁺ interferes with early VS⁺CCs and GABA_{AR} 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.

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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	adenylyl 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 _R s	<i>N</i> -methyl-D- <i>aspartate</i> receptors
Mg ²⁺	magnesium
Na ⁺	sodium
CREB	cyclic AMP response element binding protein
MAPK	mitogen activated protein kinase

GABA	<i>gamma-aminobutyric acid</i>
CAMKII	Ca ²⁺ /calmodulin-dependent kinase
CAMKIV	Ca ²⁺ /calmodulin-dependent kinase-IV
ERK	extracellular-regulated kinases
NKCC1	Na+-K+-Cl-Cl-cotransporter
KCC2	K+-Cl-Cl-cotransporter
Cl ⁻	chloride
GDPs	Giant Depolarization Potentials
AMPAs _S	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
Ach	acetyl choline
LTD	long term depression
CA	<i>Cornu Ammonis</i>
Efcab1	extracellular face hand calcium binding domain
BDNF	brain derived neurotrophic factor
DREAM	downstream regulatory element antagonist modulator
CCAT	Ca ²⁺ channel associated transcriptional regulator
GAD	<i>glutamate decarboxylase</i>
LE	Long Evans Norwegian hooded rats
EDTA	ethylenediaminetetraacetic acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
IHC	immunohistochemistry
Lux	luminescence
OF	open field test

EPM	elevated plus maze
LD	light/dark test
HB	hole board test
CFC	context fear conditioning test
ACFC	auditory cued fear conditioning test
ITI	inter-trial-interval
Acq	acquisition learning
Retent	retention learning
Ext	extinction learning
ASR	acoustic startle response test
dB	decibel
ITI	inter-trial-interval
PPI	pre-pulse inhibition

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.

1.0 - Background and Significance:

1.1-Blood lead levels and their relation to neurotoxicity

Lead (Pb²⁺) 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 "*Pb²⁺ may negatively affect early synaptogenesis disrupting early brain plasticity during critical stages of development.*" Children are most vulnerable to Pb²⁺ 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 Pb²⁺ is cleared in urine via filtration in the kidneys (Chisolm et al., 1991). Absorbed Pb²⁺ 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 Pb²⁺ in blood is ~ 35 days, ~ 2 years in brain, and dependent upon the size and amount of calcium (Ca²⁺) accumulation in bone, it can remain circulating for decades (Lidsky and Schneider, 2003). In addition, Pb²⁺ is transported into the brain by calcium-adenosine triphosphate enzyme pumps (Ca²⁺-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 Pb²⁺ toxicity. However, it fails to address the neurological sequelae associated with childhood Pb²⁺ poisoning (Toscano and Guilarte, 2001) due to continuous need for invasive procedures that children find aversive. Moreover, it does not prevent mobilization of skeletal Pb²⁺ stores that can persist years following exposure (e.g. the femur has a duration of Pb²⁺ mobilization from bone over a course of ~30 years post initial exposure) (Gulson et al., 2003). Moreover, Pb²⁺ exposed mothers

bearing children can produce increased toxicity to their children by Pb²⁺ ability to enter the placenta (Hu and Hernandez-Avila, 2002). Children chronically exposed to Pb²⁺ endure brain damage as evidenced by reductions of 0-5 intelligent quotient (IQ) scaled points for every increase in 10µg/dL of Pb²⁺ 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 µg/dL; which may have already caused irreparable IQ deficits ranging from 15-20 points (Lidsky and Schneider, 2006; Bellinger, 1995) depending on the initial Pb²⁺ concentration and duration of exposure. Moreover, reduced IQ scores induced by Pb²⁺ 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 Pb²⁺ burden the more observable symptomatic conditions were observed.

The body of literature on Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ 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.

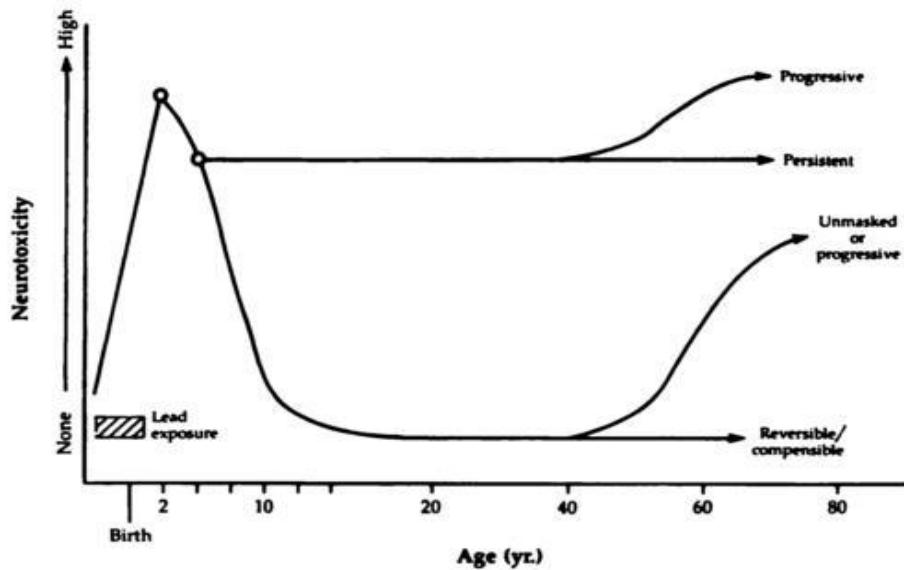


Figure 1. Suggested developmental timeline for unmasking Pb^{2+} symptoms as a function of age dependent biological compensation and neurodegeneration prior to the advancement of technology and more sensitive research studies [Adapted from Silbergeld (1992)].

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 $10\mu\text{g}/\text{dL}$ (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**.).

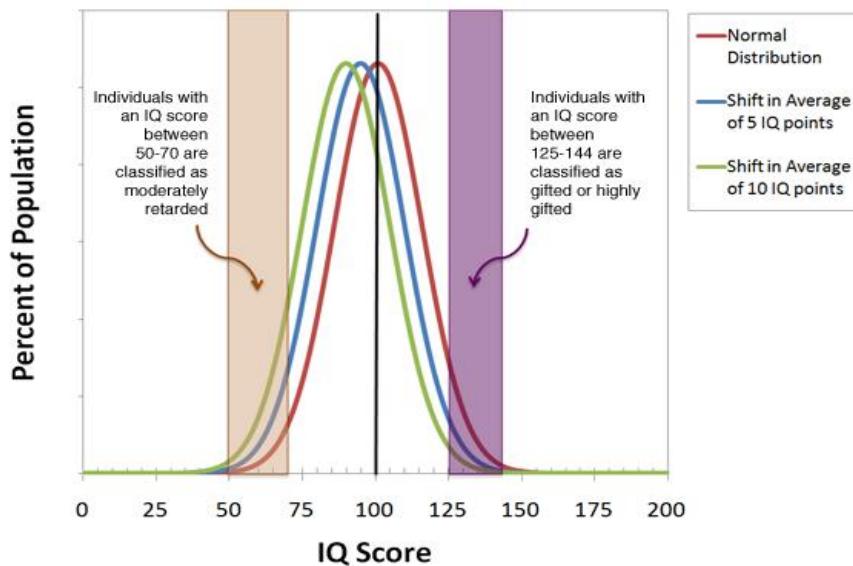


Figure 2. Simulation of governmental financial burden as a function of Pb^{2+} induced IQ reductions in children [Adapted from Godwin, 2009].

In the United States from 1976-1980 it was estimated that 88% of children between 1-5 years old had elevated BLLs $>10\mu\text{g}/\text{dL}$ (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**.).

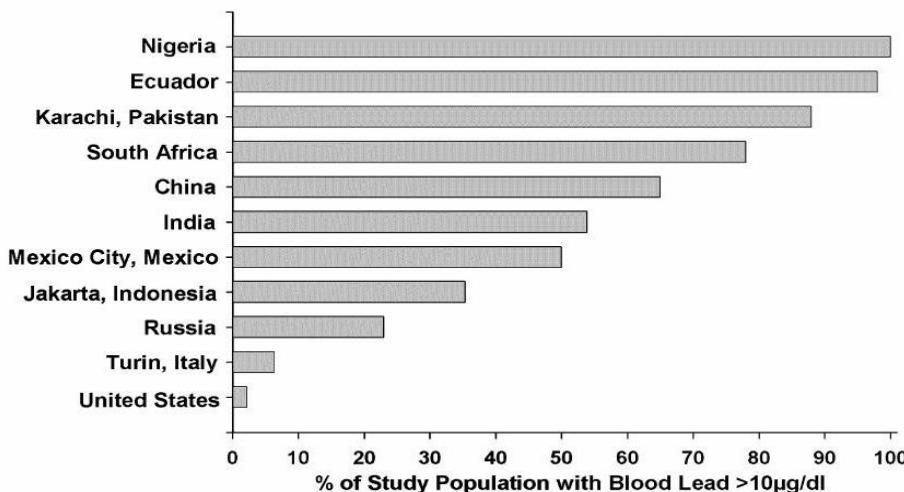


Figure 3. Comparison of elevated BLL prevalence between the US and other countries
[Adapted from Toscano and Guilarte, (2005)].

Interpretation of data from Pb²⁺ 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 Kellogg, 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 Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ have resulted in unrefined animal models (Spence et al., 1985; Minnema and Michaelson, 1986; Strużyńska and Rafalowska, 1994; Gilbert et al., 1999). Effects of Pb²⁺ on animal tissues studied *in vitro* are also conflicting due to both poor detection methods in overcoming Pb²⁺ 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 Pb²⁺ neurotoxicity in early life, whereas young females are more susceptible to immunotoxic effects of Pb²⁺. This suggests sex differences being differentially affected in response to Pb²⁺. These findings elucidate that Pb²⁺ may disrupt specific pathways in the endocrine system with influence towards emotional learning, anxiety, and fear conditioning.

1.2-Pb²⁺ 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 Pb²⁺ in a live behaving organism with assessment of the Pb²⁺-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 Pb²⁺ consequences on neuron populations. Oligodendrocytes are most vulnerable to Pb²⁺ exposure (Tang et al., 1996) and their early progenitors far more susceptible to Pb²⁺ disruptions than mature oligodendrocytes (Deng et al., 2001). Suggesting fetal neurons are more vulnerable to Pb²⁺ toxicity than mature neurons. Pb²⁺-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. Pb²⁺ 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 Pb²⁺ exposure (Dabrowska-Brouta et al., 2000).

Tiffany-Castiglioni et al., (1989) proposed a '*lead sink*' hypothesis suggesting that Pb²⁺ accumulates in astrocytes within the developing and mature brain, indirectly perturbing neurons. Astrocytic Pb²⁺ 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 $31.9\mu\text{g}/\text{dL}$, consistent with the emergent chelation criteria for intervention at $\text{BLLs} < 39\mu\text{g}/\text{dL}$ (i.e. $\sim 1.5\text{-}1.7\mu\text{M}$ 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 Pb²⁺ competition with Ca²⁺ mishandling in neonatal and adult brains (Singh and Jiang, 1997; Silbergeld et al., 1980). However, with increased focus of Pb²⁺ 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

Ca²⁺ enters the cell via two distinct routes: the *voltage sensitive calcium* channels (VSCCs) or the glutamatergic *N-methyl-D-aspartate* receptors (NMDA_{Rs}); which are both ligand and voltage-sensitive resulting in widespread changes in gene expression through NMDA_R Ca²⁺ influx. Of the Glutamatergic receptors the NMDA_R have higher selective permeability to Na⁺ and Ca²⁺; which produce fast activation and slow inactivation channel kinetics. The activation of NMDA_{Rs} require large membrane depolarizations (above ~50mV), as well as, the binding of glutamate and its co-agonist glycine to remove the magnesium (Mg²⁺) ion depolarization block which resides in the ion channel pore (Gallin and Greenberg, 1995). Once Mg²⁺ is removed, sodium (Na⁺) and Ca²⁺ influx increase membrane excitability and Ca²⁺-dependent second messenger signals.

In contrast to NMDA_{Rs}, VSCCs have selectivity for only Ca²⁺ 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. Ca²⁺ 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 Ca²⁺ 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 α1, β, α2, δ and γ (See **Figure 4**).

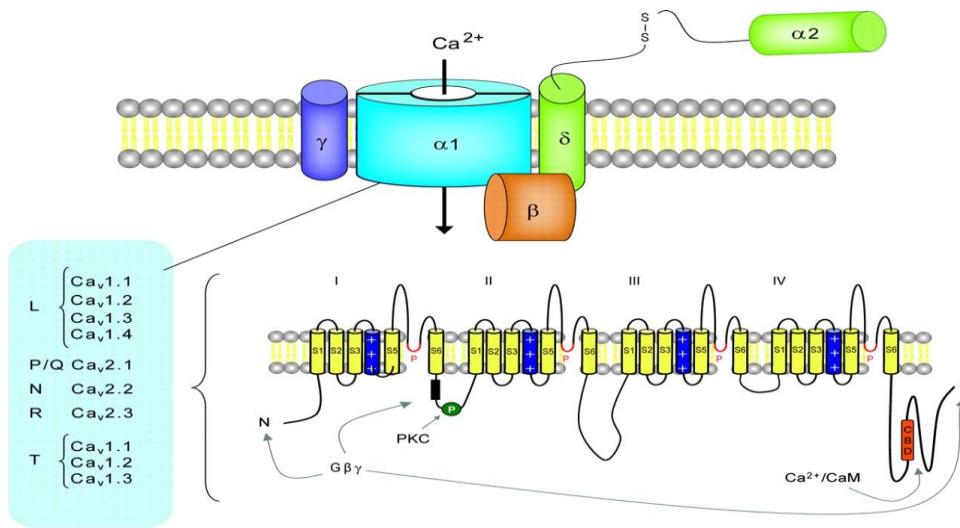


Figure 4. The L-Type VSCCs main $\alpha 1$ subunit and its β , δ and γ ancillary subunits. Note that the β subunit is the only intracellular target of this channel in which nuclear translocation of the C-terminus has been reported to augment and maintain Ca^{2+} homeostasis in neurons [Adapted from Benarroch, (2010)].

From these arrangements, $\alpha 1$ 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 , $\alpha 2$, δ and γ) are responsible for intracellular and extracellular transmembrane VSCC modulation. VSCC subunits encoded by $\text{Ca}_{\text{v}1.1}$ are expressed in skeletal muscle and cardiomyocytes (Hase et al., 1994; Mejia-Alvarez et al., 1994; Klugbauer et al., 2002), The subunits $\text{Ca}_{\text{v}1.4}$ are expressed in retinal tissues and dorsal root ganglia (Morgans, 2001; Yusaf et al., 2001). Notably, two particular subunits encoded by $\text{Ca}_{\text{v}1.2}$ and $\text{Ca}_{\text{v}1.3}$ mRNA in brain tissue contributes to $\alpha 1$ 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 $\text{Ca}_{\text{v}1.2}$ and $\text{Ca}_{\text{v}1.3}$ α 1 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.**).

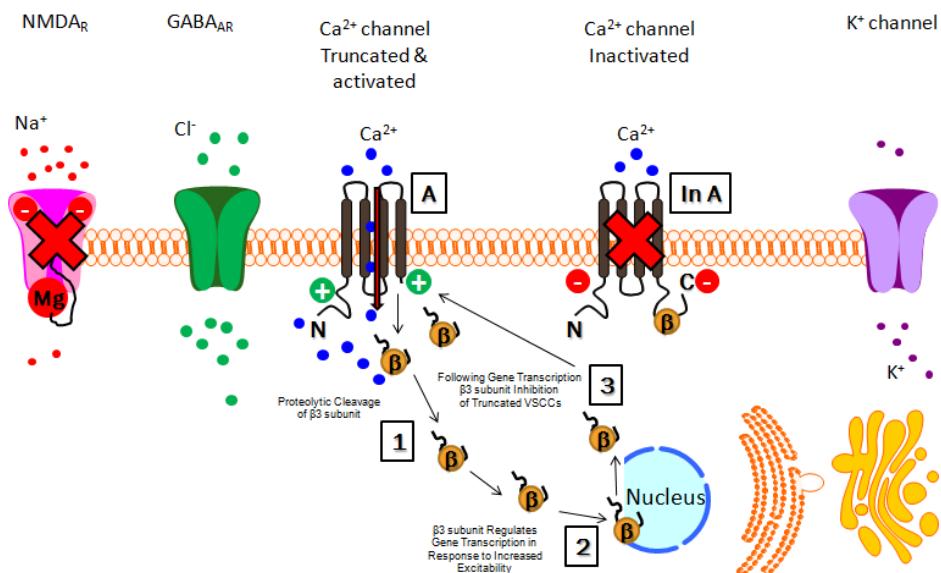


Figure 5. The L-Type VSCCs $\beta 3$ subunit autoregulation of cell excitability in early immature neurons.

L-Type VSCCs are critical for early immature neurotransmitter release, facilitating specific developmental events that dictate the temporal and spatial expression patterns of $\text{Ca}_{\text{v}1.2}$ and $\text{Ca}_{\text{v}1.3}$ mRNA. This developmental $\text{Ca}_{\text{v}1.2}$ and $\text{Ca}_{\text{v}1.3}$ mRNA expression trajectory may be susceptible for altered expression of the VSCC auxiliary subunits in response to Pb^{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 $2\text{mM}:[\text{Ca}^{2+}]_e$ vs. $50\text{-}200\text{nM}:[\text{Ca}^{2+}]_i$), 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* (nACh_R), 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

patterns consistent with early theories of Pb^{2+} -induced disruption of synaptogenesis proposed by Goldstein (1992).

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_{RS} 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_{RS}, GABA_{ARS}, 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.**).

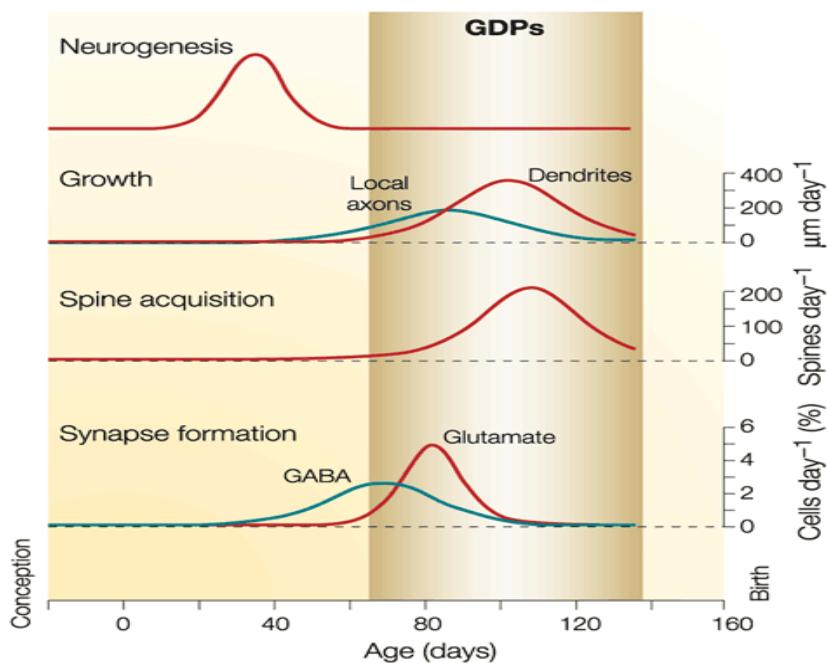


Figure 6. Neurodevelopmental trajectory and GDP critical period illustrating GABAergic neurons preceding the onset of functional glutamatergic synapse formation [Adapted from Ben-Ari et al., (2007)].

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

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 Pb²⁺ inhibits GABA neurotransmission, nerve terminal preparation studies, otherwise known as synaptosomes, have been widely used to focus solely on transsynaptic communication. Ca²⁺ influx through VSCC regulates the liberation of neurotransmitters from presynaptic nerve terminals (Turner et al., 1993). In synaptosomal preparations, Pb²⁺ enters isolated nerve terminals and is further sequestered in mitochondria (Silbergeld et al., 1977). Previously, Regunathan and Sundaresan, (1985) conducted synaptosomal studies under Pb²⁺ treatments and observed reduced postsynaptic glutamate binding and presynaptic uptake from rat cortex, cerebellum, and brainstem.

Jablonska et al., (1994) showed that chronically Pb²⁺-treated rats have decreased GABA uptake, increased dopamine uptake that were independent of Ca²⁺ concentrations, with morphological changes in the synaptic vesicles and mitochondria. Pb²⁺ 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 Pb²⁺ increases spontaneous neurotransmitter release and block depolarization dependent neurotransmitter release across transmitter systems (Goldstein, 1980).

To further elucidate Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ exposure reduced KCl dose-dependent GABA release from spinal cord synaptosomal preparations. Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ 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⁺-dependent glutamate transporter. These results may also explain the increase in spontaneous neurotransmitter release under Pb²⁺ 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 Pb²⁺ induces direct inhibition of VSCC in developing hippocampal slices attenuating action potential dependent GABA release as a more precise mechanism for Pb²⁺ neurotoxic cognitive impairments in early development.

1.6-The GABA shift is developmentally regulated by the Ca²⁺-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 Ca²⁺-dependent inducible *Slc* genes (i.e. solute carriers that transport molecules across the cell membrane) critical for developmentally regulating the GABA shift: (1) the gene that encodes for the Na⁺-K⁺-Cl⁻Cl⁻cotransporter (NKCC1) is *Slc12a2* and (2) *Slc12a5* for the K⁺-Cl⁻Cl⁻ 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 $[Cl^-]_i$ when compared to the $[Cl^-]_e$ (Ben-Ari, 2002; Ben-Ari et al., 2007) (See **Figure 7**).

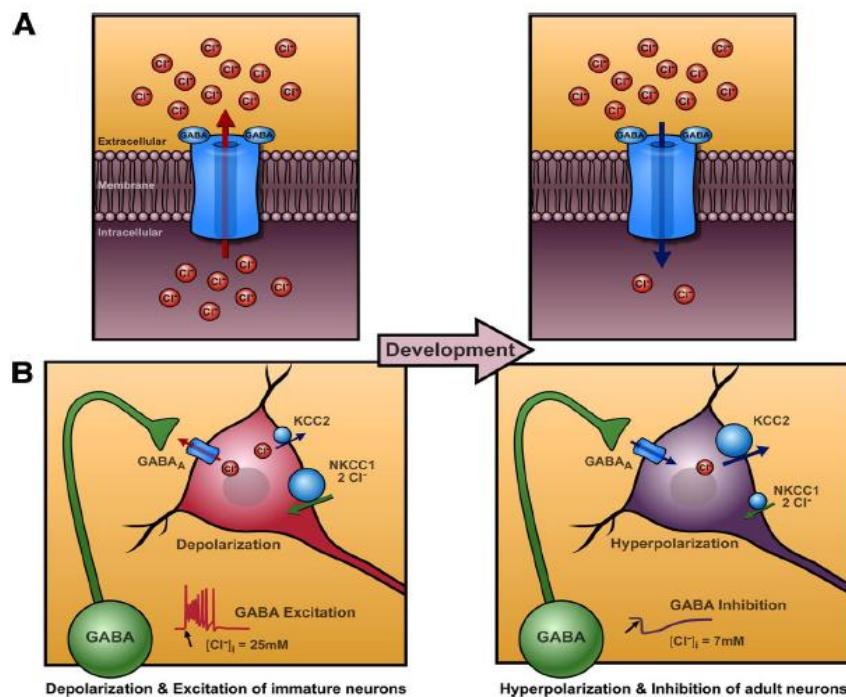


Figure 7. The Cl^- concentration and electrochemical gradients that maintain early GABAergic excitation and mature inhibition based on the NKCC1 and KCC2 Cl^- transport proteins [Adapted from Ben-Ari et al., (2007)].

Therefore, presynaptic GABA secreting neurons can excite immature post synaptic neurons expressing GABA_{AR}'s, as long as the threshold is below E_{Cl^-} and the driving force of Cl^- remains at higher intracellular concentrations. This critical developmental process is regulated by synergistic activity between VSCC and GABA_{AR}'s. Ca^{2+} influx through VSCCs depolarize neurons, activate GABA receptor permitting the efflux of Cl^- inducing further depolarization

evidencing synergistic activity between VSCCs and GABA_{AR}'s. Following VSCC and GABA_{AR} synergistic action, GABA neurotransmission excites post synaptic cells, in turn, activating NMDA_{Rs}. NMDA_{Rs} facilitate synchronized activity between the Ca²⁺ 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 Ca²⁺-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 Pb²⁺ 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 Ca²⁺ influx due to the cooperativity from the initially inactivated *α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor* (AMPA_{Rs}) and NMDA_{Rs}.

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_{ARS} (Ben-Ari, 2002). Interestingly, when immature neurons were exposed to GABA_{AR} 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 Pb²⁺ at nanomolar concentrations (Bressler et al., 1999) prematurely establishing inappropriate neural network signals altering GABA excitation-to-inhibition.

