An age-dependent seizure vulnerability of hippocampal CA2: finding the targets to rescue seizure-induced social recognition memory deficits

By

JEFF CORREA

A Thesis Submitted to Carleton University Graduate Affairs in Fulfilment of the Requirements for the Degree of

Masters of Science

in

Neuroscience

Carleton University
Ottawa, ON Canada

© 2022
Jeff Correa
Acknowledgements

I would like to first thank all the incredible individuals that I have had the pleasure working with and learning from during this and other related projects. This project would not have been possible without the support and extensive work done by Aycheh Al-chami, Ting Ting Wang and Chris Correa. Aycheh has helped immensely to collect majority of the electrophysiology data with the support of Ting Ting. Chris Correa, our lab volunteer, has helped analyze all the behavior data. I owe a deep sense of gratitude to these individuals who never give up during hard times and are extremely persistent to completing the work they have set out to do. I would also like to thank Alysia Ross for her help with the generation of our unique transgenic mice. Additionally, I would like to give tremendous thanks to Dr. Shawn Hayley and Teresa Fortin for generously sharing their equipment and resources, without their help this project would not be successful or even possible.

I cannot express enough thanks and gratitude to my mentor, Dr. Hongyu Sun. His continued encouragement, guidance, dedication and overwhelming attitude to support his students is responsible for the completion of this work. For every step throughout my Masters degree, Dr. Sun has provided his knowledge and expertise while motivating me to pursue my future career, which I am extremely grateful for.

I would also like to thank Dr. Matthew Holahan, Dr. Shawn Hayley, Dr. William Willmore, and Dr. Hongyu Sun, for serving as my committee members and offering their time, advice, and support.

Lastly, I would like to thank my parents for their constant support, guidance and inspiration. Their endless encouragement to help me pursue my goals has driven me thus far.
Abstract

Neurons in the immature brain are hyperexcitable due to their elevated ratio of excitation to inhibition, which can lead to excessive excitability and vulnerability to seizures. Early life seizure (ELS) poses a significant threat to developing neurons and often results in later-life epilepsy and cognitive deficits. Sitting between CA3 and CA1, hippocampal CA2 has recently emerged as a critical region in processing hippocampal-dependent memory, including social recognition memory. Little is known about the effects of ELS on CA2 pyramidal neurons in the developing hippocampus. Here, we hypothesize that ELS can regulate the excitability of immature CA2 pyramidal neurons during the critical period of development, influencing CA2 function and therefore social recognition memory. ELS was induced by pentylentetrazol (55mg/kg, i.p.) in P10 mouse pups. Our results indicate an increase GFP+ neurons in the CA2 post seizure in p10 mice. We found that ELS significantly increased the frequency of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) mediated sEPSCs in CA2 pyramidal neurons in hippocampal slices one hour post PTZ-induced seizure in p10 mice, mediated by a presynaptic change through the Perforant pathway evidenced by increased paired pulse ratio of evoked AMPAR eEPSCs. Furthermore, using the pre-weaning social recognition memory test, we have identified impaired functionality of social recognition memory at p12 post PTZ-induced seizure at p10. These data strongly support ELS-induced dysregulation of hippocampal CA2 pyramidal neurons in the developing hippocampus and result in social recognition memory deficits. Owing to the evidence of enhanced AMPAR activity, NBQX, an AMPAR antagonist, was administered at p10 immediately after seizure which rescued social recognition memory of the dam in p12 pups. Similarly, as per evidence of selective neuronal activation at p10 post PTZ, our novel triple-transgenic c-Fos-GFP/c-Fos-tTA/TRE-hM4Di mouse model was used to suppress selectively activated neurons through the administration of CNO from p10-12, which also rescued the social recognition memory deficits. Our results identify the potential effects of ELS on the function of CA2 neurons and help determine a novel target for intervention for reversing the long-term effects of ELS.
Table of Contents

ACKNOWLEDGMENTS .......................................................................................................................... 1
ABSTRACT ............................................................................................................................................. 2
ABBREVIATIONS ................................................................................................................................. 7
LIST OF TABLES AND FIGURES ........................................................................................................... 9

1. INTRODUCTION ............................................................................................................................... 10