1.7-Pb²⁺ 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 Ca²⁺ influx in response to changes in membrane potential, (b) they are highly permeable to Ca²⁺ in contrast to other potential ions, (c) VSCCs must be localized in areas on neurons where they can easily access relevant regulatory targets of Ca²⁺-dependent signaling cascades; thus allowing for rapid localized increases in [Ca²⁺]_i, (d) lastly, they must undergo modulation and diverse conformations to permit selective regulation of different classes of VSCCs (Tsien and Barrett, 2005).

Moreover, Pb²⁺ can influx into the cytosol in bovine adrenal medullary cells through L-type VSCCs (Sun and Suszkiw, 1995; Simons and Pocok, 1987). Once Pb²⁺ invades the intracellular cytosol it is able to perturb L-type VSCCs kinetics by increasing I_{Ca} through attenuation of the calcium-dependent steady state inactivation of the L-type VSCCs (Sun and Suszkiw, 1995). In response to cytosolic Pb²⁺ increasing Ca²⁺ conductance, the subsequent increase in [Ca²⁺]_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 Pb²⁺ exposure. In addition, internal endoplasmic reticulum stored of Pb²⁺ may be released into the cytosol activating second messenger systems altering gene activity; otherwise, cytosolic Pb²⁺ 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 Pb²⁺ diet in the rat and changes in hippocampal gene expression via microarray analyses. They found that Pb²⁺ 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 Pb²⁺ treatment, whereas females exhibited a significant 0.6-fold reduction in hippocampal gene expression (Schneider et al., 2011). Ca²⁺ signaling can be either indirectly activated by Ca²⁺-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 Ca²⁺ sensors (Leclerc et al., 2012).

Currently, a Ca²⁺-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 (Carrión et al., 1999; Mellstrom and Naranjo, 2001). Moreover, a unique concept for VSCCs arose, whereby the Ca²⁺ 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 *Ca²⁺-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 Pb²⁺ and gender further altered expression patterns, which may have resulted from Pb²⁺ alterations in CCAT regulation. NMDA_{R2A} mRNA in the developing hippocampus of gestational Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ (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 Ca²⁺ channels being localized near NMDA_R in cells and their synergistic actions in learning and memory, NMDA_{RS} during early development are present but inactive leaving only GABA_{ARS} and VSCCs as mediators of excitation. Not until the GABA shift is completed in early development do the NMDA_{RS} take on a sequential role in regulating Ca²⁺ 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^{2+} to alter the expression of genes critical in early GABA development prior to the activation of the NMDA_Rs and the establishment of functional neuronal circuits. Here, we hypothesize that in response to gestational Pb^{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_R system being activated would suggest that not only are glutamatergic neurons susceptible to Pb^{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^{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^{2+} is responsible for immediate-early gene induction, indicating that any disruption of this signaling pathway, such as those produced by Pb^{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^{2+} resulting in altered gene induction during early development (See **Figure 8.**):

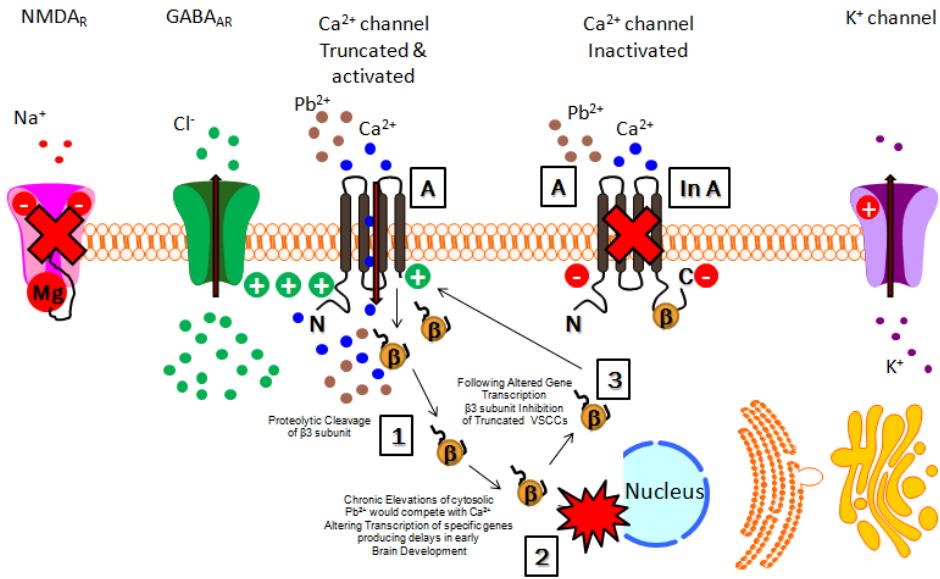


Figure 8. Hypothesis Model Pb^{2+} Competition Through VSSCs Reduces $\beta 3$ Subunit Autoregulation of Cell Excitability Reducing Gene Regulation Resulting in Developmental Delays.

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 $[\text{Ca}^{2+}]_i$ 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- $\beta 3$ subunit (CCAT) upon neuronal excitation, translocates from the membrane to the nucleus and transcribes mRNA coding from the VSCCs gene CACNB $\beta 3$ (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- $\beta 3$ subunit transcription factor in response neuronal excitability (Gomez-Ospina et al., 2006). Moreover, VSCCs- $\beta 3$ activity increases as a function of post natal development and is highly expressed in GABAergic neurons in early development (Gomez-Ospina et al., 2006). Thus, Pb^{2+} may disrupt this VSCCs- $\beta 3$ 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 Pb²⁺ 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 Pb²⁺ 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 Pb²⁺, through competition with initial Ca²⁺ influx and inhibition of Ca²⁺-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 Pb²⁺ exposure may significantly alter the Ca²⁺-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_{AR} expression in cultured cerebellar neurons to test our hypothesis. We hypothesize that Pb²⁺ attenuation of VSCC's in early development produces an upregulation in CACNB β3 mRNA expression channelopathies and a down regulation of GABA_{ARS} 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 KCC₂ 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⁻ importers regulating GABAergic excitation), *KCC2* (i.e. a marker for late Cl⁻ exporters regulating GABAergic inhibition), *GABA_{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 Pb²⁺ exposure.

- We predict that the effects of Pb²⁺ on these genes are age- and brain region-dependent. We will investigate the Pb²⁺-induced changes in the whole frontal cortex and hippocampus to assess the spatial and temporal distribution of *CACNB β3* and *GABA_{AR} β-3* across development.
- We postulate that the aforementioned altered molecular mechanisms of action in response to Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ 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) (Burkowski, 1964) behavioral consequences are expected. In addition, we will assess the potential recovery on Pb²⁺ induced disruptions by GABAergic agonists as a potential pharmacotherapeutic intervention for Pb²⁺ toxicity. Together the aforementioned effects may help explain the initial stages of Pb²⁺ induced perturbations of early GABAergic development prior to the onset of the AMPA_{Rs} and NMDA_{Rs} role in learning and memory.

The delicate interplay between both the GABA_{ARS} and VSCCs when challenged by chronic gestational low level Pb²⁺ exposure do not result in organism death. Thus, biochemical compensations exist in response to this Pb²⁺ neurotoxicity that, in turn, produces altered brain wiring and synaptogenesis underlying the aforementioned behavioral outcomes produced in our rat model.

2.0-Chronic Gestational Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ toxicity. Additionally, rat brains were harvested, Cryoprotected (or homogenized) and stored at -80°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 1 μ g/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 \pm SEM.

2.2.-Results

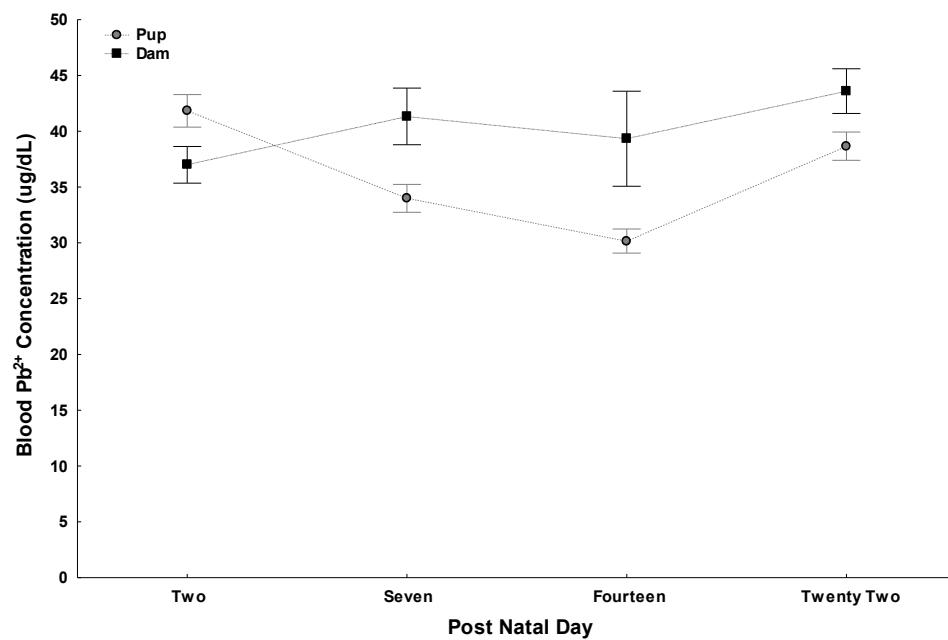


Figure 9. Dams and pups BLL subject comparisons as a function of age. Pups at PND 2 had increased BLLs when compared to their dams. Following PND 2 a reversal of BLL profiles were observed and remained until PND 22 where both subjects leveled off.

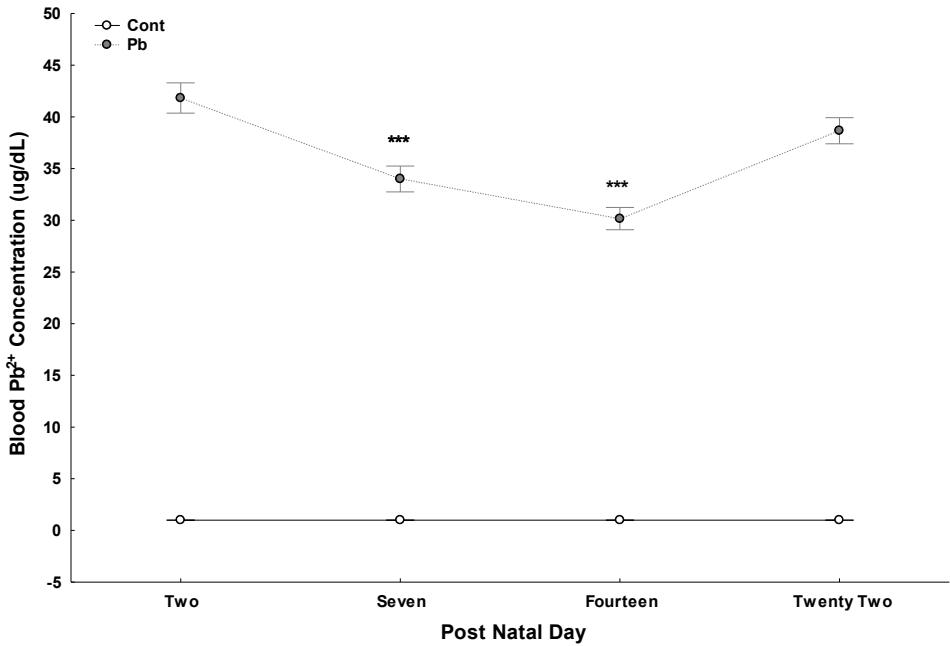


Figure 10. BLL treatment comparisons as a function of age during development. Control rats were Pb²⁺ negative throughout development. There was a significant difference in Pb²⁺ treated rats in all ages when compared to controls ($p<0.001^{***}$). Pb²⁺ treated rats showed significant differences at PND 7 ($p<0.001^{***}$) and 14 ($p<0.001^{***}$) when compared to PND 2 rats. In addition, the Pb²⁺ 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 Pb²⁺ gestational diet (i.e. from birth to sacrifice) resulted in pup mean blood lead levels (BLLs measured in $\mu\text{g}/\text{dL}$) at PND 2= 41.83 SEM \pm 14.17, PND 7= 34 SEM \pm 10.4, PND 14= 30.17 SEM \pm 7.77 and PND 22= 38.67 SEM \pm 10.67. The dams at corresponding Pb²⁺ exposure times evinced mean BLLs at PND 2= 37 SEM \pm 9, PND 7= 41.33 SEM \pm 21.33, PND 14= 39.33 SEM \pm 60.33 and PND 22= 43.6 SEM \pm 22.3 respectively.

In contrast, control pups and dams were Pb²⁺ 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 ($\sim 42\mu\text{g}/\text{dL}$) in contrast to their dams ($\sim 37\mu\text{g}/\text{dL}$) and at PND 7 these observations reversed in that the dam had elevated BLLs ($\sim 42\mu\text{g}/\text{dL}$) when compared to their pups, whose BLL dropped remarkably ($\sim 34\mu\text{g}/\text{dL}$). These reversed BLLs/age ratios persisted throughout postnatal development (PND 7-14), whereas at weaning (PND 22) pups BLLs were quite similar ($\sim 39\mu\text{g}/\text{dL}$) to that of their respective dams ($\sim 43\mu\text{g}/\text{dL}$). 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 $1\mu\text{g}/\text{dL}$ 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-Pb²⁺ exposure alters Ca²⁺-dependent GABA shift: Novel mechanism for early Pb²⁺ 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 $n = 12$; Pb²⁺ $n = 12$; $n = 3$ for PND 2, 7, 14, and 22) were maintained under controlled temperature ($24 \pm 1^\circ\text{C}$) 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°C until use.

3.1.3-RNA preparation

RNA was prepared from brain tissue samples in accordance with manufacturers' protocol (TRIzol Reagent; Invitrogen 15596-026). Briefly, 50-60mg of wet brain tissues were weighed and homogenized in 1ml of TRIzol reagent 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 100 μl of DEPC-treated H₂O.

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 <i>ORF</i>	
Forward primer	5'-ACAGGGTGGTGGACCTCATG-3'
Reverse primer	5'-GTTGGATAGGGCCTCTCTTG-3'
GAD 80 <i>ORF</i>	
Forward primer	5'-AGTGTGGCCTCCAGAGGTTC-3'
Reverse primer	5'-TGGATATGGCTCCCCCAGGAG-3'
GAD 86 <i>ORF</i>	
Forward primer	5'-TGGCCTCCAGAGGTGATGGT -3'
Reverse primer	5'-TGGATATGGCTCCCCCAGGAG -3'
GAD 65 <i>ORF</i>	
Forward primer	5'-GGCTCTGGCTTTGGTCCTTC -3'
Reverse primer	5'-TGCCAATTCCCAATTATACTCTTGA -3'
GAD 67 <i>ORF</i>	
Forward primer	5'-GCTGGAAGGCATGGAAGGTTTA-3'
Reverse primer	5'-AATATCCCACCATCTTTATTGACC -3'
GABA _A β3 <i>ORF</i>	
Forward primer	5'-CCACGGAGTGACAGTGAAAA-3'
Reverse primer	5'-CACGCTGCTGTCGTAGTGAT-3'
CACNB β3 <i>ORF</i>	
Forward primer	5'-TGGATCGGGAGGCTAGTGAA-3'

Reverse primer	5'-CACGCTGCTCGTAGTGAT-3'
<i>NKCC₁ ORF</i>	
Forward primer	5'-ATGAGTCTTCCAGTTGCCCG-3'
Reverse primer	5'-GCAACGTGTCCATGTGCTTT-3'
<i>KCC₂ ORF</i>	
Forward primer	5'-GGACCCCCGCATACAAAGAA-3'
Reverse primer	5'-CCTCCAGACCTTGTGGTGAC-3'
<i>MECP2ORF</i>	
Forward primer	5'-CAGGTCATGGTGATCAAACG -3'
Reverse primer	5'-CCACACTCCCAGGCTTTCTA-3'
<i>FMR1ORF</i>	
Forward primer	5'-GTGAGATTCCCACCACCTGT -3'
Reverse primer	5'-CCAACAGCAAGGCTTTTT -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 ΔC_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 \pm SEM.

3.2-Results

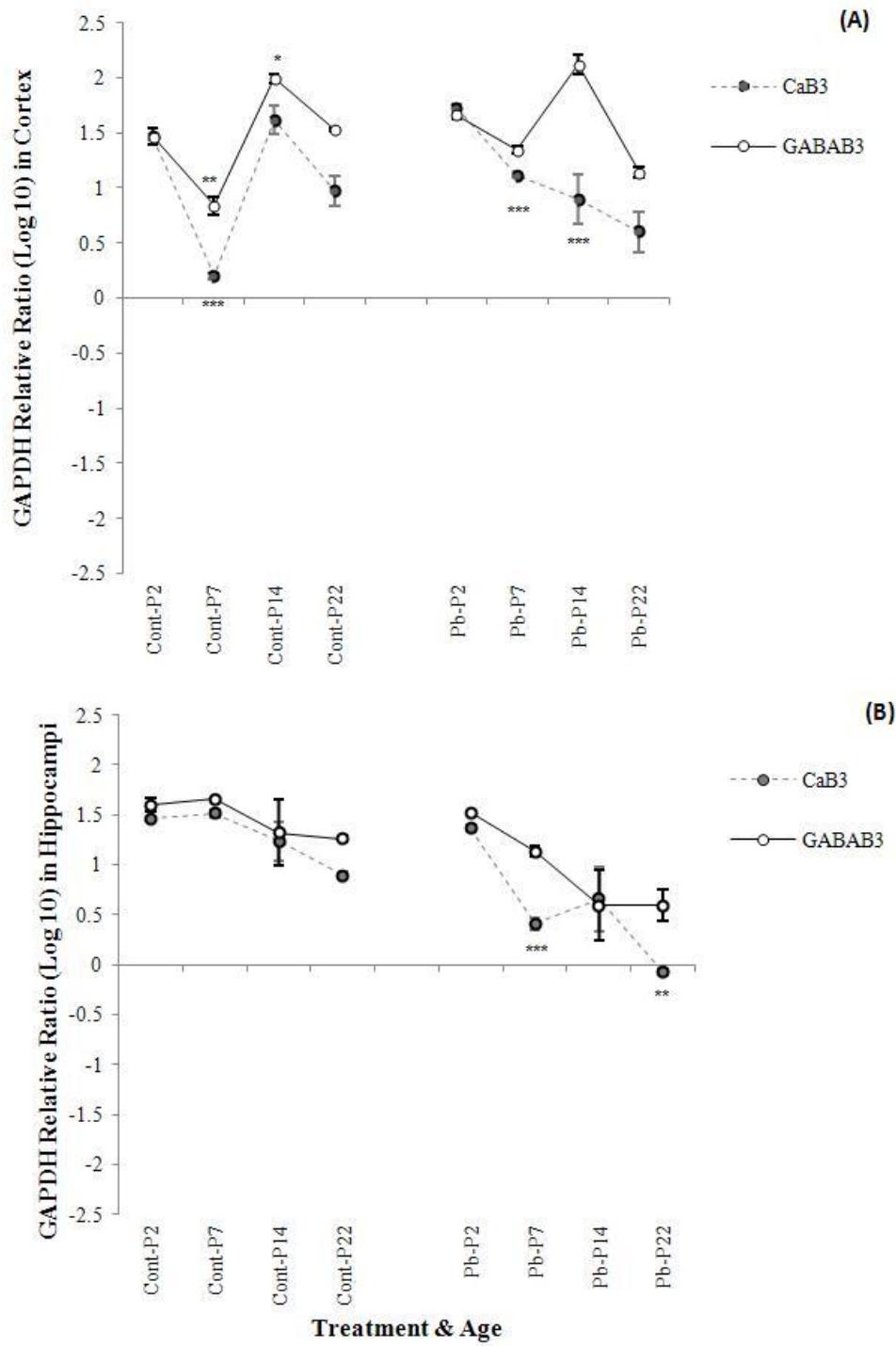


Figure 11. Chronic gestational Pb $^{2+}$ exposure down regulated Ca β 3 mRNA expression in cortex and hippocampus during early brain development. Cortical Ca β 3 mRNA was down regulated from PND 7 through PND22 ($p<0.001***$) (A). Hippocampal Ca β 3 mRNA was down regulated affected at PND 7 and 22 ($p<0.001***$), but recovered at PND 14 (B). GABA β 3 mRNA was down regulated at PND 22 in cortex (A) and hippocampus (B) in response to Pb $^{2+}$ ($p<0.01**$).

Figure 11. GABA_{AR}'s showed that in cortex GABA_{AR}-β3 and Ca β3 mRNA expression changes induced by Pb²⁺ exposure caused a *Gene* effect (DF=1, MS=2.31, F=82.69, p<0.001***), an *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***) and 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_{AR}-β3 and Ca β3 mRNA expression changes induced by Pb²⁺ 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**).

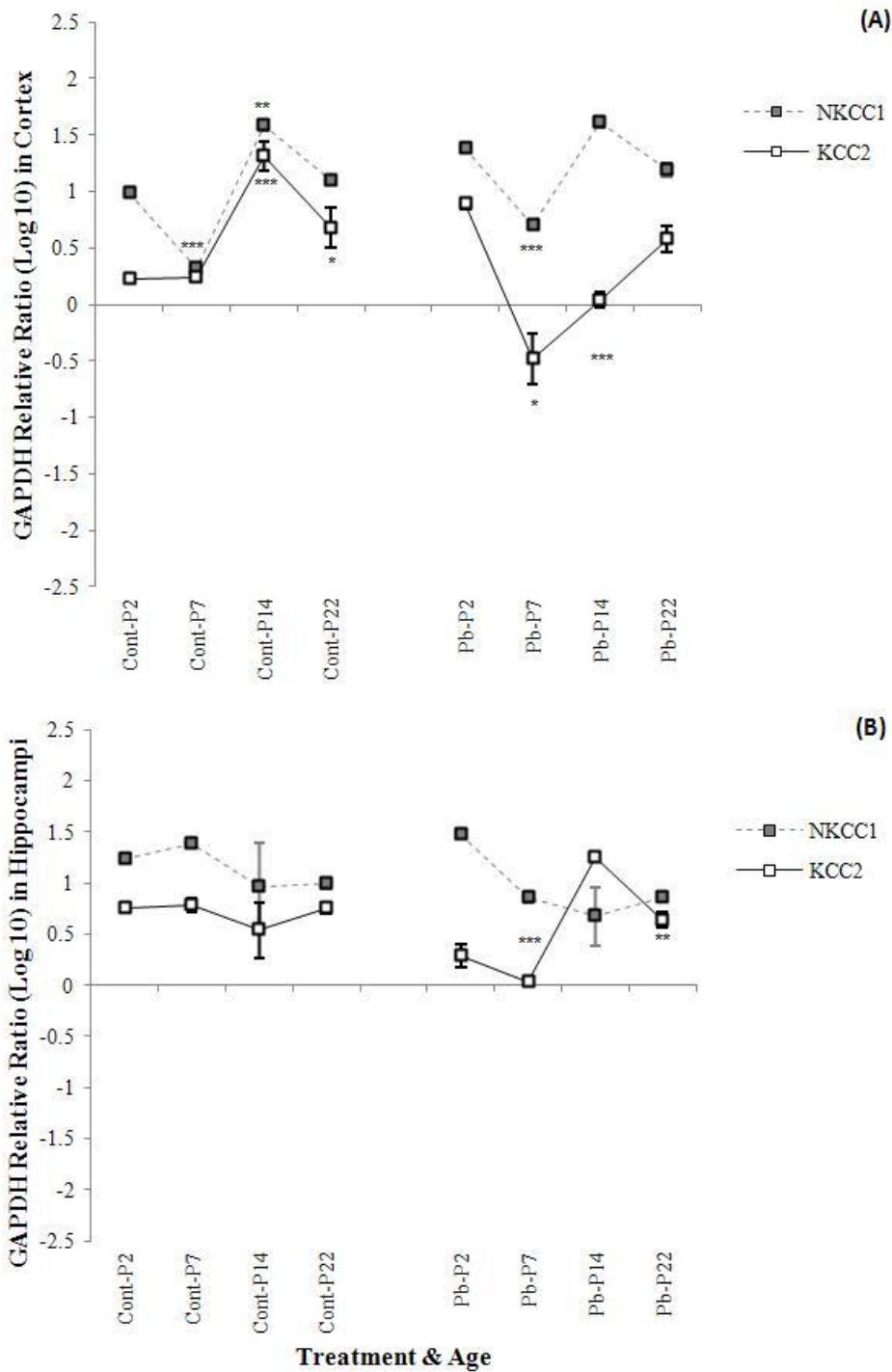


Figure 12. Chronic gestational Pb²⁺ exposure decreases KCC2 and increases NKCC1 mRNA in cortex and hippocampus during early brain development. Cortical KCC2 mRNA is down regulated from PND 7 ($p<0.05^*$), through PND 14 ($p<0.001^{***}$), whereas NKCC1 mRNA is up regulated at PND 2 and 7 (A). Interestingly, 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 Pb²⁺ 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 Pb²⁺ 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**).

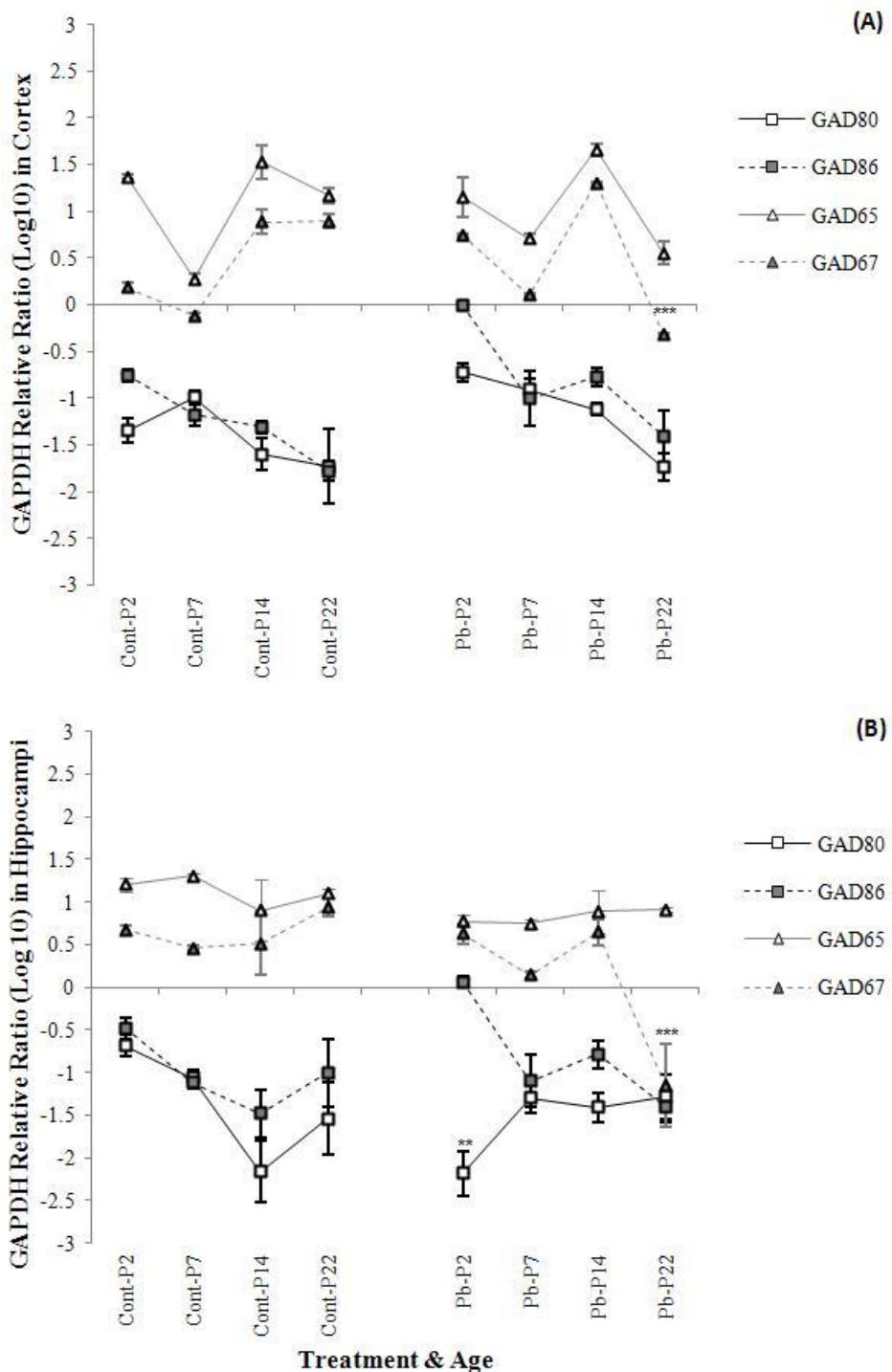


Figure 13. Chronic gestational Pb²⁺ altered early and late GAD mRNA expression levels. Cortical Pb²⁺ exposure disrupted early GAD 80 and 86 and late GAD 67 mRNA expression at PND 2 ($p<0.01**$) and altered late GAD 65 and 67 mRNA at PND 22 ($p<0.001***$) (A). Hippocampal, early GAD 86 mRNA expression 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 Pb²⁺ 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***) and 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 Pb²⁺ 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**).

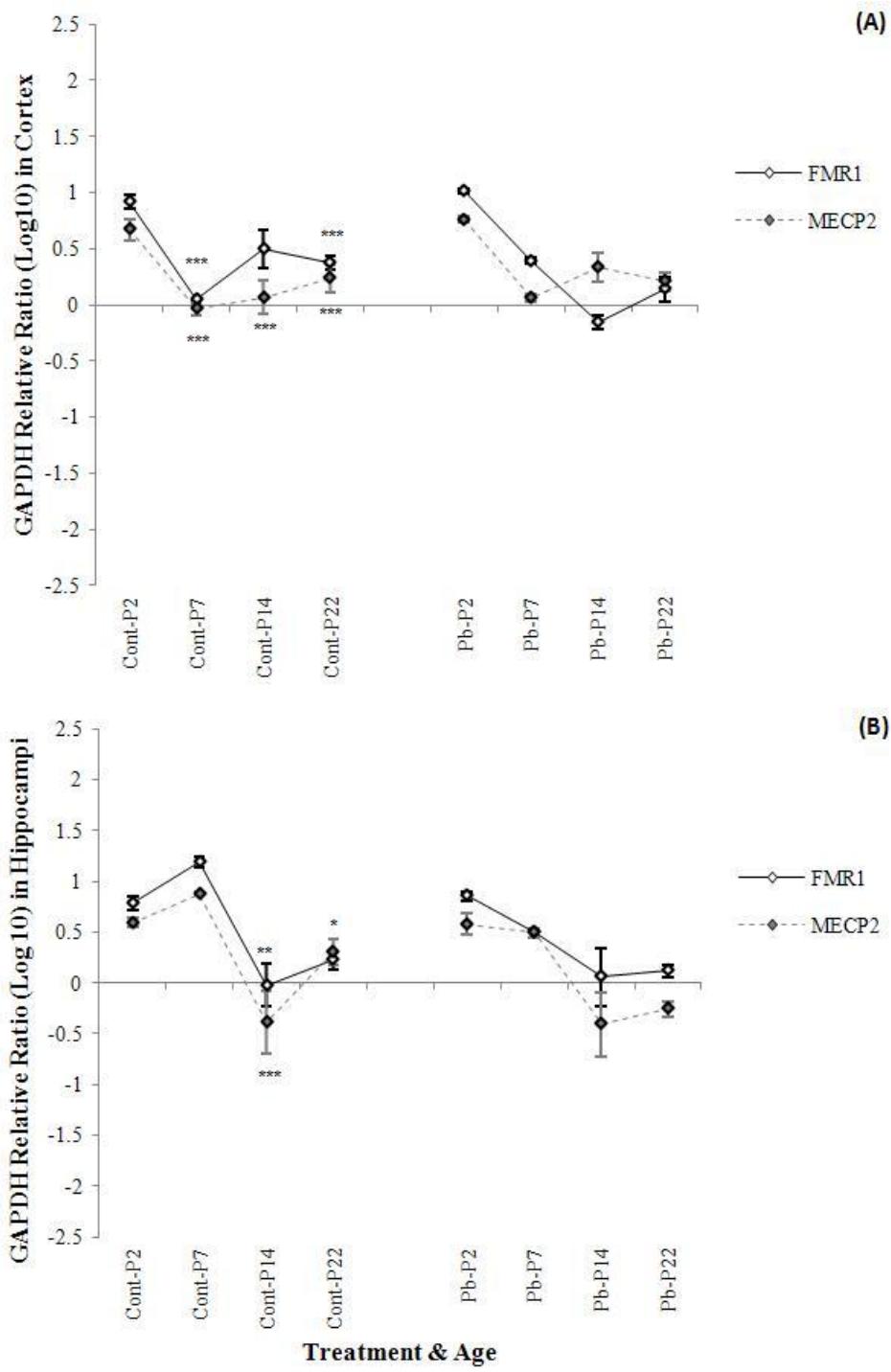


Figure 14. Chronic gestational Pb²⁺ effects on synaptic vesicle release MECP2 and synaptic plasticity FMR1 mRNA expression levels. Pb²⁺ up regulated FMR1 mRNA expression levels at PND 2 through 7 ($p<0.001^{***}$) and down regulated expression levels at PND 14 ($p<0.05^*$) in cortex (G). Interestingly, FMR1 mRNA expression of MECP2 mRNA expression reversed at PND 14 when comparing Pb²⁺ rats to controls (A). Pb²⁺ 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 Pb^{2+} effects on gene expression during early development in cortex and hippocampus. The qRT-PCR data revealed that Pb^{2+} decreases VS β 3 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.**). Pb^{2+} effected these genes differentially resulting in the VS β 3 mRNAs down regulation in expression, whereas the GABA_{AR}- β 3 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_{AR}- β 3 mRNA expression was decreased at PND 7 and 22 under Pb^{2+} treatment (**Figure 11-B.**). These findings suggest that GABA_{AR}- β 3 mRNA increases expression when VS β 3 mRNA decreases in response to Pb^{2+} 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 Pb^{2+} 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 Pb²⁺ exposure. However, the ratio of the NKCC1 and KCC2 transport proteins and their functional activation through phosphorylation remain to be elucidated under Pb²⁺ exposure.

To determine how Pb²⁺ 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 (**Figure 13-A.**). In hippocampus, GAD 80 mRNA expression decreased at PND 2, whereas GAD 86 increased at PND 2 in response to Pb²⁺ treatment (**Figure 13-B.**). At PND 14 both GAD 80 and 86 increased in hippocampus when compared to control rats (**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 Pb²⁺ exposure (**Figure 13-A.**). In hippocampus, GAD 65 showed decreased mRNA expression at PND 2 and 7 in response to Pb²⁺ (**Figure 13-B.**). In cortex GAD 67 mRNA expression was observed to increase at PND 7 and decrease at PND 22 (**Figure 13-A.**), whereas in hippocampus a decrease in GAD 67 mRNA expression was observed at PND 7 and PND 22 (**Figure 13-B.**). These findings suggest that Pb²⁺ interferes with early GAD 80 and 86 that prolong early GABAergic excitation during development. Together, these findings show a direct effect of Pb²⁺ 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 Pb²⁺ 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, Pb²⁺ exposure caused increased MECP2 mRNA expression at PND 14 when compared to controls (**Figure 14-A.**). In hippocampus Pb²⁺ decreased mRNA expression in PND 7 and 22 (**Figure 14-B.**). In cortex FMR1 mRNA expression was observed to increase in PND 2 and 7 followed by a decrease at PND 14 (**Figure 14-A.**), whereas in hippocampus decreased mRNA expression was shown at PND 7 in response to Pb²⁺ (**Figure 14-B.**). These findings indicate that in cortex at PND 14 Pb²⁺ perturbs vesicular release and post synaptic plasticity. In hippocampus at PND 22 Pb²⁺ disrupts vesicular release.

4.0- Chronic gestational Pb²⁺ exposure reduces 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 $n = 12$; Pb²⁺ $n = 12$; $n = 3$ 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® 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 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') 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 1.5×10^6 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. 0.05 μM, 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 AOBS 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 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 \pm SEM.

4.2-Results

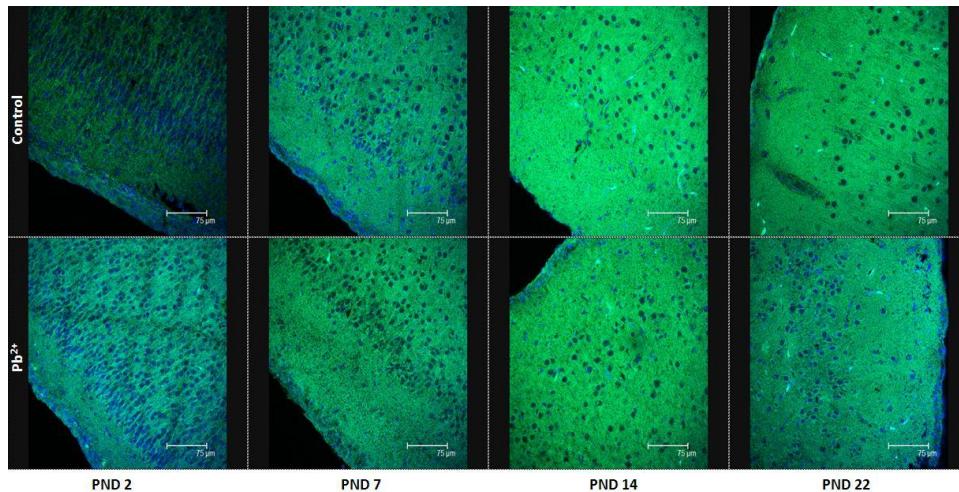


Figure 15. Pb^{2+} effects on KCC2 expression (Green) patterns in cortex as a function of age.

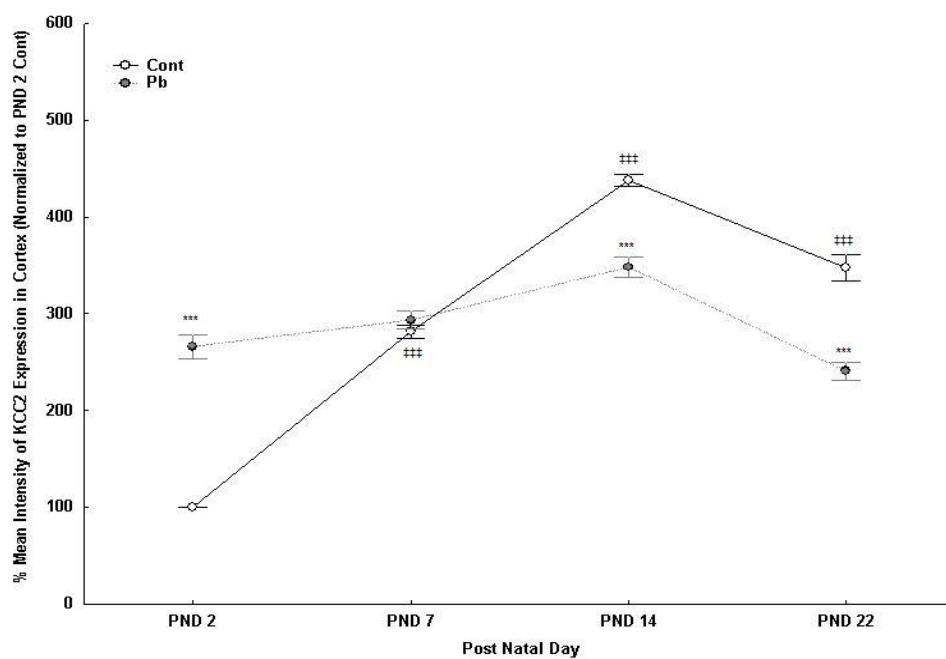


Figure 16. Pb^{2+} effects on KCC2 IHC mean intensity expression in cortex as a function of age.

Figures 15-16 showed an *Age* effect ($DF=3$, $MS=146,787$, $F=150.895$, $p<0.001^{***}$) and an *Age* X *Treatment* interaction ($DF=3$, $MS=78,563$, $F=80.762$, $p<0.001^{***}$).

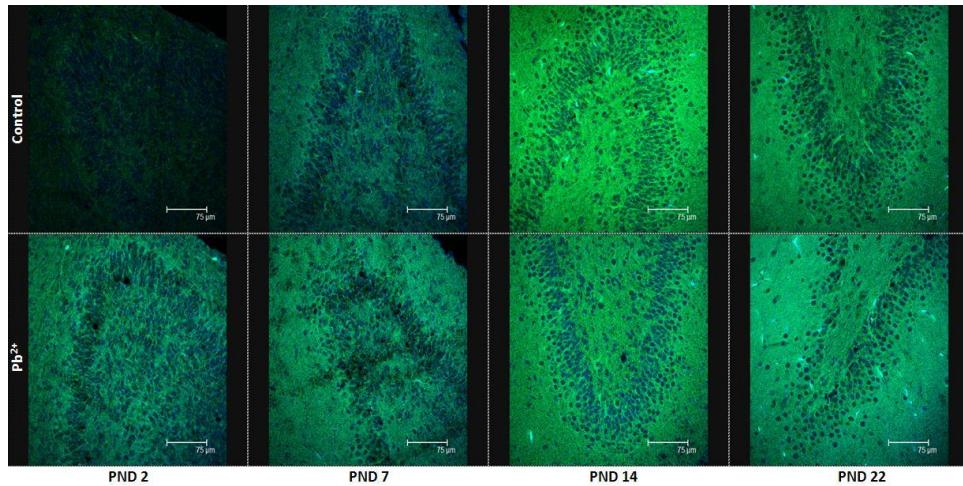


Figure 17. Pb^{2+} effects on KCC2 expression (Green) patterns in dentate gyrus as a function of age.

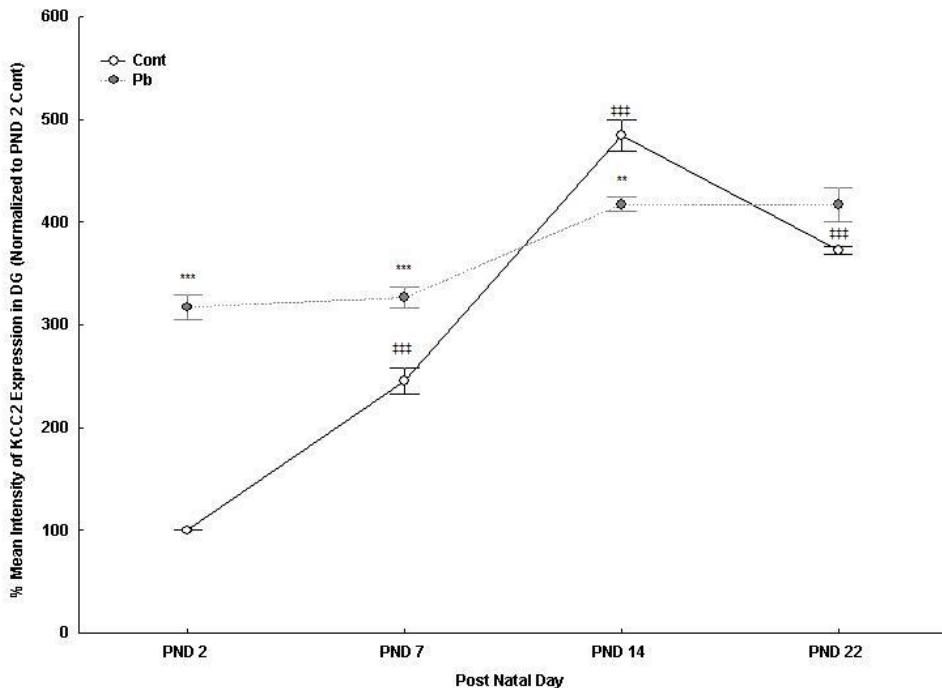


Figure 18. Pb^{2+} effects on KCC2 IHC mean intensity expression in dentate gyrus as a function of age.

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 ($DF=3$, $MS=51,263$, $F=48.796$, $p<0.001^{***}$).

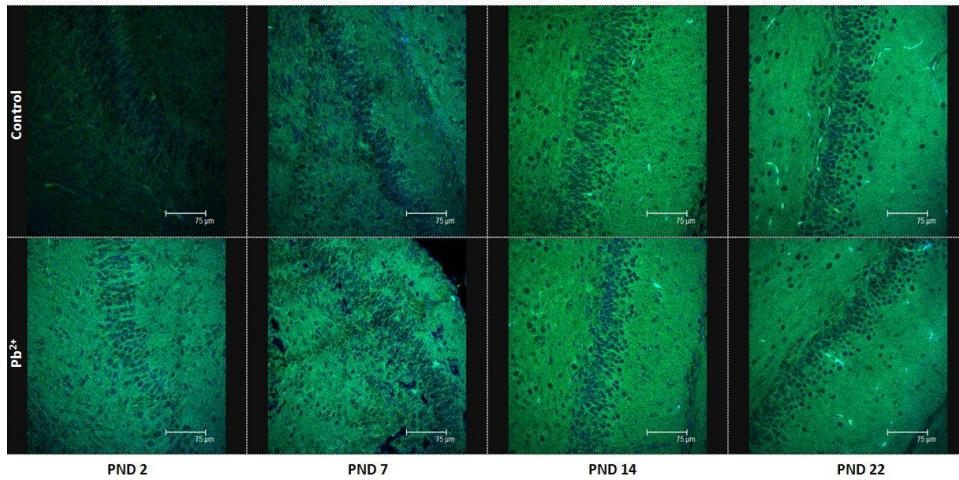


Figure 19. Pb^{2+} effects on KCC2 expression (Green) patterns in CA3 as a function of age.

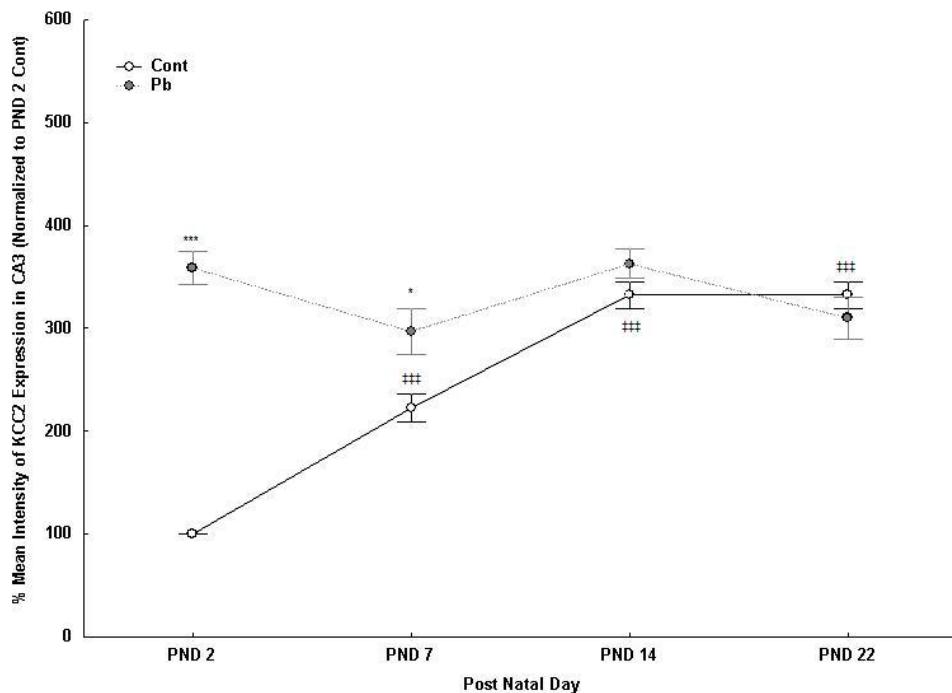


Figure 20. Pb^{2+} effects on KCC2 IHC mean intensity expression in CA3 as a function of age.

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 ($DF=3$, $MS=37,357$, $F=28,491$, $p<0.001^{***}$).

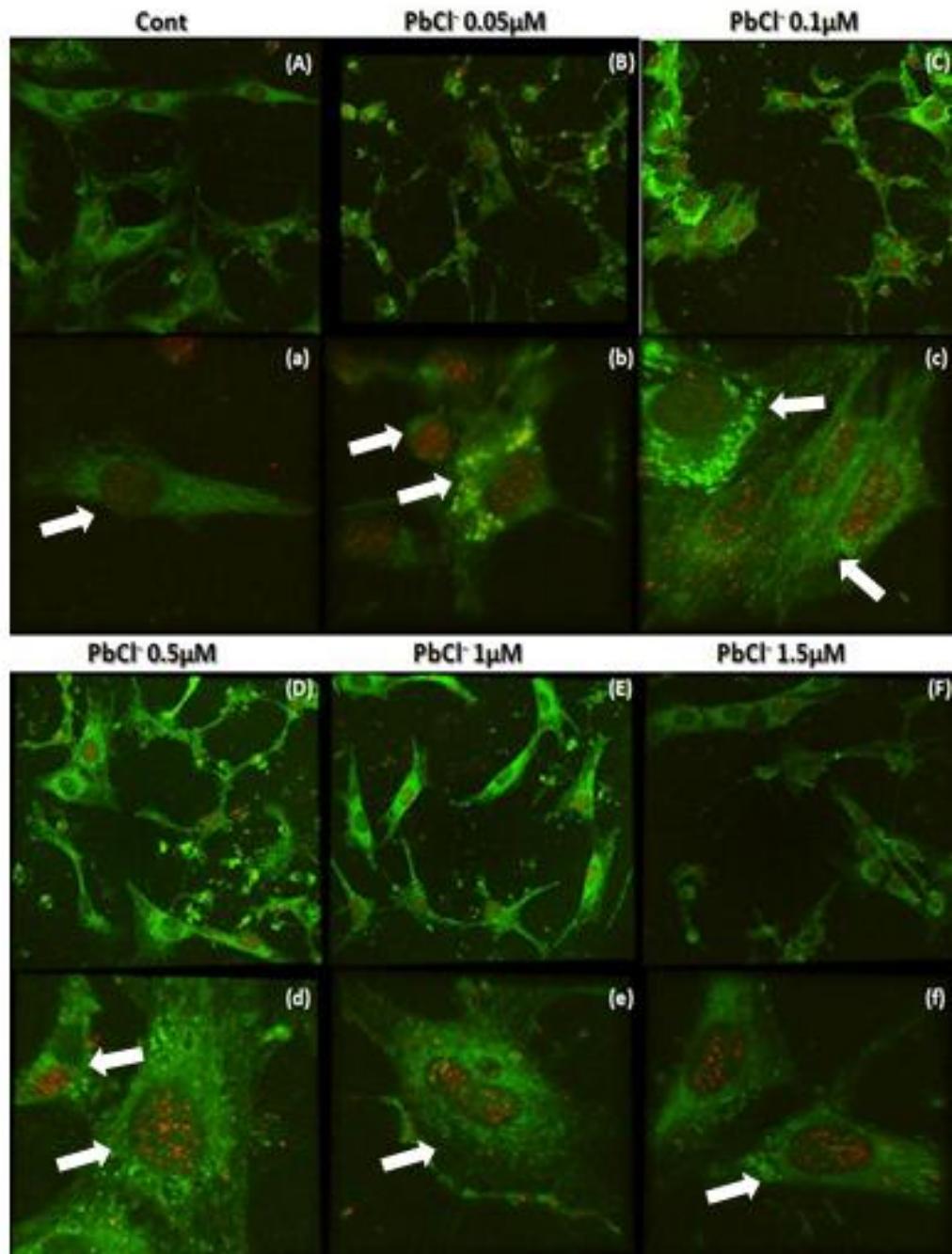


Figure 21. PbCl_2 alters GABA_{AR} expression (Green) in culture and VSCC- $\beta 3$ nuclear translocation (white arrow pointing to Red) in cerebellar granule cell cultured neurons. (uppercase letter shows field and lower case letter shows magnified cell). Images were taken with a 40x oil objective.

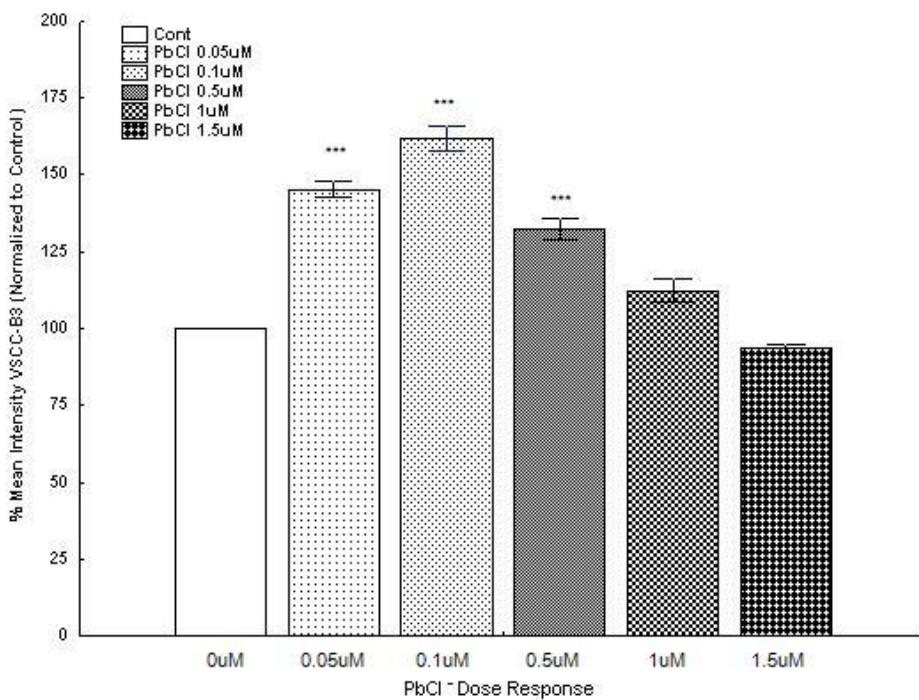


Figure 22. PbCl⁻ induces a curvilinear effect on VSCC-β3 nuclear expression in cerebellar granule cell cultured neurons.

Figure 22. 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.

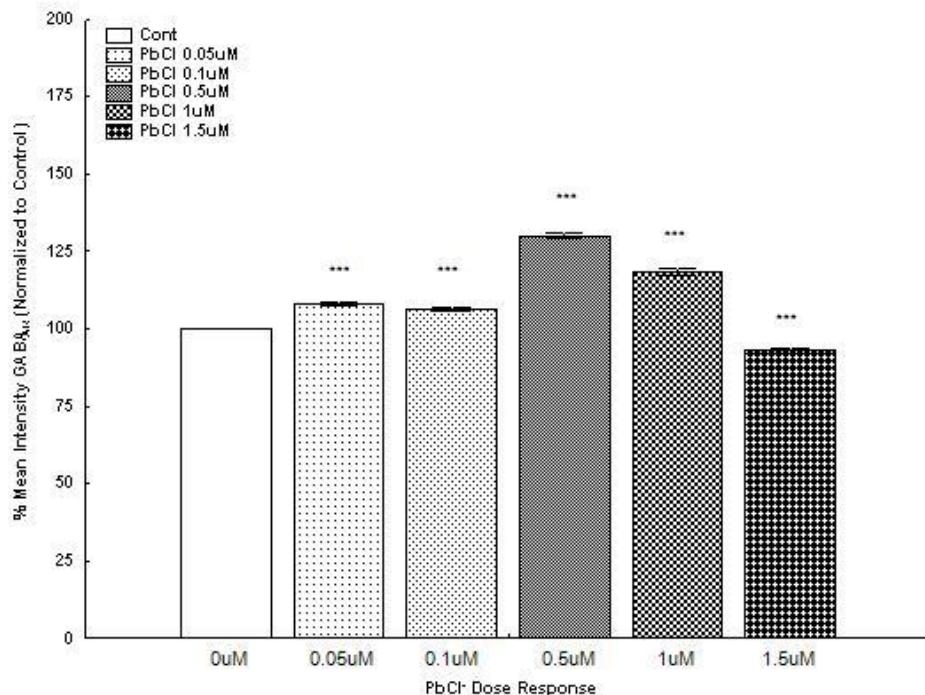


Figure 23. PbCl⁻ induces a curvilinear effect on GABA_{AR} expression in cerebellar granule cell cultured neurons.

Figure 23. shows a curvilinear relationship between increased dose responses of PbCl⁻ on GABA_{AR} expression in cerebellar granule cell cultured neurons. PbCl⁻ 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.

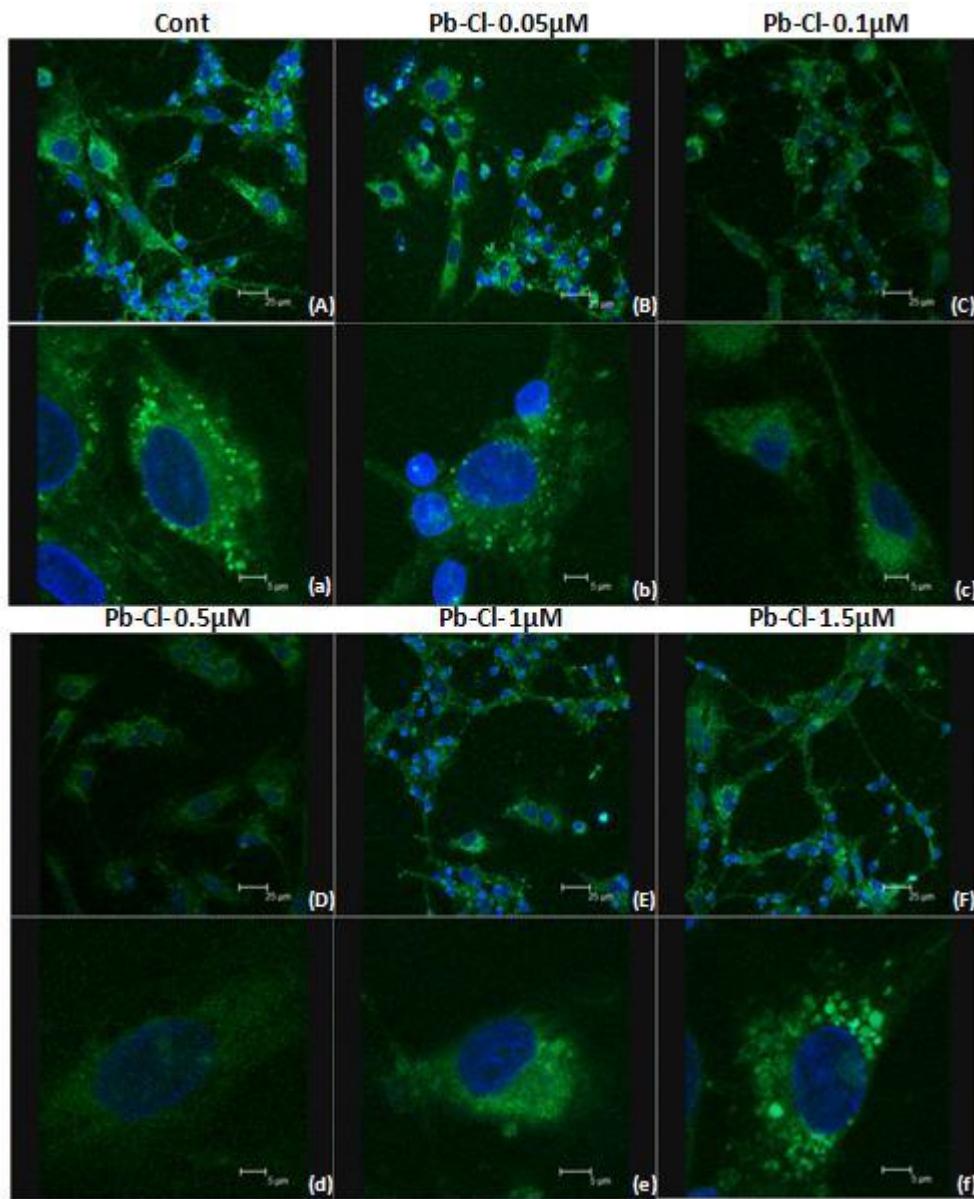


Figure 24. PbCl_2 decreases KCC2 expression (Green) in cerebellar granule cell cultured neurons regardless of dose (uppercase letter shows field and lower case letter shows magnified cell). Images were taken with a 40x oil objective.

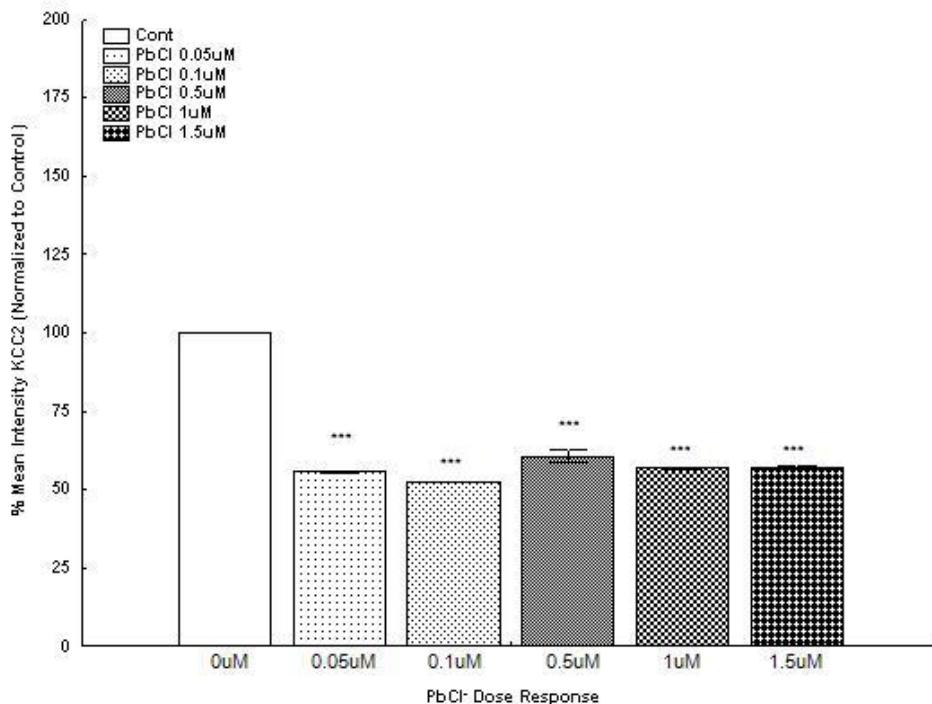


Figure 25. PbCl^- reduces KCC2 expression in cerebellar granule cell cultured neurons regardless of dose.

Figure 25. shows a decreased KCC2 expression in cerebellar granule cell cultured neurons in response to PbCl^- regardless of dose. PbCl^- 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^{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^{2+} exposure reduced KCC2 immunoreactivity at PND14 and PND 22 when compared to controls (**Figures 15-18.**). In the DG Pb^{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^{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 VS⁺CC-β3 and GABA_{AR} expression as a function of increased Pb²⁺ exposure we examined the dose response effects of PbCl⁻ in primary cerebellar granule cell cultured neurons 3 days in vitro (3 DIV). Our results showed that PbCl⁻ at low concentrations increases VS⁺CC-β3 and at high concentrations reduced immunoreactivity with peak expression at PbCl⁻ 0.1μM in contrast to control conditions (*Figures 21-23.*). These findings describe an atypical dose response curve where higher doses of PbCl⁻ 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⁻, whereas at low doses such compensation may not be possible.

Interestingly, the cerebellar granule cells GABA_{AR} immunoreactivity showed similar effects as did the VS⁺CC-β3 (*Figures 21-23.*); however, peak expression of GABA_{AR} was observed at PbCl⁻ 0.5μM. These findings showed that VS⁺CC-β3 is more sensitive to PbCl⁻ insult and the early GABAergic system is altered suggesting interference with increased VS⁺CC-β3 and GABA_{AR} synergistic activity at low and reduced synergistic activity with high doses of PbCl⁻.

When we applied the same dose response of PbCl⁻ to the cerebellar granule cells we observed a drastic decrease in KCC2 immunoreactivity (i.e. ~40-50%) irrespective of dose; indicating that PbCl⁻ produced delays in the GABA shift in isolated cultured neurons when compared to control conditions (*Figures 24-25.*). These findings indicate that Pb²⁺ can interrupt early GABAergic development through perturbing the precise interplay between the VS⁺CC-β3 and GABA_{AR}; 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,-³H-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 $n = 96$; Pb²⁺ $n = 96$) 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 Pb²⁺ 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-centrifuged 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 centrifuged 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-2,3,³H-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 500 μ l of aCSF. A radioactive glutamate solution was prepared with 2 μ l 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 200 μ l pipette in 1ml of oxygenated aCSF solution. Then 200 μ l 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. 1-3 μ M) 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 200 μ l 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 100 μ M glutamate. The radioactive (i.e. hot) D-2, 3,3H-aspartic acid solution was made by taking 5 μ l of D-2, 3,3H-aspartic acid and adding it to 5 μ l of 1mM cold glutamate. The cold glutamate sample contained 200 μ l of synaptosomes, 200 μ l of D-2, 3,3H-aspartic acid solution, 300 μ l of binding buffer and 80 μ l of 1mM cold glutamate. The hot glutamate sample contained 200 μ l of synaptosomes, 200 μ l of D-2, 3,3H-aspartic acid solution, 380 μ l 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 ($\mu\text{g}/\mu\text{l}$) 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 $2\mu\text{g}/\mu\text{l}$ respectively. Standards were run in triplicates simultaneously with unknown proteins from the synaptosomal experiments. Using a 96 well plate, 5 μl of standards and unknown protein samples were added into each well, followed by 25 μl of working reagent A+S, and finally 200 μl of reagent B were added (i.e. total volume 230 $\mu\text{l}/\text{well}$). 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 ($R^2 > 0.9$) as a function of protein concentration ($\mu\text{g}/\mu\text{l}$) 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 Pb²⁺ exposed samples were normalized against controls.

In the release studies the CPM/mg protein were collected from the 2 minute incubated samples and Pb²⁺ 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 Ca⁴⁵ 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 $\alpha= 0.05\%$ with a $95\% \pm \text{SEM}$. Significant differences were determined by equal and unequal Tukey's HSD post hoc comparisons test.

5.2-Results

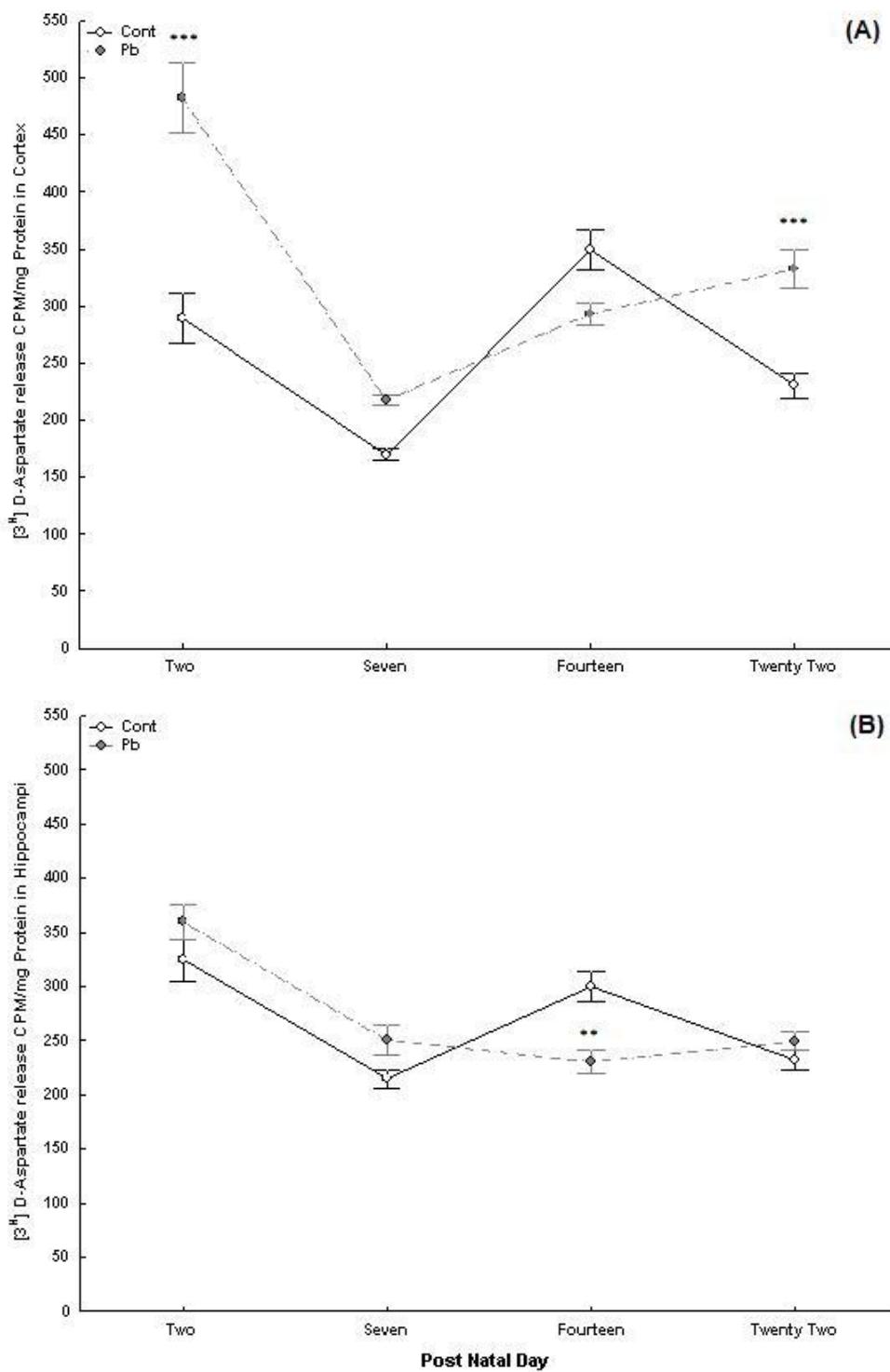


Figure 26. Effects of chronic gestational Pb^{2+} exposure on spontaneous $[^3\text{H}]\text{-D-Aspartate}$ release in cortex (A) and hippocampus (B) as a function of age during early post natal development. Pb^{2+} increases spontaneous release at PND 2, 7, and 22 in cortex (A) and PND 14 in hippocampus (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).

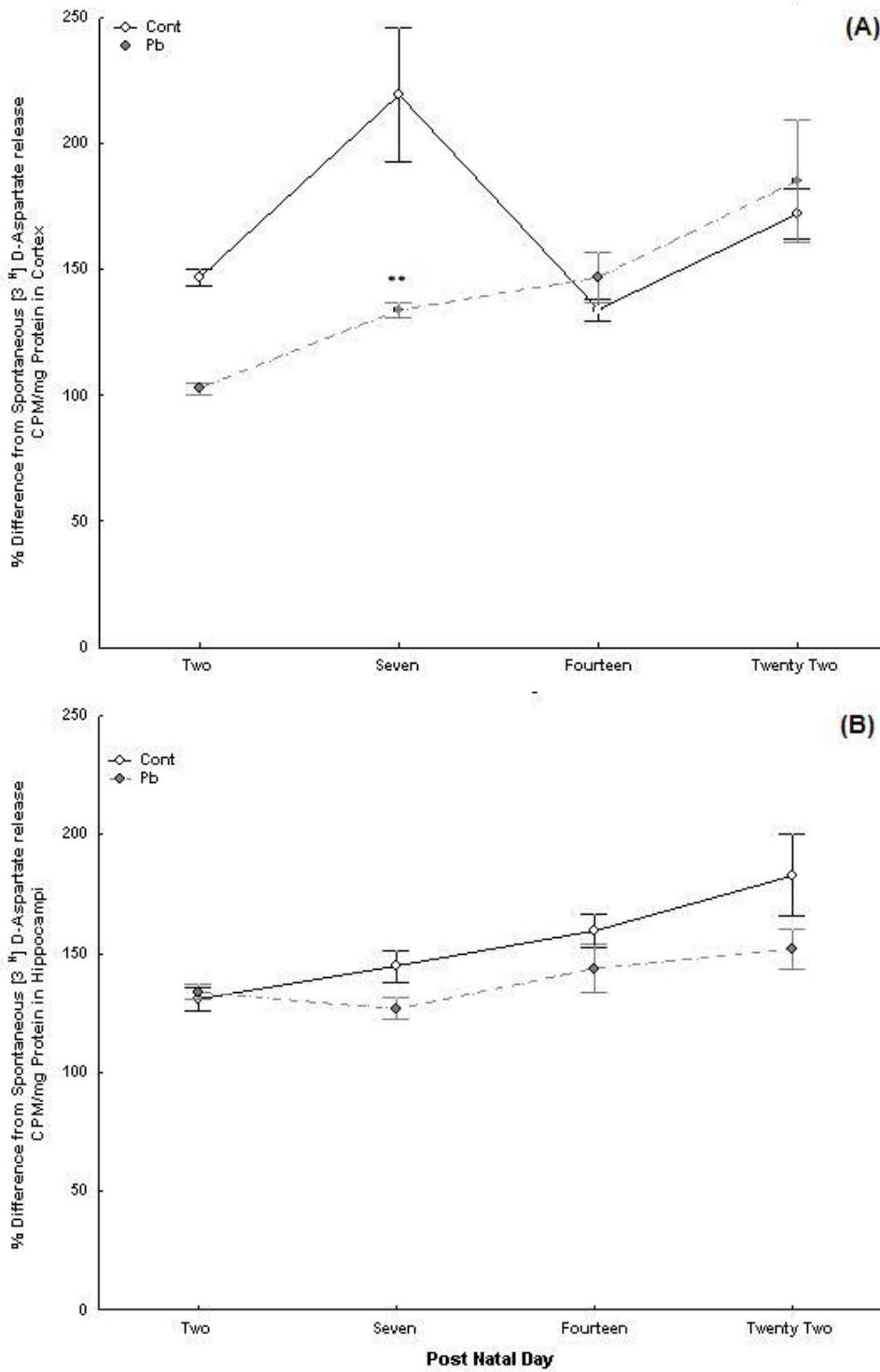
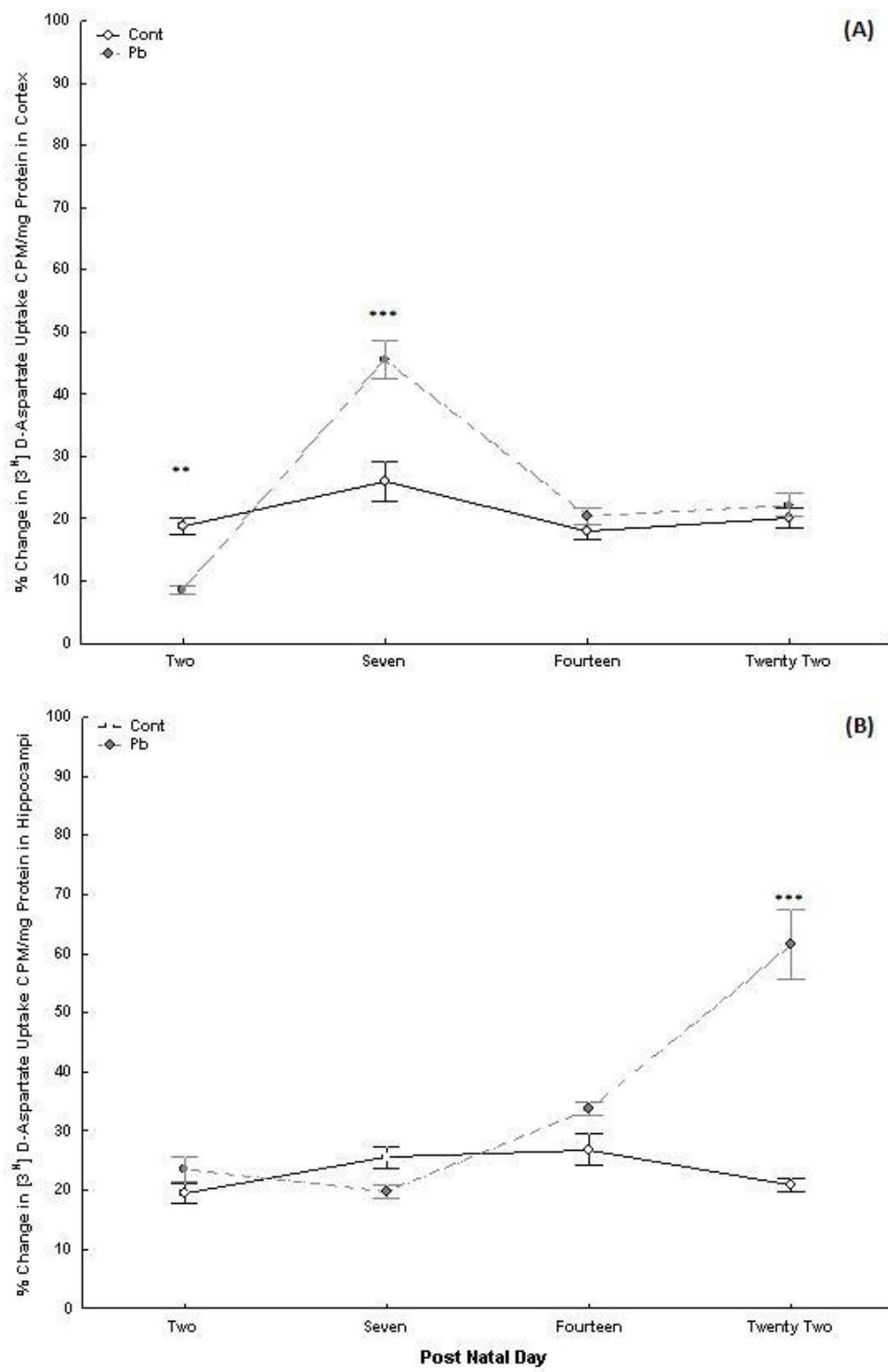


Figure 27. Effects of chronic gestational Pb^{2+} exposure on evoked ${}^3\text{H}$ -D-Aspartate release in cortex (**A**) and hippocampus (**B**) as a function of age during early post natal development. Pb^{2+} inhibits evoked neurotransmitter release at PND 2 and 7 in cortex (**A**) and PND 2-22 in 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**).



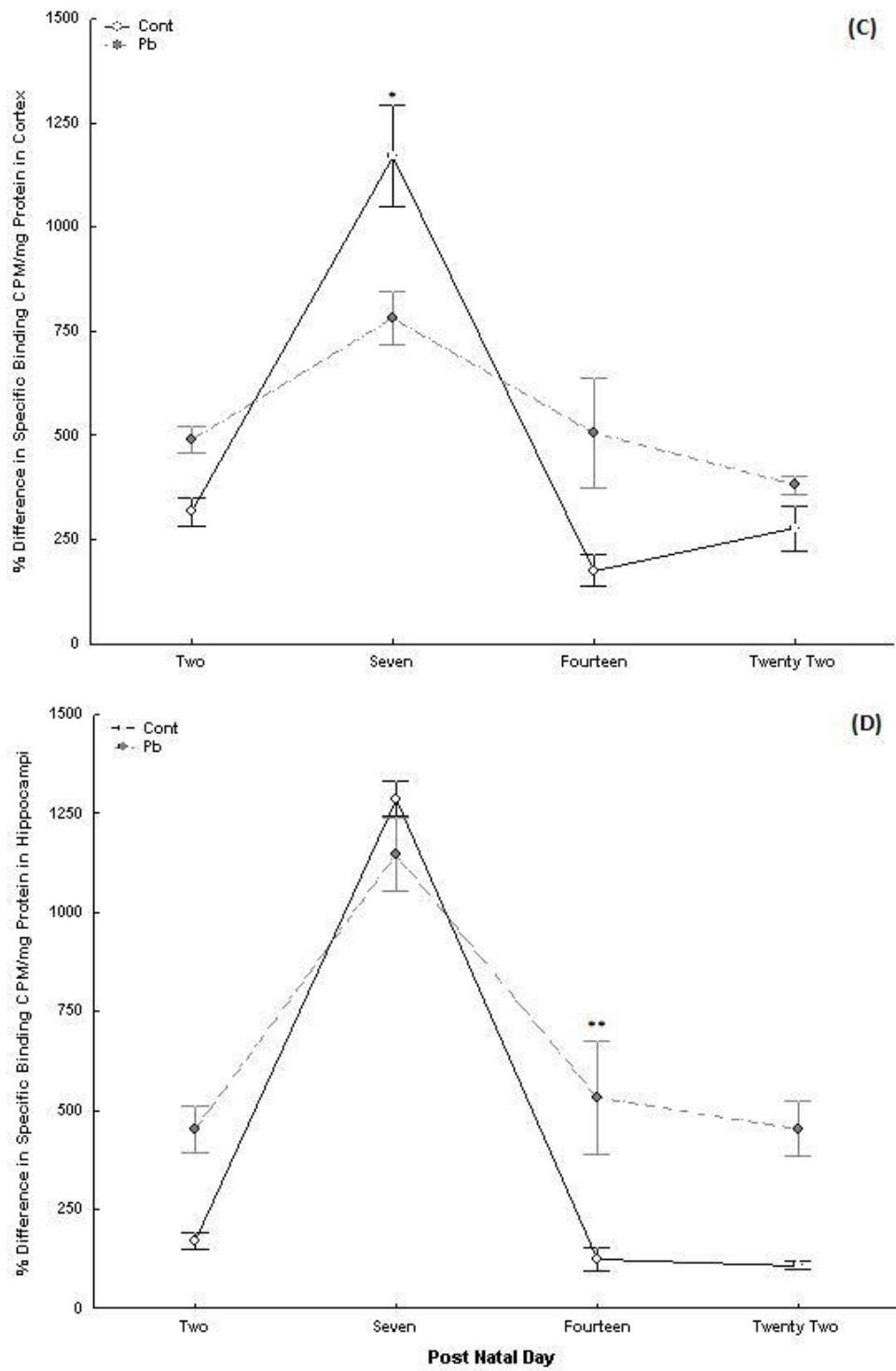


Figure 28. Effects of chronic gestational exposure of Pb^{2+} on ${}^3\text{H}$ -D-Aspartate uptake and binding in cortex (**A & B**) and hippocampus (**C& D**) as a function of age during early post natal development. Pb^{2+} decreases neurotransmitter uptake at PND2 and increases uptake at PND 7 in cortex (**A**). In hippocampus uptake is increased at PND 22 (**B**). In cortex Pb^{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-Aspartate 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-Asparte 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 Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ treated rats at this age. In contrast, in hippocampus Pb²⁺ 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 Pb²⁺ treated rats.

In cortex peak binding was observed at PND 7 in both treatment groups. Notably, Pb²⁺ treatment increased binding at PND 2, 14, and 22, while at PND 7 resulted in a decrease in binding (**Figure 28-C**). Pb²⁺ 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 Pb²⁺ were observed in hippocampi, with the exception that at PND 7 no Pb²⁺ effects were noted (**Figure 28-D**).

We evaluated gestational Pb²⁺ effects on cortical and hippocampal synaptosomal spontaneous and evoked release, uptake, and binding of glutamate analog, D-2,3,-³H-aspartic acid which we considered as indicative of changes at Glutamatergic synapses. D-2,3,-³H-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 Pb²⁺ insult, rather than an acute *in-vitro* test solution application. Interestingly, these lifelong changes in neurotransmitter properties by Pb²⁺ 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. Pb²⁺ 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 Pb²⁺ 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. Pb²⁺ 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 Pb²⁺ resulted in reduced neuronal survival during development. Increasing glutamate post synaptic receptor expression would increase the probability of neuron survival in response to Pb²⁺ 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.

Pb²⁺ 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 Pb²⁺-induced cortical aberrations at PND 7 and at PND 14 in hippocampi. Pb²⁺ 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 Pb²⁺ exposure causes imprecision of cortical and hippocampal signal-mediated pruning post nataally 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 Pb²⁺ 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 exposer 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 $95\% \pm \text{SEM}$. Significant differences were determined by equal and unequal Tukey's HSD post hoc comparisons test.

6.2-Results

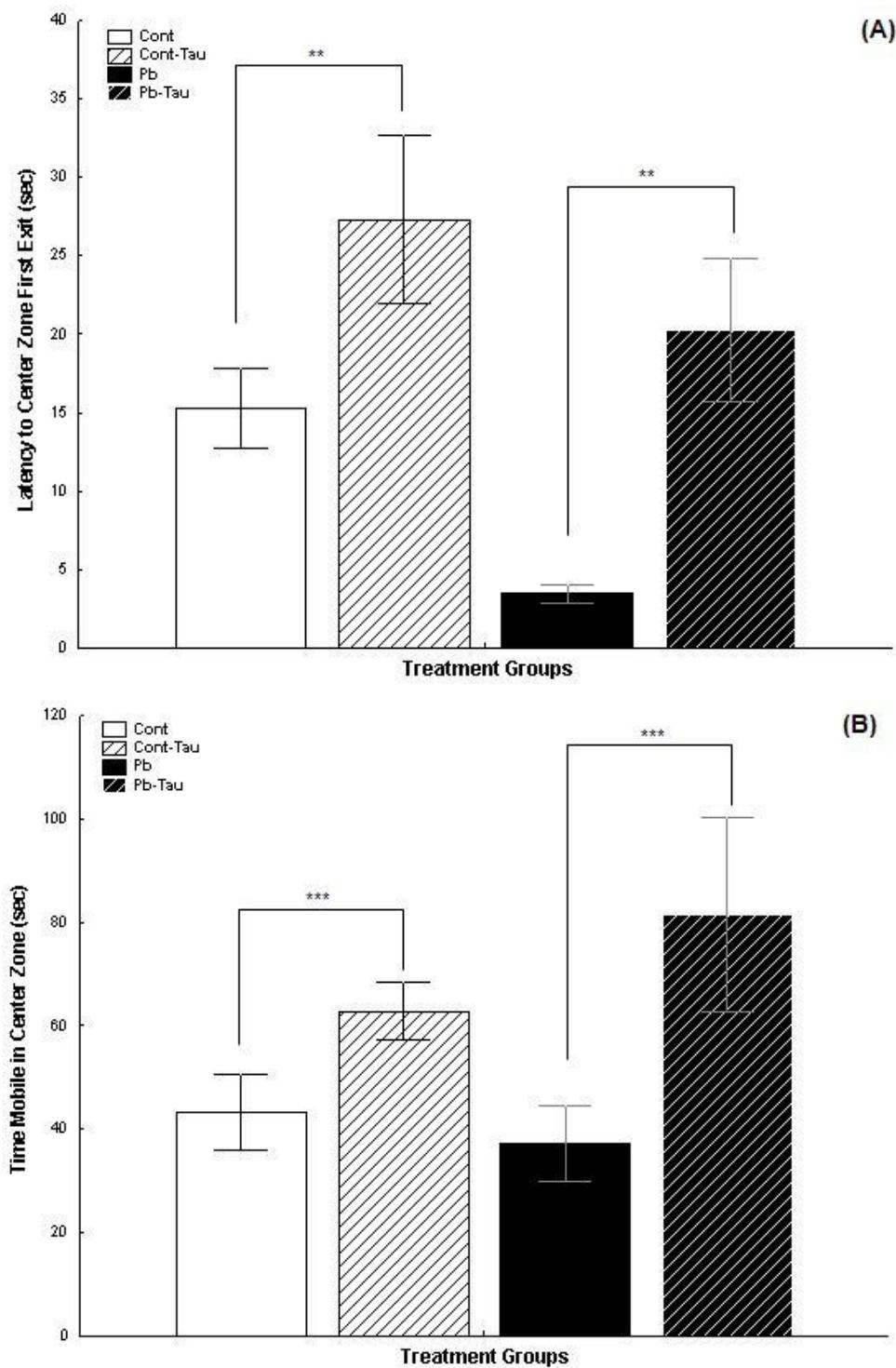


Figure 29. Pb^{2+} increased anxiety measures in OF and taurine reduced anxiety through GABAergic modulation in latency to exit center zone (A) and time mobile in center zone (B).

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 Pb²⁺ 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).

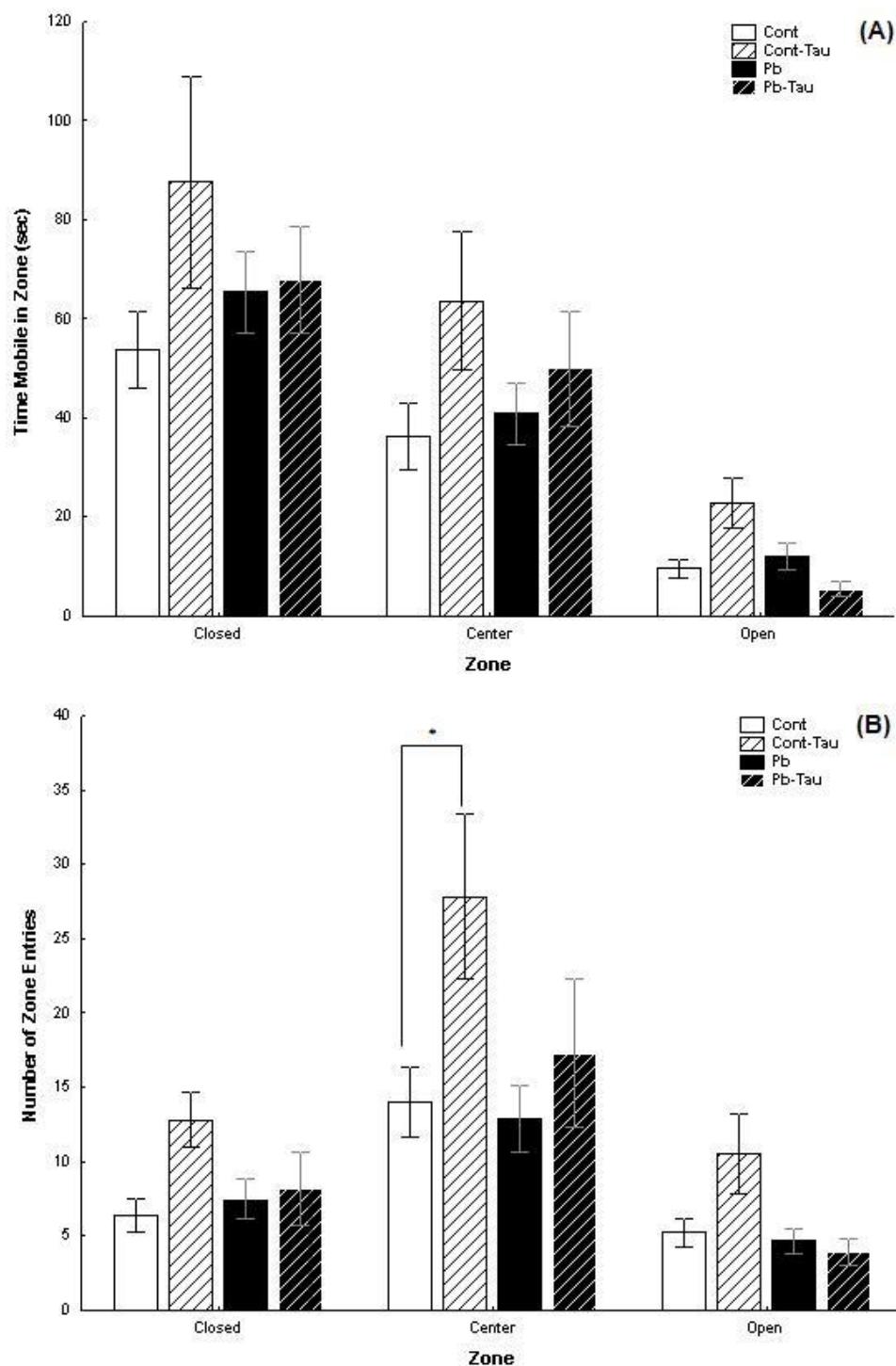


Figure 30. Pb^{2+} showed no difference on EPM anxiety measures, but taurine decreased anxiety in control rats and increased anxiety in Pb^{2+} treated rats (**A&B**).

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**).

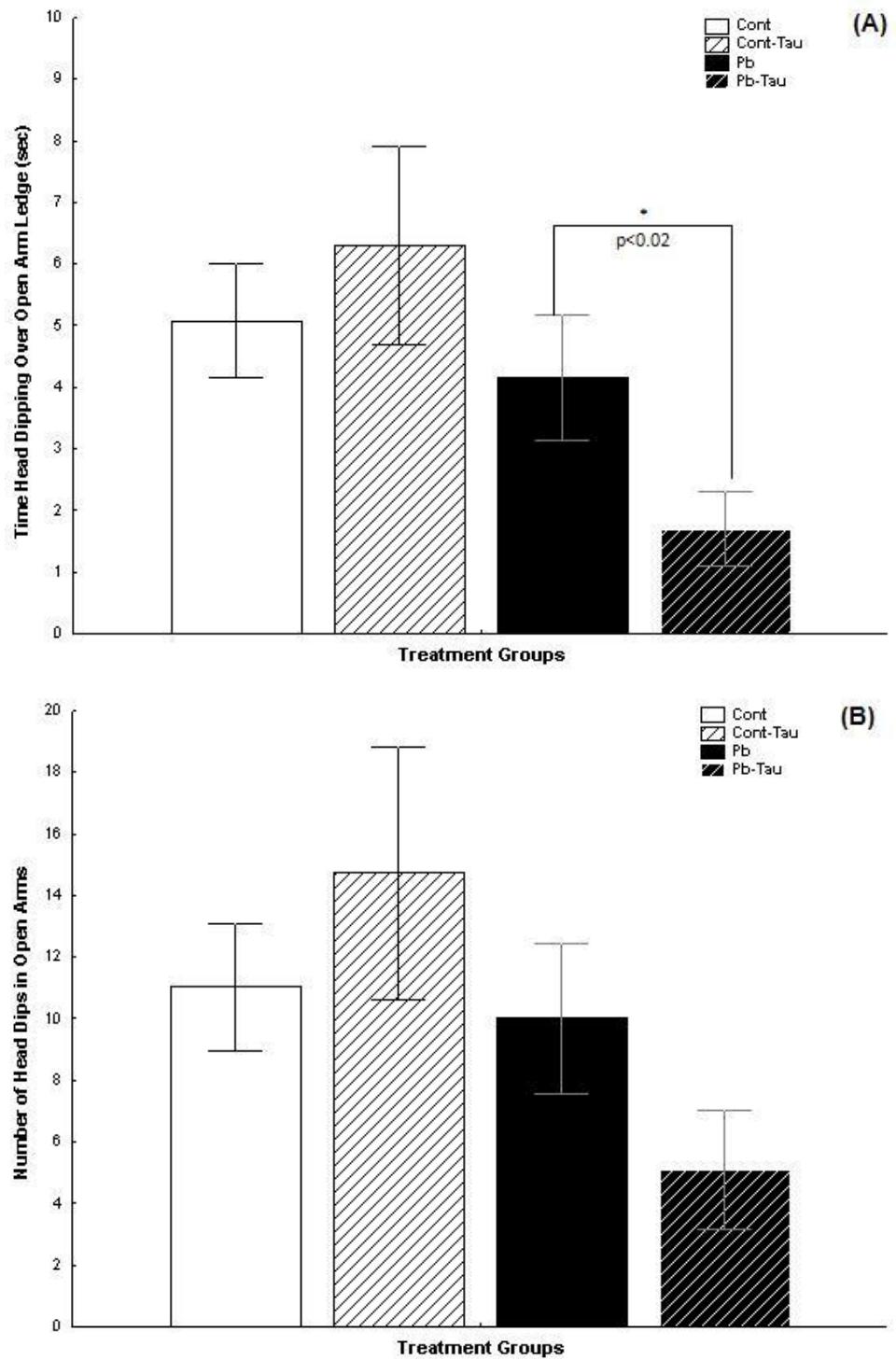


Figure 31. Taurine treatment increased anxiety in Pb^{2+} rats evidenced by reduced time head dipping over the open arm ledge (**A**) but showed no difference in total number of head dips in the EPM (**B**). **Figure 31.** The EPM time head dipping over the open arm ledge showed a Pb^{2+} effect (DF=1, MS=116.21, F=4.93, p<0.03*) (**A**). **Figure 31.** Revealed no significant differences (**B**).

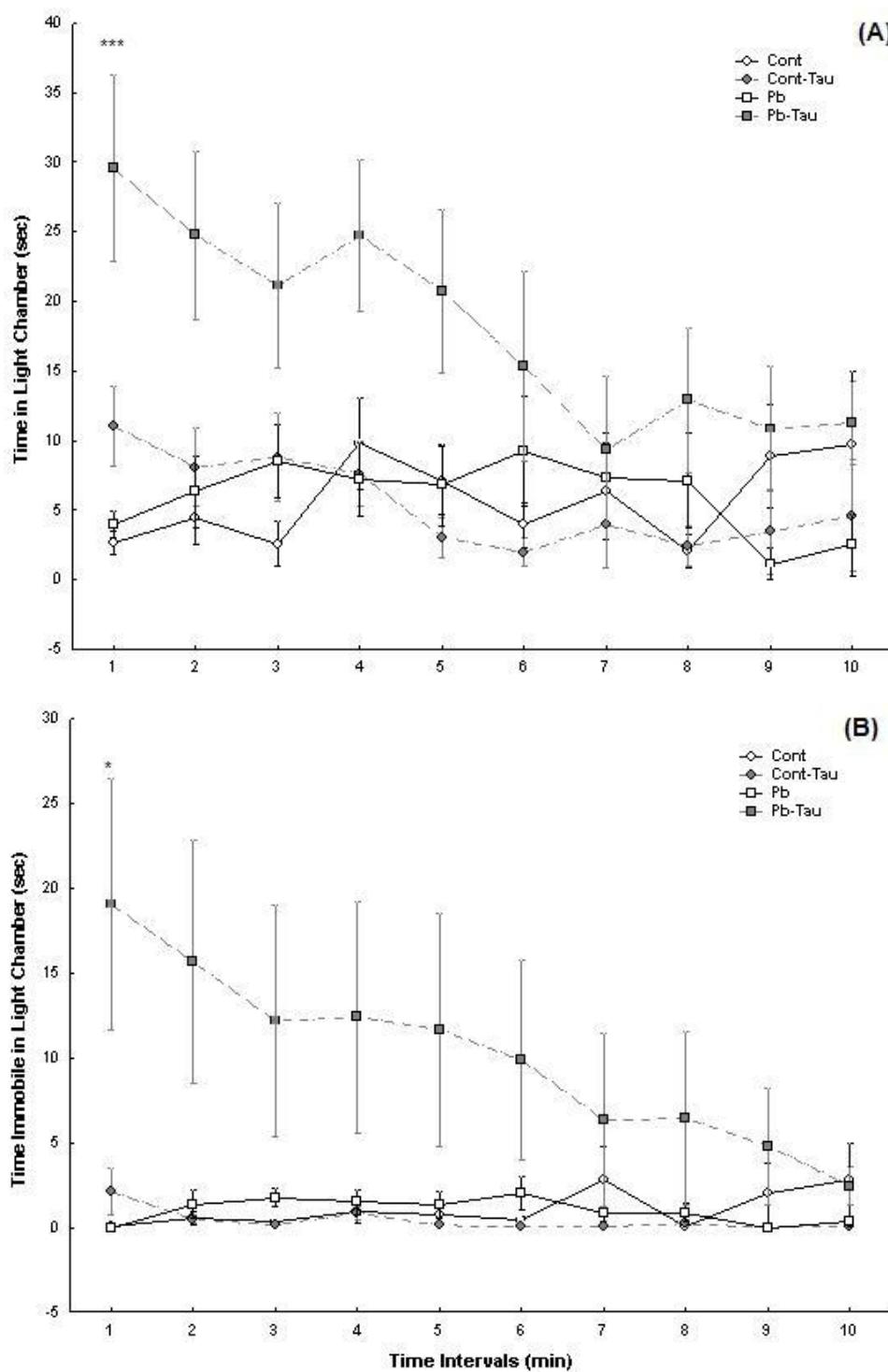


Figure 32. Taurine treatment increased anxiety in Pb^{2+} rats evidenced by increased acclimation time in light chamber during the first minutes in Pb^{2+} rats and for first 5 minutes in Pb^{2+} & Taurine rats in the LD test (A). However, this time spent in the light chamber was time spent freezing 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***), 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***), 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**).

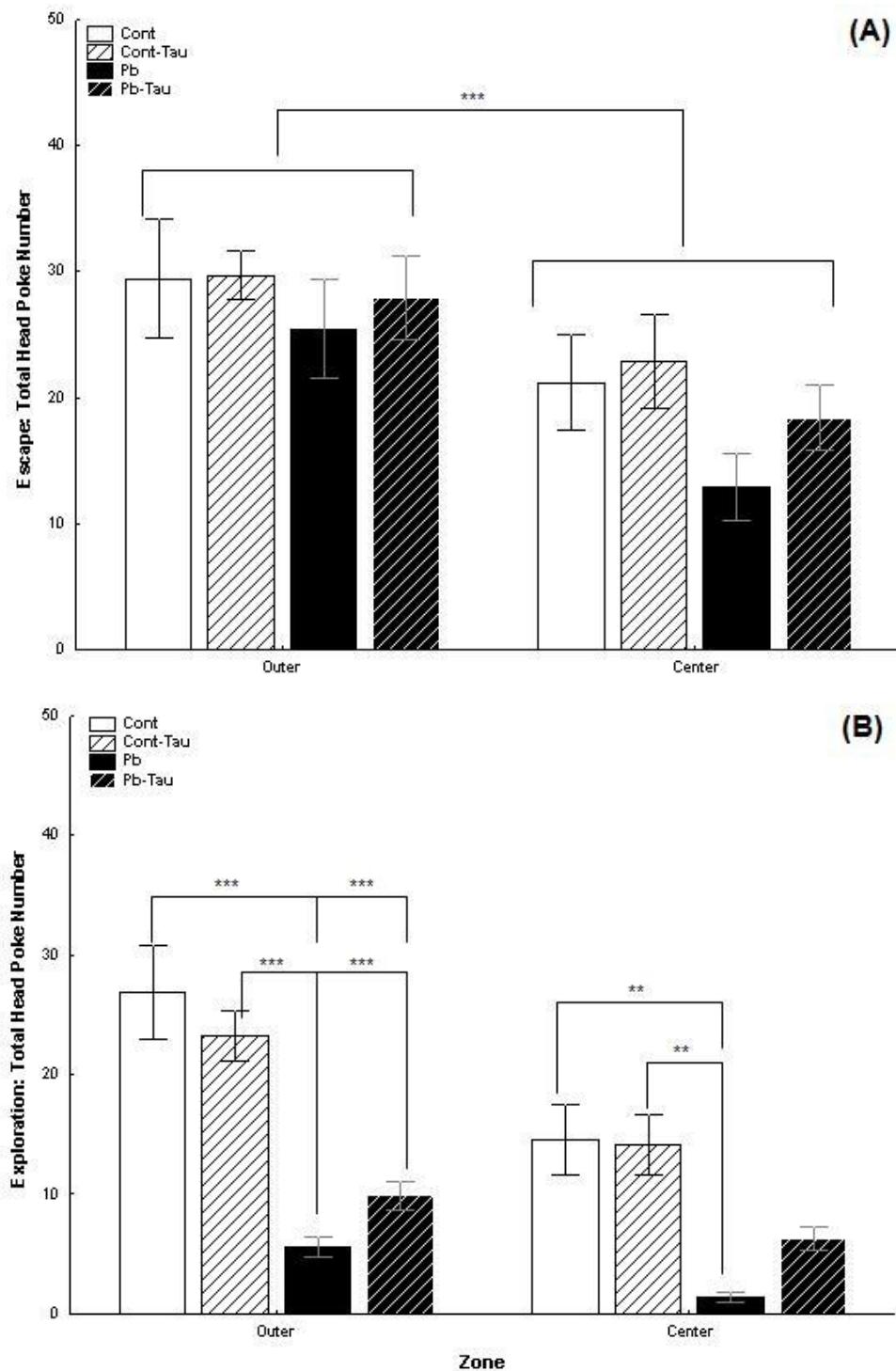


Figure 33. Frequency comparisons between the traditional HB test for anxiety escape behaviors (A) vs. a novel exploration test (B). The HB test showed a zone effect in which Pb^{2+} rats had reduced head pokes in the center zone, but this trait improved with taurine treatment (A). However, the day two's 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*).

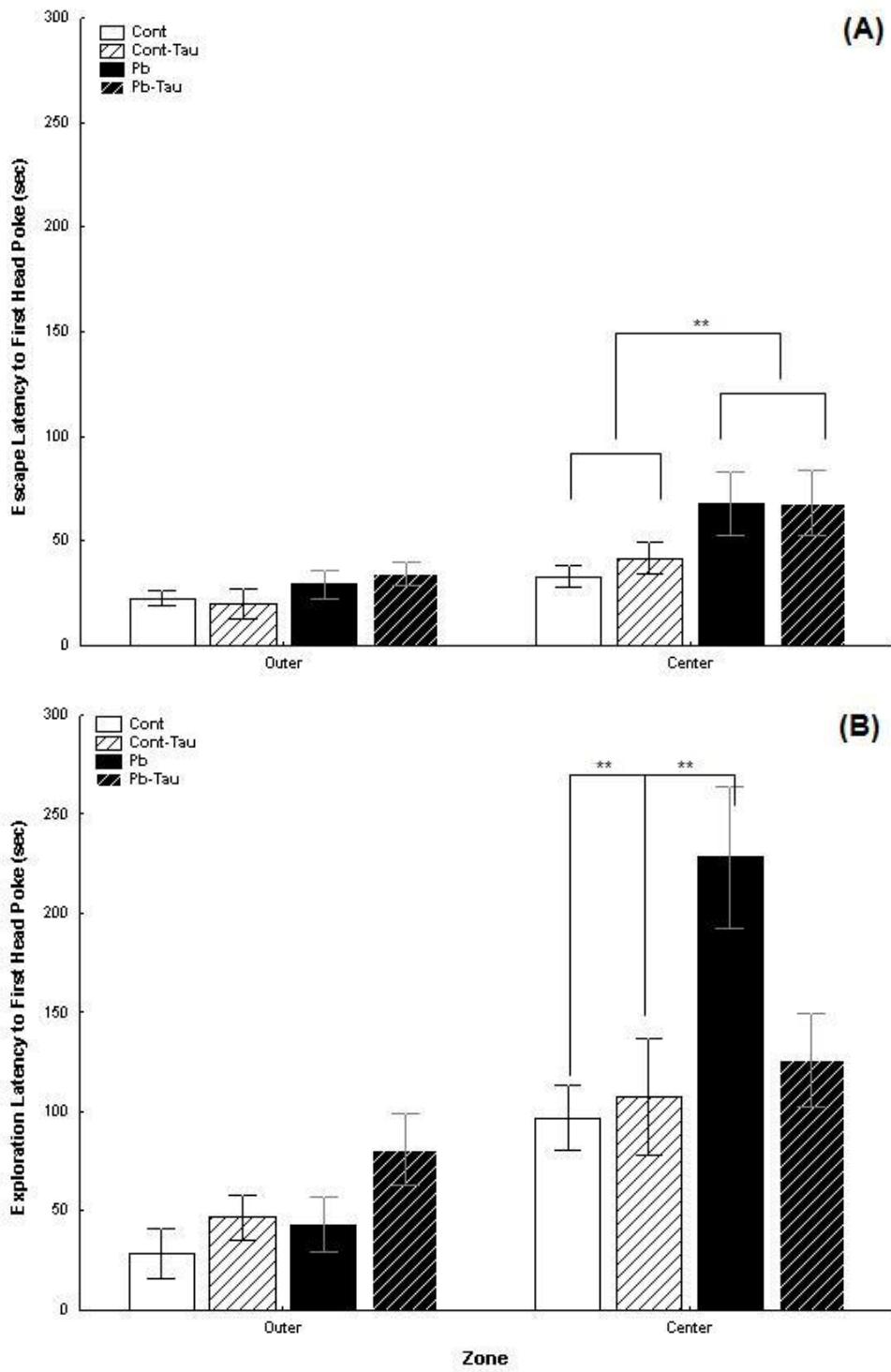


Figure 34. Latency to first head poke comparisons between the traditional HB test for anxiety escape behaviors (A) vs. a novel exploration test (B). The HB test showed a zone and Pb^{2+} effect in the escape condition, with no taurine differences noted (A). However, the day two's testing procedures were 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 Pb²⁺ X Taurine* interaction (DF=1, MS=18, 395.4, F=4.36, p<0.04*).

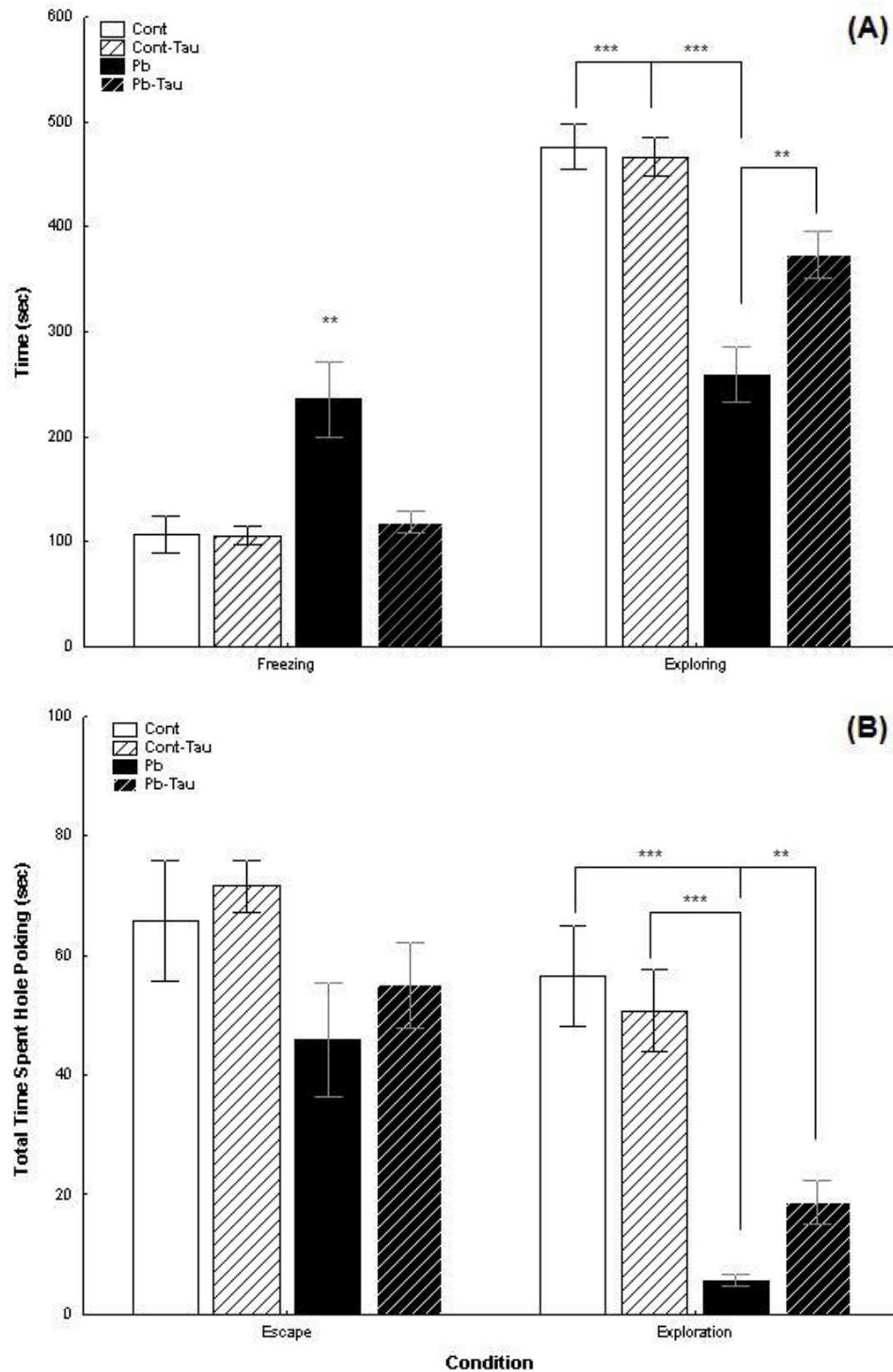


Figure 35. Time comparisons between freezing and exploring behaviors (A) and total time engaged in hole poking behaviors during these test conditions (B). The HB test showed a Pb^{2+} and a taurine effect in which Pb^{2+} rats had increased time freezing in the escape condition and decreased time freezing 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 Pb²⁺* 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 Pb²⁺ 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 Pb²⁺* interaction (DF=1, MS=2,241.7, F=5.04, p<0.03*).

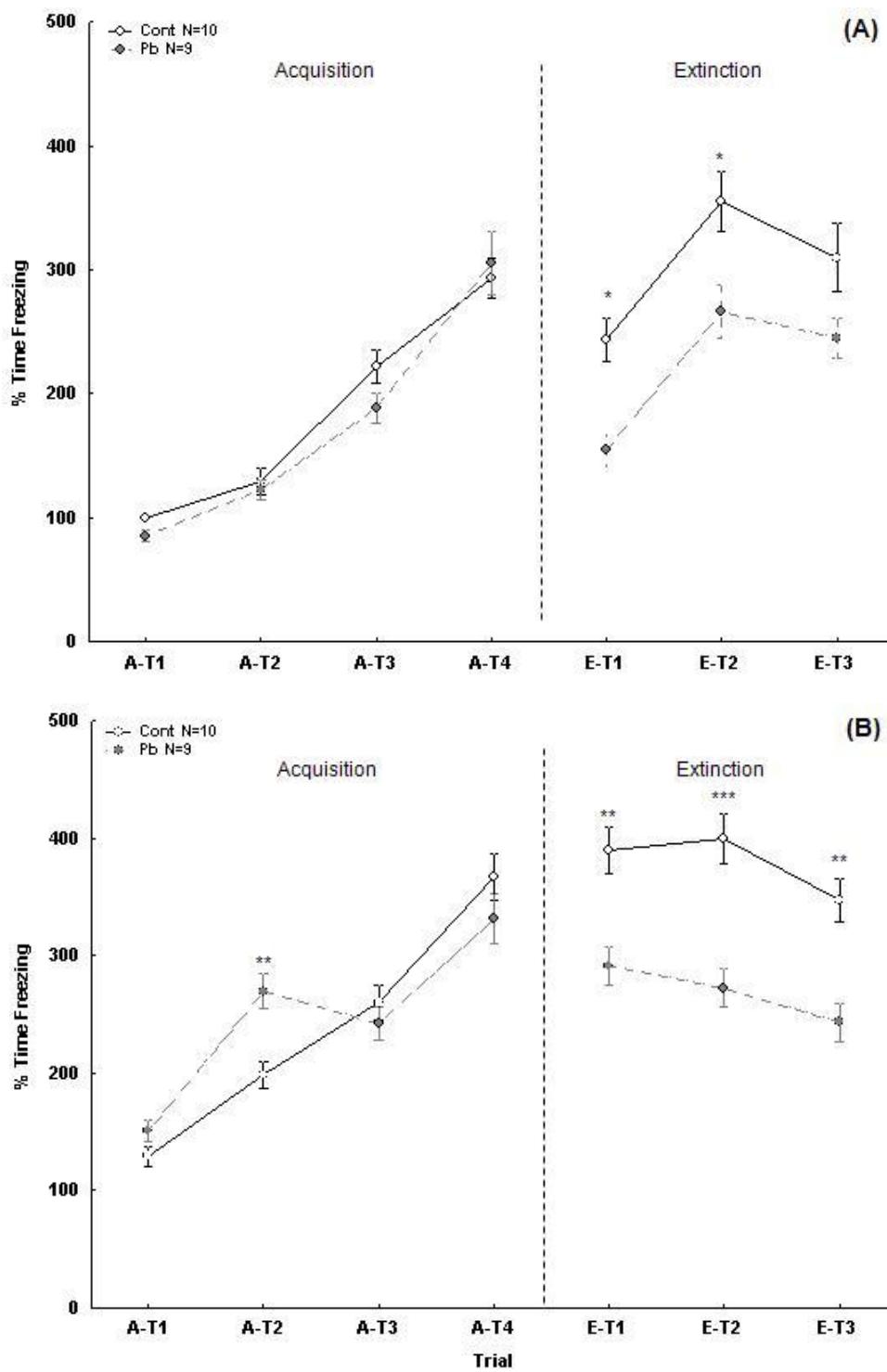


Figure 36. Differences in Context Fear acquisition and extinction learning with respect to context **(A)** and aversive stimuli **(B)**. Pb²⁺ treated rats show no differences in fear learning, but have reduced memory consolidation of the prior days learning as evidenced by extinction trials in both context **(A)** and aversive **(B)** conditions. In addition, Pb²⁺ 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 Pb²⁺* 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).

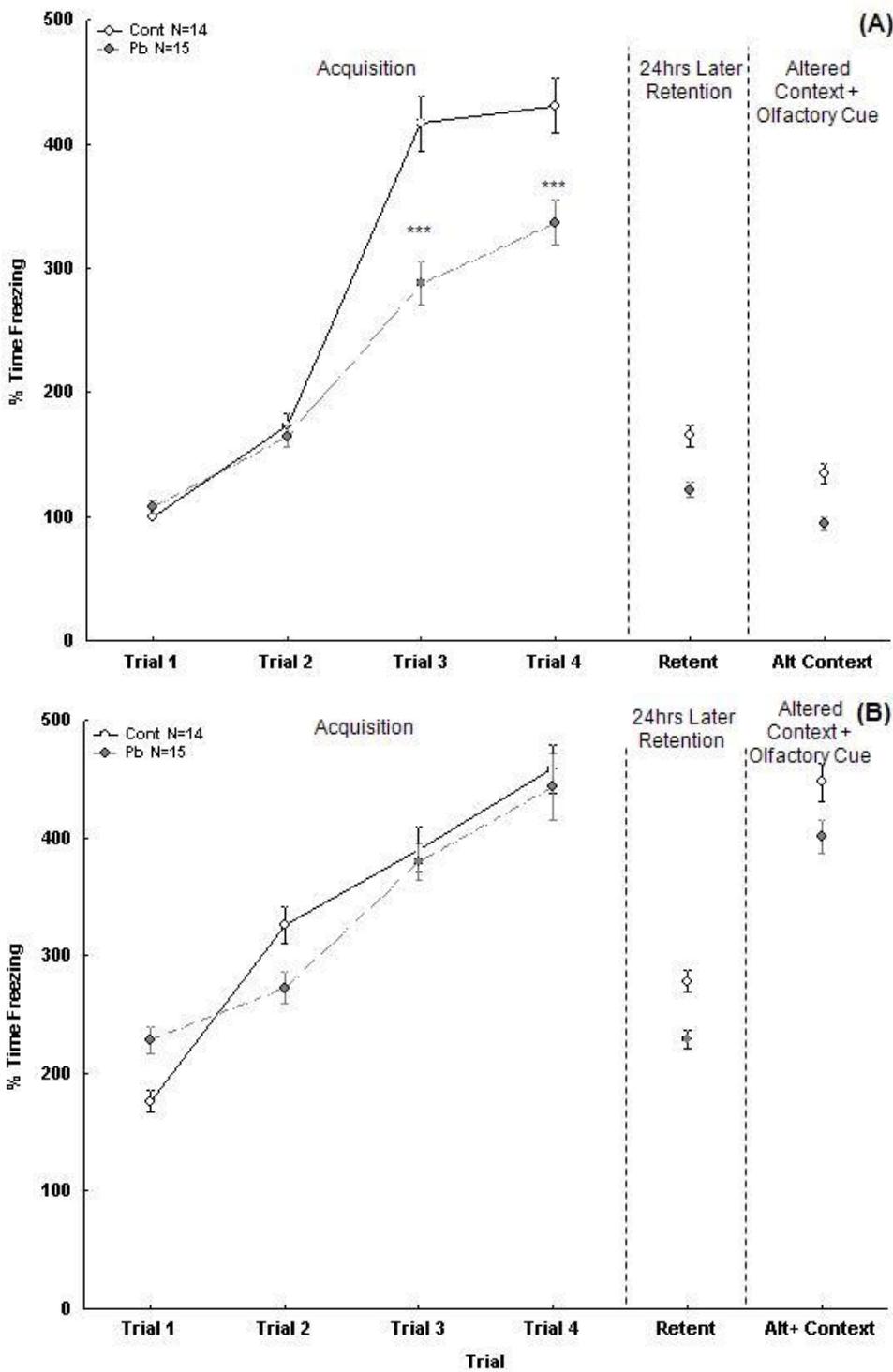


Figure 37. Differences in ACFC acquisition and retention learning with respect to context **(A)** and aversive stimuli **(B)**. Pb²⁺ treated rats showed reduced context learning **(A)**, and initial sensitization to aversive fear learning indicating stressful conditions that may impeded memory consolidation 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**).

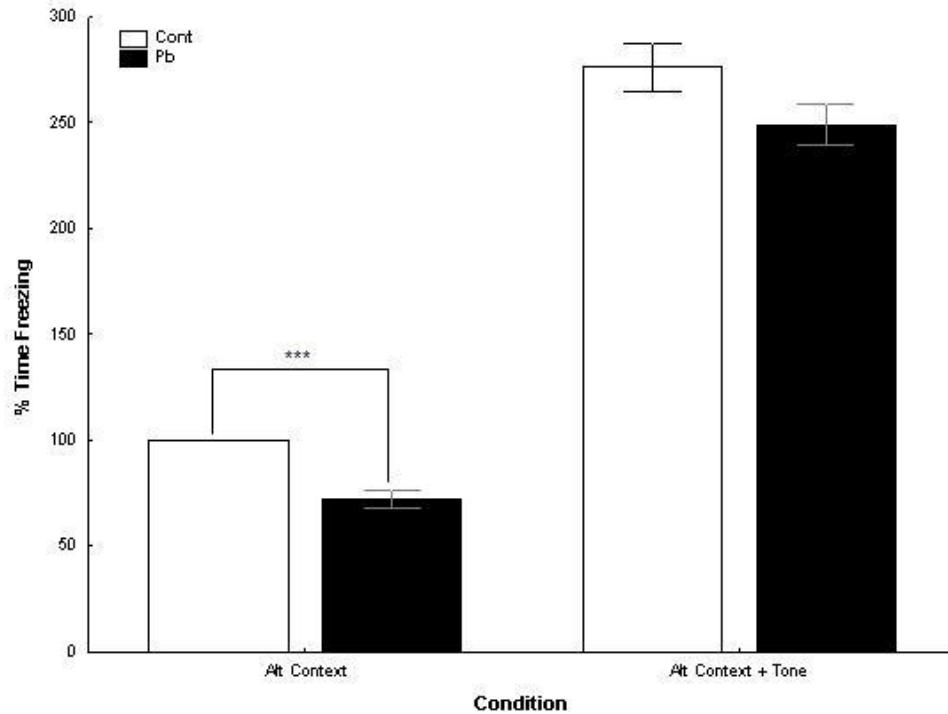


Figure 38. Differences in Auditory Cued Context Conditioning and generalization into alternate contexts. Pb^{2+} treated rats showed reduced freezing in an altered context when compared to controls. However, once the tone previously paired with aversive foot shock was presented both control and Pb^{2+} rats froze equivalently. 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***).

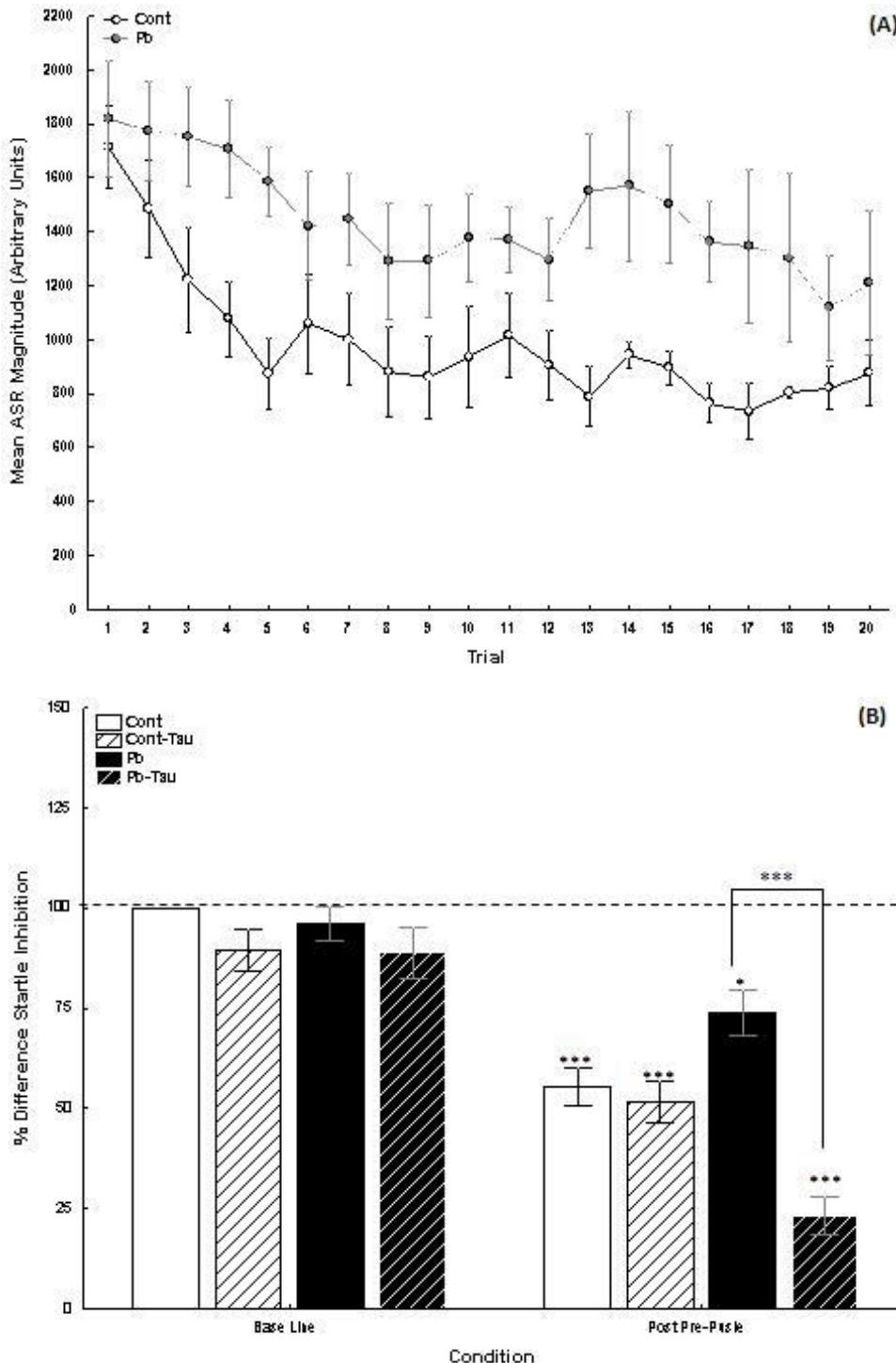


Figure 39. Effects of Pb^{2+} on non-associative startle habituation (A) and the effects of Pb^{2+} treated rats PPI learning through a pre-post startle 115dB intervention and the use of taurine (B). The graph illustrates that Pb^{2+} rats are hypersensitive to auditory stimuli and they have delays in 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 $Pb^{2+} \times Taurine$ interaction ($DF=1$, $MS=8,193$, $F=8.50$, $p<0.01^{**}$) and a *Condition X Pb²⁺ X Taurine* interaction ($DF=1$, $MS=10,982$, $F=11.4$, $p<0.001^{***}$) (B).

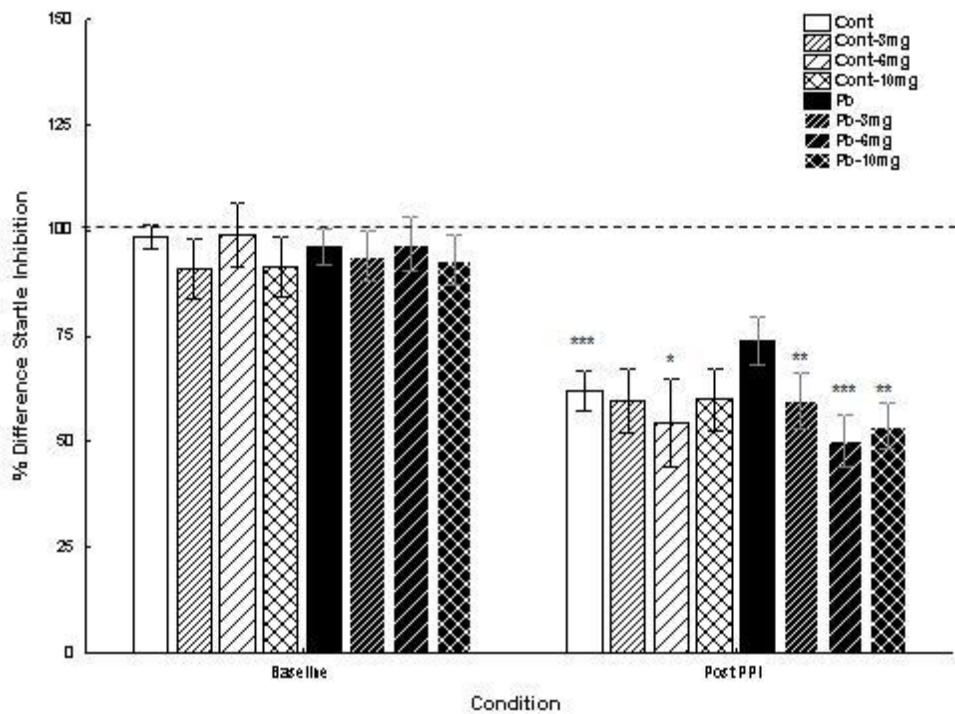


Figure 40. Effects of Pb^{2+} treated rats PPI learning through a pre-post startle 115dB intervention and the use of Baclofen.

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 Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ in the cortex and hippocampus at the aforementioned ages. Our results indicate a Pb²⁺ 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 Pb²⁺ inhibits Caβ3 gene regulation in both brain regions beginning at PND 7.

Notably, the GABA_{AR}-β3mRNA 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 Pb²⁺ 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 Pb²⁺. This suggests a Pb²⁺ 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 Pb²⁺ 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 Pb²⁺. This suggests that in cortex Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ 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 Pb²⁺.

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 Pb²⁺. Interestingly, we observed FMR1 mRNA down regulation (i.e. reduced postsynaptic plasticity) at PND 14. Together these results indicate that Pb²⁺ 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 Pb²⁺ alters signaling based on these molecular changes.

Following these qRT-PCR findings we evaluated whether Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ 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 Pb²⁺. 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 Pb²⁺ induces brain region specific alterations between cortex and hippocampus and inter region changes within the hippocampus in response to Pb²⁺.

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⁻, whether or not Pb²⁺ can induce VSCC-β3 nuclear translocation consistent with our hypothesis indicating the mechanism by which Pb²⁺ would alter GABAergic synergistic signaling via the GABA_{AR} and the VSCCs. Results show that cultured neurons treated with PbCl⁻ 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 Pb²⁺ exposure and as these levels increase neurons may appear to be insensitive, but more practically

mechanisms involved in clearing/removing Pb²⁺ 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 Pb²⁺ influences the increased expression of GABA_{ARS} to most likely compensate for the increased neuron excitability produced by Pb²⁺. In addition, this increased excitability produces synergistic effects with VSCCs having increased sensitivity to low doses of Pb²⁺, 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 Pb²⁺ interferes with physiological functions at these respective ages. To address this question we evaluated the spontaneous and evoked release of glutamate analog, ³H-D-Asparate, its binding and uptake in synaptosomal tissue fractions from cortex and hippocampus to assess neuronal communication in response to Pb²⁺. Results showed that in cortex Pb²⁺ increased ³H-D-Asparate spontaneous release at PND 2, 7 and 22 and was reduced at PND 14. In contrast, in hippocampus ³H-D-Asparate spontaneous release was slightly

increased at PND 7 and decreased at PND 14. Comparatively, $^{3\text{H}}$ -D-Aspartate evoked release in the cortex diminished at PND 2 and 7; whereas in hippocampus showed a trend of decreased $^{3\text{H}}$ -D-Aspartate 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-Aspartate uptake was reduced at PND 7 and increased at PND 14 and 22. Lastly, in both cortex and hippocampus $^{3\text{H}}$ -D-Aspartate 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 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 Pb^{2+} having altered the synaptic proteins involved in neurotransmission from development rather than being added *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 Pb^{2+} , synaptosomal fractions had difficulty accumulating 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 PND7 in cortex. These findings suggest that the cortex and hippocampus are negatively affected by 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 Pb²⁺. In addition, these Pb²⁺ 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 Pb²⁺ 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 prepulse 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, Pb²⁺ 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 Pb²⁺ 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, Pb²⁺ rats were not affected by taurine, with the exception of head dips. Taurine reduced Pb²⁺ rats time and number of head dips suggesting an anxiogenic, rather than anxiolytic effect.

In the light: dark test, control and Pb²⁺ 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 Pb²⁺ rats. Notably, the Pb²⁺ 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 Pb²⁺ induced effects on freezing behavior absent of any painful aversive stimuli and if we could further augment the Pb²⁺ 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.

Pb²⁺ 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 Pb²⁺ rats exploration latency on day 2 when compared to Pb²⁺ rats and were equivocal to controls. Taurine did not produce an effect on control rats. Pb²⁺ rats administered taurine also exhibited less time freezing on day 1 and more

time exploring on day 2 when compared to Pb²⁺, 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 Pb²⁺ rats may respond differently to contextual environmental cues. The state of anxiety or distress that the rat undergoes in novel testing situations may be over active in Pb²⁺ 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 Pb²⁺ rats may have an altered GABAergic system which is more sensitive than the control rats.

In order to further assess whether Pb²⁺ rats respond differently to contextual environmental cues we subjected another set of rats to a classical Pavlovian conditioning paradigm. This test revealed that Pb²⁺ rats learning abilities are not different from controls, but Pb²⁺ rats are more sensitive to aversive stimuli (i.e. Pb²⁺ makes them more reactive to stressors) and they are less inclined to pick up the contextual environmental cues due to this elevate stress response. The consequence of these Pb²⁺ 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 Pb²⁺ rats exhibited similar problems to the previous groups where reduced contextual learning was observed and poor retention; whereas, Pb²⁺ rats exhibited increased sensitivity to aversive conditions, were more reactive and still produced poor retention. Notably, Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ treated rats. Moreover, there were no control (+) Baclofen effects when compared to Pb²⁺ (+) 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 than its possible ratio of GABA_{BRs}. In addition, GABA_{BRs} 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_{BRs} 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 Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ interferes with immature neuronal VSCCs and GABA_{AR} mediated gene regulation in early development which has never been reported in this framework we present here.
- Specifically, gestational Pb²⁺ alters the expression pattern of the genes regulating the GABA-shift in cortex and hippocampus. Brain regions are differentially affected.
- Gestational Pb²⁺ induces developmental delays through altered expression of KCC2 differentially affecting cortex and hippocampus excitation-to-inhibition signaling.
- Gestation Pb²⁺ induces altered neurotransmitter signaling resulting in increased brain excitability post synaptically.
- Gestational Pb²⁺ 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 Pb²⁺ exposure in potentially ameliorating Pb²⁺ induced gene dysfunction in early development.
- Assess in primary neuronal cultures taurine and Pb²⁺ 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 Ca²⁺ homeostasis under Pb²⁺ altered gene expression.
- Assess seizure susceptibility as a result of increased brain excitability induced by Pb²⁺ under GABAergic and Glutamatergic pharmacology.
- Assess more advanced cognitive behavioral tests to understand Pb²⁺ 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 Pb²⁺ on GABAergic neural development in the rat model. Our data suggest that Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ may produce varied effects; however our model was restricted to a 956ppm Pb²⁺ 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 Pb²⁺ 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

- Abazyan, B., Dziedzic, J., Hua, K., Abazyan, S., Yang, C., Mori, S., Pletnikov, M.V., & Guilarte, T.R. (2013). Chronic exposure to mutant DISC1 mice to lead produces sex-dependent abnormalities consistent with schizophrenia and related mental disorders: A gene-environment interaction study. *Schizophrenia Bulletin*, 1-10.
- Andrew, H., Zhang, A., Ke, Y., El Idrissi, A., & Shen, C.H. (2012). Decreased expression of GABA_Aβ subunits in the brains of mice lacking the Fragile X mental retardation protein. *J. Mol. Neurosci.* 46, 272-275.
- Atchison, W.D. and Narahashi, T. (1984). Mechanism of action of lead on neuromuscular junctions. *Neurotoxicology*, 5, 247.
- Atkins, C.M., Selcher, J.C., Petraitis, J.J., Trzaskos, J.M. & Sweatt, J.D. (1998). The MAPK cascade is required for mammalian associative learning. *Nature Neuroscience*, 1, 602-609.
- Audesirk, G.J & Tjalkens R.B. (2004). In vitro studies of neurotoxicant effects on cellular homeostasis. In: *In Vitro Neurotoxicology: Principles and challenges* (Ed. Evelyn Tiffany-Caastiglioni). pp 59-94, Humana Press.: Totowa. N.J.
- Averill, D., and Needleman, H. (1980). Neonatal lead exposure retards cortical synaptogenesis in the rat. In: *Low Level Lead Exposure: The Clinical Implications of Current Research* (Needleman, H. ed.) New York: Raven Press, 210.
- Bai, F. and Witzmann, F.A. (2007). Synaptosome proteomics. In: *Subcellular Proteomics*, E. Bertrand and M. Faupel, (Eds.), Springer, New York: N.Y., pp.77-98.
- Beal, M.F., Brouillet, E., Jenkins, B.G., Ferrante, R.J., Kowall, N.W., Miller, J.M., et al. (1993). Neurochemical and histological characterization of striatal excitotoxic lesions produced by the mitochondrial toxin 3-nitropropionic acid. *J Neurosci*, 13, 4181-4192.

Bellinger, D.C., Stiles, K.M., & Needleman, H.L. (1992). Low-level lead exposure, intelligence and academic achievement: a long-term follow up study. *Pediatrics* 90: 855-861.

Bellinger, D.C. and Dietrich, K.N. (1994). Low-level lead exposure and cognitive function in children. *Pediatr. Ann.* 23: 600-605.

Bellinger, D. (1995). Lead and neuropsychological function in children: progress and problems in establishing brain-behavior relationships. *Adv. Child. Neuropsychol.* 3, 12-45.

Bellinger, D. (1996). Learning and behavioral sequelae of lead poisoning. In. S.M. Pueschel, J.G. Linakis, & A.C. Anderson (Eds.), *Lead poisoning in childhood*. (pp 97-116). Baltimore, MD: Brookes.

Ben-Ari, Y. (2002). Excitatory actions of GABA during development: The nature or the nurture. *Nature Reviews Neuroscience*, 9, 728-739.

Ben-Ari, Y., Gaiarsa, J.L., Tyzio, R., & Khazipov, R. (2007). GABA: A pioneer transmitter that excites immature neurons and generates primitive oscillations. *Physiol Rev*, 87, (4), 1215-1284.

Ben-Ari, Y., Khalilov, I., Kahle, K.T., & Cherubini, E. (2012). The GABA excitatory/inhibitory shift in brain maturation and neurological disorders. *The Neuroscientist*: 1-20.

Benarroch, E.E. (2010). Neuronal voltage-gated calcium channels: Brief overview of their function and clinical implications in neurology. *Neurology*, 74 (16), 1310-1315.

Bliss, T.V.P. and Collingridge, G.L. (1993). A synaptic model of memory: Long-term potentiation in the hippocampus. *Nature*, 361, 31-39.

Borisova, T., Krisanova, N., Sivko, R., Kasatkina, L., Borysov, A., Griffin, S., & Wireman, M. (2011). Presynaptic malfunction: The neurotoxic effects of cadmium and lead on the proton

gradient of synaptic vesicles and glutamate transport. *Neurochemistry International*, 59, 272-279.

BourJeily, N. and Suszkiw, J.B. (1997). Developmental cholinotoxicity of lead: loss of septal cholinergic neurons and long-term changes in cholinergic innervation of the hippocampus in perinatally lead exposed rats. *Brain Res.*, 771, 319-328.

Braga, M.F., Pereira, E.F., Albuquerque, E.X (1999). Nanomolar concentrations of lead inhibit glutamatergic and GABAergic transmission in hippocampal neurons. *Brain Res.*, 826, 22-34.

Bragin, A. Jando, G., Nadasdy, Z., Hetke, J., Wise, K., & Buzaki, G. (1995). Gamma (40-100Hz) oscillation in the hippocampus of the behaving rat. *J. Neurosci.*, 15, 47-60.

Bressler, J.P. and Goldstein, G.W. (1991). Mechanisms of lead neurotoxicity. *Biochem Pharmacol*, 41, 479-494.

Bressler, J.P., Kim, K.A., Charaboti, T., & Goldstein, G. (1999). Molecular mechanisms of lead neurotoxicity. *Neurochem Res*, 24, 595-600.

Bros, M., Dexheimer, N., Ross, R., Trojandt, S., Höhn, Y., Tampe, J., Sutter, A., Jährling, F., Grabbe, S., & Reske-Kunz, A.B. (2011). Differential gene expression analysis identifies murine Cacnb3 as strongly upregulated in distinct dendritic cell populations upon stimulation. *Gene*, 472, 18-27.

Brutkowski, S. (1964). Prefrontal cortex and drive inhibition. In : *The Frontal Granular Cortex and Behavior*, J.M. Warren and K. Akert, (Eds.), McGraw-Hill, New York: N.Y., pp. 242-270.

Büsselberg, D., Evans, M.L., Rahmann, H., and Carpenter, D.O. (1991). Lead and zinc block a voltage activated calcium channel of *Aplysia* neurons. *J. Neurophysiol.* 65, 786-795.

Buzaki, G., and Chrobak, J.J. (1995). Temporal structure in spatially organized neuronal ensembles: A role for interneuron networks. *Curr. Opin. Neurobiol.* 5, 504-510.

Canfield, R.L., Henderson Jr., C.R., Cory-Slechta, D.A., Cox, C., Jusko, T.A., & Lanphear, B.P. (2003). Intellectual impairment in children with blood lead concentrations below 10 microg per deciliter. *N Engl J Med*, 348, (16), 1517-1526.

Carrion, A.M., Link, W., A., Ledo, F., Melstrom, B., & Naranjo, J.R. (1999). DREAM is a Ca^{2+} -regulated transcriptional repressor. *Nature*, 398, 80-84.

Cecil, K.M., Brubaker, C.J., Adler, C.M., Dietrich, K.N., Altaye, M., et al., (2008). Decreased brain volume in adults with childhood lead exposure. *PLoS Med*, 5, e 112. doi. 10.1371/journal.pmed. 0050112.

CDC, 1991. Preventing lead poisoning in young children: A statement by the centers for disease control. Atlanta, GA: *Centers for Disease Control*.

Coria, F. Berciano, M.T., Berciano, J. & LaFarga, M. (1984). Axon membrane remodeling in the lead-induced demyelinating neuropathy of the rat. *Brain Res*, 291, 369-372.

Chisolm, J., Angle, C., Bornschein, R., Graziano, J., Keck, J., Mortenson, M., & Needleman, H. (1991). *A new look at lead toxicity: Conference on childhood lead toxicity [pamphlet]*. Comtack Corporation.

Dabrowska-Bouta, B., Sulkowski, G., Bartosz, G., Walski, M., & Rafalowska, U. (1999). Chronic lead intoxication affects the myelin membrane status in the central nervous system of adult rats. *J Mol Neurosci*, 13, 127-139.

Dabrowska-Bouta, B., Sulkowski, G., Walski, M. Struzynska, L., Lenkiewicz, A., & Rafalowska U. (2000). Acute lead intoxication *in vivo* affects myelin membrane morphology and CNPase activity. *Exp Toxicol Pathol*, 52, 257-263.

Deisseroth, K., Bito, H., and Tsien, R.W. (1996). Signaling from synapse to nucleus: Postsynaptic CREB phosphorylation during multiple forms of hippocampal synaptic plasticity. *Neuron*. 16, 89-101.

Deng, W., McKinnon, R.D., & Portez, R.D. (2001). Lead exposure delays the differentiation of oligodendroglial progenitors in vitro. *Toxicol Appl Pharmacol*, 174, 235-244.

Drew, C.A., Spence, I., & Johnston, G.A. (1989). Effects of lead salts on the uptake, release, and binding of gamma-aminobutyric acid: The importance of buffer composition. *J Neurochem*, 52, 433-440.

Dolmetsch, R.E., Pajvani, U., Fife, K., et al. (2001). Signaling to the nucleus by an L-Type calcium channel-calmodulin complex through the MAP kinase pathway. *Science*, 294, 333-339.

Dyck, P.J., O'Brien, P.C., & Ohnishi, A. (1977). Random distribution of segmental demyelination among 'old internodes' of myelinated fibers. *J Neuropathol Exp Neurol*, 36, 570-575.

Evans, M.L., Büsselberg, D., and Carpenter, D.O. (1991). Pb^{2+} blocks calcium currents of cultured dorsal root ganglion cells. *Neuroscience Letters*. 129, 103-106.

Faust, D. and Brown, J. (1987). Moderately elevated blood lead levels: effects on neuropsychologic functioning in children. *Pediatrics* 80: 623-629.

Fergusuon, C., Kern, M., & Audesirk, G. (2000). Nanomolar concentrations of inorganic lead increase Ca^{2+} efflux and decrease intracellular free Ca^{2+} ion concentrations in cultured rat hippocampal neurons by a calmodulin-dependent mechanism. *Neurotoxicology*, 21, 365-378.

Ford, J., Odeyale, O. Eskandar, A., Kouba, N., & Shen, C.H. (2007). A SWI/SNF-and INO80-dependent nucleosome movement at the INO1promoter. *Biochem. Biophys. Res. Commun.* 361: 974-979.

Finkelstein, Y., Markowitz, M.E., & Rosen, J.F. (1998). Low-level lead-induced neurotoxicity in children: an update on central nervous system effects. *Brain Research Reviews*. 27, 168-176.

Gamba, G. (2005). Molecular physiology and pathophysiology of electroneutral cation chloride cotrans-porters. *Physiol Rev*, 85, 423-493.

Ganguly, K., Schinder, A.F., Wong, S.T., & Poo, M. (2001). GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. *Cell Press* 105: 521-532.

Gilbert, M.E., Mack, M.C., & Lasley, S.M. (1996). Chronic developmental lead exposure increases the threshold for long-term potentiation in rat dentate gyrus in vivo. *Brain Res*, 736, 118-124.

Gilbert, M.E., Mack, C.M., & Lasley, S.M. (1999). Chronic developmental lead exposure and long term potentiation: Biphasic does response relationship. *Neurotoxicology*, 20 (1), 71-82.

Giustizieri, M., Bernardi, G., Mercuri, N.B., Berretta, N. (2005). Distinct mechanisms of presynaptic inhibition at GABAergic synapses of the rat substantia nigra pars compacta. *J. Neurophysiol.*, 94, 1992-2003.

Gomez-Ospinosa, N., Tsuruta, F., Barreto-Chang, O., Hu, L., & Dolmetsch, R. (2006). The C terminus of the L-type voltage gated calcium channel $\text{Ca}_{v1.2}$ encodes a transcription factor. *Cell*, 127, 591-606.

Greenberg, M.E., Ziff, E.B., and Green, L.A. (1986). Stimulation of neuronal acetylcholine receptors induces rapid gene transcription. *Science*. 234, 80-83.

Guilarte, T.R. and McGlothan, J.L. (1998). Hippocampal NMDA receptor mRNA undergoes subunit specific changes during developmental lead exposure. *Brain Res*. 790, 98-107.

Guilarte, T.R., McGlothan, J.L., & Nihei, M.K. (2000). Hippocampal expression of N-methyl-D-aspartate receptor (NMDAR1) subunit splice variant mRNA is altered by developmental Pb²⁺ exposure. *Molecular Brain Research*, 76, 299-305.

Guilarte, T.R., and McGlothan, J.L. (2003). Selective decrease in NR1 subunit splice variant mRNA in the hippocampus of Pb²⁺-exposed rats: implications for synaptic targeting and cell surface expression of NDMAR complexes. *Molecular Brain Research*, 113, 37-43.

Gulson, B.L., Mizon, K.J., Korsch, M.J., Palmer, J.M., & Donnrlly, J.B. (2003). Mobilization of lead from human bone tissue during pregnancy and lactation--a summary of long-term research. *Sci Total Environ*, 303 (1-2), 79-104.

Hasse, H., Wallukat, G., Flockerzi, V., et al., (1994). Detection of skeletal muscle calcium channel subunits in cultured neonatal rat cardiac myocytes. *Receptors Channels*, 2, 41-52.

Healy, M.A., Aslam, M., Harrison, P.G., & Fernando, N.P. (1984). Lead-induced convulsions in young infants-a case history and the role of GABA and sodium valproate in the pathogenesis and treatment. *J. Clin. Hosp. Pharm.*, 9, 199-207.

Hell, J.W., Westenbroek, R.E., Warner, C., et al. (1993). Identification and differential subcellular localization of the neuronal class C and class D L-Type calcium channel alpha 1 subunits. *J Cell Biol*, 123, 949-962.

Herlitze, S. and Mark, M.D. (2005). Distribution and targeting mechanisms of voltage activated Ca²⁺ channels. In: *Voltage-Gated Calcium Channels*, G. Zamponi (Ed.), Kluwer Academic / Plenum Publishers, New York: N.Y., pp. 113-140.

Hu, H. and Hernandez-Avila, M. (2002). Invited commentary: lead, bones, women, and pregnancy--the poison within? *Am J Epidemiol*, 156 (2), 1088-1091.

Hussain, R.J., Parsons, P.J., & Carpenter, D.O. (2000). Effect of lead on long-term potentiation hippocampal CA3 vary with age. *Brain Res Dev Brain Res*, 121, (2), 243-252.

Hyde, T.M., Lipska, B.K., Ali, T., Matthew, S.V>, Law, A.J., Metitiri, O.E., Straub, R.E., Ye, T., Colantuoni, C., Hermna, M.M., Bigelow, L.B., Weinberger, D.R., & Kleinman, J.E. (2001). Expression of GABA signaling molecules KCC2, NKCC1, and GAD1 in cortical development and schizophrenia. *The Journal of Neuroscience*, 31, (30), 11088-11095.

Jablońska, L., Walski, M., & Rafalowska, U. (1994). Lead as a n inductor of some morphological and functional changes in synaptosomes from rat brain. *Cell Mol Neurobiol.*, 14, (6), 701-709.

Jett, D.A., Kuhlmann, A.C., Farmer, S.J., & Guilarte, T.R. (1997). Age-dependent effects of developmental lead exposure on performance in the Morris water maze. *Pharmacol Biochem Behav*, 57 (1-2), 271-279.

Jason, K.M. and Kellogg, C.K. (1980). Behavioral neurotoxicology of lead. In. *Lead toxicity*. R.L. Singhal and J.A. Thomas (Eds.). (pp.241-272). Baltimore, MD: Urban & Schwarzenberg.

Kahle, K.T., Rinehart, J., de los Heros, P., Louvi, A. Meade, P., Vazquez, N., et al. (2005). WNK3 modulates of Cl⁻ transport in and out of cells: Implications for control of cell volume and neuronal excitability. *Proc. Natl. Acad. Sci. USA* 102: 16783-16788.

Kahle, K.T., Staley, K.J., Nahed, B.V., Gamba, G., Hebert, S.C., Lifton, R.P. & Mount, D.B. (2008). Roles of the cation-chloride cotransporters in neurological disease. *Nature Clin Prac Neurology* (4) 9: 490-503.

Kahle, K.T., Rinehart, J., & Lifton, R.P. (2010). Phosphoregulation of the Na-K-2Cl and K-Cl cotransporters by the WNK kinases. *Biochimica et Biophysica Acta*, 1802, 1150-1158.

Kahle, K.T., Rinehart, J., & Lifton, R.P. (2012). Phosphoregulation of the Na-K-2Cl cotransporters by the WNK kinases. *Biochem. Biophys. Acta*. 1802 (12): 1150-1158.

Kawamoto, J.C., Overmann, S.R., Woolley, D.E., & Vijayan, V.K. (1984). Morphometric effects of preweaning lead exposure on the hippocampal formation of adult rats. *Neurotoxicology*, 5, 125-148.

Kern, M. and Audersirk, G. (2000). Stimulatory and inhibitory effects of inorganic lead on calcineurin. *Toxicology*, 150, 171-178.

Kerpler, L.E. and Hinkle, P.M. (1997a). Lead uptake in brain capillary endothelial cells: Activation by calcium store depletion. *Toxicol. Appl. Pharmacol.*, 146, 127-133.

Kerpler, L.E. and Hinkle, P.M. (1997b). Cellular uptake of lead is activated by depletion of intracellular calcium stores. *J. Biol. Chem.*, 272, 8346-8352.

Klugbauer, N., Welling, A., Specht, V., et al. (2002). L-Type Ca(2+) channels of the embryonic mouse heart. *Eur J Pharmacol*, 447, 279-284.

Kolb, B. and Gibb, R. (2001). Early brain injury, plasticity and behavior. In. *Handbook of Developmental Cognitive Neuroscience*, C.A. Nelson and M. Luciana (Eds.), The MIT Press, Cambridge: MA, pp.175-190.

Koller, K., Brown, T., Spurgeon, A. & Levy, L. (2004). Recent developments in low level lead exposure and intellectual impairment in children. *Environmental Health Perspectives*, 12 (9), 987-994.

Kornhauser, J.M., Cowan, C.W., Shaywitz, A.J., Dolmetsch, R.E. Griffith, E.C., Hu, L.S., Haddad, C., Xia, Z., & Greenberg, M.E. (2002). CREB transcriptional activity in neurons is regulated by multiple, calcium-specific phosphorylation events. *Neuron*, 34, 221-233.

Kostial, K. and Vouk, V.B. (1957). Lead ions and synaptic transmission in the superior cervical ganglion of the cat. *Br. J. Pharmacol.* 12, 219.

Kovatsi, L., Georgiou, E., Ioannou, A., Haitoglou, C., Tzimagiorgis, G., Tsoukali, H., & Koidou, S. (2010). P16 promoter methylation in Pb²⁺-exposed individuals. *Clinical Toxicology*, 48 (2) 124-128.

Krishnamoorthy, M.S., Parthiban, N., Muthu, P., Paul, V., Balagopal, G., Kumaravel, T.S. (1993). Effect of acute pretreatment of lead on picrotoxin-induced convulsions in rats. *J. Appl. Toxicol.*, 13, 155-159.

Lanphear, B.P., Dietrich, K., Auinger, P., & Cox, C. (2000). Cognitive deficits associated with blood lead concentrations <10µg/dL in US children and adolescents. *Public Health Rep.* 115: 521-529.

Lanphear, B.P., Hornung, R., Khoury, J., Yolton, K., Baghurst, P., Bellinger, D.C. et al. (2005). Low-level environmental lead exposure and children's intellectual function: An international pooled analysis. *Environ Health Perspect*, 113, 894-899.

Lasley, S.M., Green, M.C., & Gilbert, M.E. (1999). Influence of exposure period on in vivo hippocampal glutamate and GABA release in rats chronically exposed to lead. *Neurotoxicology*, 20, 619-629.

Lasley, S.M. and Gilbert, M.E. (2000). Glutamatergic components underlying lead-induced impairments in hippocampal synaptic plasticity. *Neurotoxicology*, 21, 1057-1068.

Lasley, S.M. and Gilbert, M.E. (2002). Rat hippocampal glutamate and GABA release exhibit biphasic effects as a function of chronic lead exposure level. *Toxicol. Sci.*, 66, 139-147.

Leclerc, C., Néant, I., & Moreau, M. (2012). The calcium: An early signal that initiates the formation of the nervous system during embryogenesis. *Frontiers in Molecular Neuroscience*, 5, (64), 1-12.

Lidsky, T.I. and Scheneider, J.S. (2003). Lead neurotoxicity in children: basic mechanisms and clinical correlates. *Brain*, 126, 5-19.

Lidsky, T.I. and Schneider, J.S. (2006). Adverse effects of childhood lead poisoning: The clinical neuropsychological perspective. *Environmental Research*. 100, 284-293.

Lindahl, L.S., Bird, L., Legare, M.E., Mikeska, G., Bratton, G.R., & Tiffany-Castiglioni, E. (1999). Differential ability of astroglia and neuronal cells to accumulate lead: Dependence on cell type and on degree of differentiation. *Toxicol Sci*, 50, 236-243.

Liu, J., Shi, L., Wan, B., & Li, B. (2000). Effects of chronic exposure to lead on long-term potentiation in hippocampal CA1 area on young rats in vivo. *Chinese Journal of Preventative Medicine*, 34 (1), 34-36.

Lu, J., Karadsheh, M., & Delpire, E. (1999). Developmental regulation of the neuronal-specific isoform of K-Cl cotransporter KCC2 in postnatal rat brains. *J. Neurobiol.* 39: 558-568.

Manalis, R.S. and Cooper, G.P. (1973). Presynaptic and postsynaptic effects on synaptic transmission. *Neurotoxicology*, 5, 247.

Matton, M.P. (1988). Neurotransmitters in the regulation of neuronal cytoarchitecture. *Brain Res. Rev.*, 13, 179.

Mazzolini, M., Traverso, S., & Marcherri, C. (2001). Multiple pathways of Pb²⁺ permeation in rat cerebellar granule neurons. *J. Neurochem.*, 79, 407-416.

McCauley, P.T., Bull, R.J., Tonti, A.P., Lutkenhoff, S.D., Meister, M.V., Doerger, J.U., & Stober, J.A., (1982). The effect of prenatal and postnatal lead exposure on neonatal synaptogenesis in rat cerebral cortex. *J Toxicol Environ Health*, 10, 639-651.

Mejia-Alvarez, R., Tomaselli, G.F., Marban, E. (1994). Simultaneous expression of cardiac and skeletal muscle isoforms of the L-Type Ca^{2+} channel in a rat heart muscle cell line. *J Physiol*, 478, 315-329.

Mellstrom, B. and Naranjo, J.R. (2001). $\text{Ca}(2+)$ -dependent transcriptional repression and depression: DREAM, a direct effector. *Semin. Cell Dev. Biol.*, 12, 59-63.

Minnema, D.J., Greenland, R.D., & Manalis, R.S. (1986). Effect of in vitro inorganic lead on dopamine release from superfused rat striatal synaptosomes. *Toxicol. Appl. Pharmacol.*, 92, 351.

Minnema, D.J. and Michaelson, I.A. (1986). Differential effects of inorganic lead and delata-aminolevulinic acid in vitro on synaptosomal gamma-amminobutyric acid release. *Toxicol. Appl. Pharmacol.* 86, 437-447.

Minnema, D.J., Michelson, I.A., & Cooper, G. P. (1988). Calcium efflux and neurotransmitter release from rat hippocampal synaptosomes exposed to lead. *Toxicol. Appl. Pharmacol.*, 92, 351.

Morgans, C.W. (2001). Localization of the alpha(1F) calcium channel subunit in the rat retina. *Invest Ophthalmol Vis Sci*, 19, 264-267.

Murphy, T.H., Worley, P.F., and, Baraban, J.M. (1991). L-type voltage-sensitive calcium channels mediate synaptic activation of immediate early genes. *Neuron*. 7, 635-635.

Murphy, K.J. and Regan, C.M. (1998). Low-level lead exposure in the early postnatal period results in persisting neuroplastic deficits associated with memory consolidation. *J. Neurochem.*, 72, 2099-2104.

Nevin, R. (2007). Understanding international crime trends: The legacy of preschool lead exposure. *Environmental Research*, 104, 315-336.

Nihei, M.K. and Guilarte, T.R. (1999). NMDAR-2A subunit protein expression is reduced in the hippocampus of rats exposed to Pb²⁺ during development. *Brain Res. Mol. Brain Res.* 66, 42-49.

Nussey, S. and Whitehead, S. (2001). *Endocrinology: An integrated Approach*. London, UK: Oxford Press.

Rashidy-Pour, A. Motaghed-Larijani, Z., & Bures, J. (1995). Reversible inactivations of medial septal area impairs consolidation but not retrieval of passive avoidance learning in rats. *Behav. Brain Res.*, 72, 185-188.

Regan, C.M. and Keegan, K.(1990). Neuroteratological consequences of chronic low level lead exposure. *Dev. Pharmacol. Ther.*, 5, 189-195.

Regunathan, S. and Sundaresan, R. (1985). Effects of organic and inorganic lead on synaptosomal uptake, release, and receptor binding of glutamate in young rats. *J Neurochem.*, 44 (5), 1642-1646.

Riberio, M.J., Schofield, M.G. Kemenes, I., O'Shea, M., Kemenes, G., & Benjamin, P.R. (2005). Activation of MAPK is necessary for long-term memory consolidation following food-reward conditioning. *Learn Mem*, 12 (5), 538-545.

Rice, D. and Barone Jr., S. (2000). Critical periods of vulnerability for the developing nervous system: Evidence from humans and animal models. *Environ Health Perspect.*, 108 (Suppl 3), 511-533.

Rinehart, J., Maksimova, Y.D., Tanis, J.E., Stone, K.L., Hodson, C.A., Zhang, J. et al. (2009). Sites of regulated phosphorylation that control K-Cl cotransporter activity. *Cell* 138: 525-536.

Rivera, C., Voipio, J., Payne, J.A., Ruusuvuori, E., Lahtinen, H., Lamsa, K., Pirvola, U., Saarma, M., & Kaila, K. (1999). The K+/Cl- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. *Nature* 397: 251-256.

Saliba, R.S., Zhenglin, G., Zhen Y., and Moss, S.J. (2009). Blocking L-type voltage gated Ca²⁺ channels with dihydropyridines reduces γ -aminobutyric acid type A receptor expression and synaptic inhibition. *The Journal of Biological Chemistry* 284: 32544-3250.

Satin, J., Schroder, E.A., & Crump, S.M. (2011). L-type calcium channel auto-regulation of transcription. *Cell Calcium*, 49, 306-313.

Schneider, J.S., Lee, M.H., Anderson, D.W., Zuck, L., & Lidsky, T.I. (2001). Enriched environment during development is protective against lead-induced neurotoxicity. *Brain Res.* 896, 48-55.

Schneider, J.S., Anderson, D.W., Sonnenahalli, H., & Vadigepalli, R. (2011). Sex-based differences in gene expression in hippocampus following postnatal lead exposure. *Toxicology and Applied Pharmacology*, 256, 179-190.

Silbergeld, E.K., Adler, H.S., & Costa, J.L. (1977). Subcellular localization of lead in synaptosomes. *Res Commun Chem Pathol Pharmacol*, 17, (4), 715-725.

Silbergeld, S.K. and Goldberg, A.M. (1980). Problems in experimental studies on lead poisoning. In. *Lead toxicity*. R.L. Singhal and J.A. Thomas (Eds.). (pp. 19-42). Baltimore, MD: Urban & Schwarzenberg.

Silbergeld, E.K. (1992). Mechanisms of lead neurotoxicity, or looking beyond the lamppost. *FASEB J*, 6, 3201-3206.

Simmons, T.J.B. and Pocock, G. (1987). Lead enters bovine adrenal medullary cells through calcium channels. *J. Neurochem.* 48, 383-389.

Singh, A.K., and Jiang., Y. (1997). Comparative effects of age and chronic low-level lead exposure on calcium mobilization from intracellular calcium stores in brain samples obtained from the neonatal and the adult rats. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol*, 117(1), 89-98.

Snutch, T.P., Tomlinson, W.J. & Leonard, J.P. (1991). Distinct calcium channels are generated by alternative splicing and are differentially expressed in the mammalian CNS. *Neuron*, 7,45-57.

Soltesz, I., and Deschenes, M. (1993). Low-and high-frequency membrane potential oscillations during theta activity in CA1 and CA3 pyramidal neurons of the rat hippocampus under ketamine-xylazine anesthesia. *J. Neurophysiol.*, 70, 97-116.

Spotts, J.M., Dolmetsch, R.E., & Greenberg, M.E. (2002). Time-lapse imaging of a dynamic phosphorylation-dependent protein-protein interaction in mammalian cells. *Proc. Natl. Acad. Sci. USA*, 99, 15142-15147.

Spence, I., Drew, C., Johnston, G.A., & Lodge, D. (1985). Acute effects of lead at central synapses in vitro. *Brain Res*, 333, 103-109.

Squire, L.R. (1992). Memory and the hippocampus: A synthesis from findings with rats, monkeys and humans. *Psychol. Rev.*, 99, 195-231.

Strużyńska, L. and Rafalowska, U. (1994). The effect of lead on dopamine, GABA and histidine spontaneous and KCl-dependent releases from rat brain synaptosomes. *Acta Neurobiol. Exp.* 54, 201-207.

Strużyńska, L. and Sulkowski, G. (2004). Relationships between glutamine, glutamate, and GABA in nerve endings under Pb-toxicity conditions. *J Inorg Biochem*, 98, (6), 951-958.

Sugiura, Y. and Ko, P. (1997). Novel modulatory effect of L-Type calcium channels at newly formed neuromuscular junctions. *J Neurosci*, 17, 1101-1111.

Sun, Y. and Poo, M.M. (1987). Evoked release of acetylcholine from the growing embryonic neuron. *Proc Natl Acad Sci USA*, 84, 2540-2544.

Sun, L.R., and Suszkiw, J.B. (1995). Extracellular inhibition and intracellular enhancement of Ca^{2+} currents by Pb^{2+} in bovine adrenal chromaffin cells. *J. Neurophysiol.* 74, 574-581.

Sun, X., Tian, X., Tomsig, J.L., & Suzkiw, J.B. (1999). Analysis of differential effects of Pb^{2+} on protein kinase C isozymes. *Toxicolo. Appl. Pharmacol.*, 156, 140-145.

Sun, Q., Tian, E., James-Turner, R., Ten-Hagen, K.G. (2010). Developmental and functional studies of the Slc12 gene family members from drosophila melanogaster. *Am J Physiol Cell Physiol*, 298, C26-C37.

Tang, H.W., Yan, H.L., Hu, X.H., Liang, Y.X., & Shen, X.Y. (1996). Lead cytotoxicity in primary cultured rat astrocytes and schwann cells. *J Appl Toxicol*, 16, 187-196.

Tiffany-Castiglioni, E. Sierra, E.M., Wu, J.N., & Rowles, T.K. (1989). Lead toxicity in neuroglia. *Neurotoxicology*, 10, 417-443.

Tiffany-Castiglioni, E., and Qian, Y. (2001). Astroglia as metal depots: Molecular mechanisms for metal accumulation, storage, and release. *Neurotoxicology*, 22, 577-592.

Tsien, R.W., Lipscombe, D., Madison, D.V., Bley, K.R., and Fox, A.P. (1988). Multiple types of neuronal calcium channels and their selective modulation. *Trends Neurosci.* 11, 431-438.

Tsien, R.W. and Barrett, C.F. (2005). A brief history of calcium channel discovery. In: *Voltage-Gated Calcium Channels*, G. Zamponi (Ed.), Kluwer Academic / Plenum Publishers, New York: N.Y., pp. 27-47.

Toscano, C.D., Hashemzadeh-Gargari, H., McGlothan, J.L., & Guilarte, T.R. (2002). Developmental Pb²⁺-exposure alters NMDAR subtypes and reduces CREB phosphorylation in the rat brain. *Brain Res. Dev. Brain Res.* 139, 217-226.

Toscano, C.D., McGlothan, J.L., & Guilarte, T.R. (2003). Lead exposure alters cyclic-AMP response element binding protein phosphorylation and binding activity in the developing rat brain. *Brain Res. Dev. Brain. Res.* 145, 219-228.

Toscano, C.D. and Guilarte, T.R. (2005). Lead neurotoxicity: From exposure to molecular effects. *Brain Research Reviews*. 49, 529-554.

Turner, T.J., Adams, M.E. & Dunlap, K. (1993). Multiple Ca²⁺ channel types coexist to regulate synaptosomal neurotransmitter release. *Proc. Natl. Acad. Sci. USA.*, 90, 9518-9522.

U.S. EPA1997. The Benefits and Costs of the Clean Air Act, 1970–1990. Washington, DC:U.S. *Environmental Protection Agency*.

Vahter, M., Akesson, A., Lidén, C., Ceccatelli, S., & Berglund, M. (2007). Gender differences in the disposition and toxicity of metals. *Environ Res*, 104 (1), 85-95.

Vitari, A.C., Thastrup, J. Rafiqi, F.H., Deak, M., Morrice N.A., Karlsson, H.K.R., et al. (2006). Functional interactions of the SPAK/OSR1 kinases with their upstream activator WNK1 and downstream substrate NKCC1. *Biochem. J.* 397: 223-231.

Vu, T.Q., Payne, J.A., & Copenhagen, D.R. (2000). Localization and developmental expression patterns of the neuronal K-Cl co-transporter (KCC2) in the rat retina. *J. Neurosci.* 20: 1414-1423.

Wake, H., Watanabe, M., Moorhouse, A.J., Kanematsu, T., Horibe, S., Matsukawa, N., et al. (2007). Early changes in KCC2 phosphorylation in response to neuronal stress result in functional downregulation. *J. Neurosci.* 27: 1642-1650.

Wasserman, G.A., Liu, X., Lolacono, N.J., Factor-Litvak, P., Kline, J.K., Popvac, D. et al. (1997). Lead exposure and intelligence in 7-year old children: The Yugoslavia prospective study. *Environ Health Perspect*, 105, 956-962.

West, A.E., Chen, W.G., Dalva, M.B., Dolmetsch, R.E., Kornhauser, J.M., Shaywitz, A.J., Takasu, M.A., Tao, X., & Greenberg, M.E. (2001). Calcium regulation of neuronal gene expression. *Proc. Natl. Acad. Sci. USA*, 98, 11204-11031.

Westenbroek, R.E., Ahlijanian, M.K. & Catterall, W.A (1990). Clustering of L-Type Ca^{2+} channels at the base of major dendrites in hippocampal pyramidal neurons. *Nature*, 347, 281-284.

Williams, M.E., Feldman, D.H., McCue, A.F., et al. (1992). Structure and functional expression of alpha 1, alpha 2, and beta subunits of a novel human neuronal calcium channel subtype. *Neuron*, 8, 71-84.

Wilson, M.A., Johnston, M.V., Goldstein G.W., & Blue, M.E. (2000). Neonatal lead exposure impairs development of rodent barrel field cortex. *PNAS*, 10 (97), 5540-5545.

Wimalarathana, R.N., Tsai, C.H., & Shen, C.H. (2011). Transcriptional regulation of genes involved in yeast phospholipid biosynthesis. *J. Microbiol.* 49: 265-273.

Xiao, C., Gu, Y., Zhou, C.Y., Wang, L., Zhang, M.M., & Ruan, D.Y. (2006). Pb^{2+} impairs GABAergic synaptic transmission in rat hippocampal slices: A possible involvement of presynaptic calcium channels. *Brain Research*, 1088, 93-100.

Yusaf, S.P., Goodman, J., Pinnock, R.D., et al. (2001). Expression of voltage-gated calcium channel subunits in rat dorsal root ganglion neurons. *Neurosci Lett*, 311, 137-141.

Zakharenko, S., Chang, S., O'Donoghue, M., et al. (1999). Neurotransmitter secretion along growing nerve processes: comparison with synaptic vesicle exocytosis. *J Cell Biol*, 144, 507-518.

Zaiser, A.E. and Miletic, V. (2000). Differential effects of inorganic lead on hippocampal long-term potentiation in young rats *in vivo*. *Brain Res*, 876 (1-2), 201-204.

Zhang, A., Shen, C.H., Ma, S.Y., Ke, Y., & El Idrissi, A. (2009). Altered expression of Autism-associated genes in the brain of Fragile X mouse model. *Biochem. Biophys. Res. Commun.* 379: 920-923.