1.1 Unprovoked seizure event ............................................................................................................. 10
1.2 Seizure induced molecular changes ............................................................................................. 10
1.3 Epilepsy .......................................................................................................................................... 12
  1.3.1 Outcomes of Temporal Lobe Epilepsy ..................................................................................... 13
1.4 Seizure effect on CA2 hippocampus ............................................................................................... 14
  1.4.1 CA2 region of the hippocampus ............................................................................................... 14
  1.4.2 CA2 and social recognition memory .......................................................................................... 15
    1.4.2a Oxytocin and vasopressin influence on CA2 neuronal firing ................................................. 15
    1.4.2b Parvalbumin-expressing interneurons in CA2 aid social memory ........................................ 16
  1.4.3 CA2 resistance to seizures ......................................................................................................... 17
    1.4.3a NKCC1 defense mechanism ................................................................................................. 18
    1.4.3b GABAergic defense mechanism ........................................................................................... 19
    1.4.3c Protein based defense mechanism ......................................................................................... 20
    1.4.3d PNN defense mechanism .................................................................................................... 21
    1.4.3e Calcium-binding protein defense mechanism ...................................................................... 22
  1.4.4 CA2 vulnerability to TLE ........................................................................................................... 23
1.5 Early life seizure .............................................................................................................................. 24
1.6 Theories of altered neuronal network after ELS ............................................................................ 25
  1.6.1 Heterogeneous population of seizure sensitive cells ............................................................... 26
1.7 ELS influence autistic-like social behavior deficits ......................................................................... 27
  1.7.1 ELS dysregulation of mTORC1 pathway ................................................................................... 29
1.8 Seizure susceptibility in immature CA2 pyramidal neurons ............................................................ 30
1.9 Animal models of early life seizures .............................................................................................. 32

2. RATIONALE AND OBJECTIVES ..................................................................................................... 33

3. METHODS .......................................................................................................................................... 37
3.1 Experimental Animals ........................................................................................................37
3.2 Early life seizure and adult seizure induction ........................................................................38
3.3 NBQX and CNO treatment ..................................................................................................39
3.4 Immunohistochemistry ........................................................................................................39
   3.4.1 Perfusion .........................................................................................................................39
   3.4.2 Immunohistochemistry staining .....................................................................................40
3.5 Behavioral testing ..................................................................................................................41
3.6 Preweaning two-choice social recognition memory test .........................................................41
3.7 Three Chamber social recognition memory test ....................................................................42
3.8 Caregiving mother/novel mother exposure for immediate early gene analysis ......................42
3.9 Hippocampal slice preparation ..............................................................................................43
3.10 Whole-Cell Patch Clamp Recording ...................................................................................44
3.11 Statistical data analysis ........................................................................................................45
   3.11.1 Analysis of GFP, RGS14 and c-Fos staining .................................................................46
   3.11.2 Analysis of behavior testing ..........................................................................................46
4. RESULTS ......................................................................................................................................47
   4.1 Acute seizures in adult mice did not affect the function of mature hippocampal CA2 ..........47
      4.1.1 Acute seizures in adult mice induced a small but significant increase in the activation of CA2 neurons ........................................................................................................47
      4.1.2 Acute seizures in adult mice did not influence social recognition memory ..................48
   4.2 RGS14 contributes to the seizure resistance in CA2 neurons ...............................................51
      4.2.1 Adult PTZ seizures did not alter RGS14 expression in CA2 ...........................................51
      4.2.2 Expression of RGS14 in CA2 neurons is developmentally regulated ............................59
   4.3 PTZ-induced early-life seizures selectively enhanced the excitability of seizure-sensitive CA2 neurons and impaired social recognition memory in immature mice ..................................59
      4.3.1 Early life seizures significantly activated CA2 pyramidal neurons ...............................59
      4.3.2 Level of RGS14 is negatively correlated to early seizure-induced neuronal activation in the immature CA2 ........................................................................................................60
      4.3.3 Early seizures impaired social behaviors in immature mice ........................................61
      4.3.4 Early life seizures cause functional changes in immature CA2 pyramidal cells ............69
4.3.4.1 Early life seizure induced an enhancement of AMPA receptor function in CA2 pyramidal neurons at P10.

4.3.4.2 Early-life seizures induced AMPAR function enhancement was through a presynaptic mechanism.

4.3.4.3 Persistent early seizure-induced AMPAR function enhancement in CA2 neurons was observed in P12 mice.

4.3.5 NBQX treatment reverses early seizure-induced social recognition memory deficits.

4.3.5.1 NBQX treatment rescued early seizure-induced social recognition memory deficits.

4.3.5.2 NBQX treatment reversed early seizure-induced enhancement of AMPAR function in CA2 pyramidal neurons.

4.3.5.3 NBQX treatment rescued early seizure-induced diminishment of CA2 function during social behaviors.

4.3.6 Early life seizure induced target specific enhancement of AMPAR function in CA2 seizure sensitive neurons.

4.3.7 Target-specific chemogenetic inhibition of seizure-sensitive neurons reverses early seizure-induced social recognition memory deficits in immature mice.

4.3.7.1 Chemogenetic suppression of the selectively activated seizure-sensitive neurons rescued early seizure-induced social deficits.

4.3.7.2 Chemogenetic suppression of the selectively activated seizure-sensitive neurons reversed early seizure-induced persistent enhancement of AMPAR function in CA2 pyramidal neurons.

4.3.7.3 Chemogenetic suppression of the selectively activated seizure-sensitive neurons rescued early seizure-induced diminishment of CA2 function during social behaviors.

4.4 Chemogenetic inhibition of seizure-sensitive neurons reverses early KA seizure-induced social deficits in immature mice.

4.4.1 KA-induced early life seizure selectively activated a subgroup of CA2 neurons.

4.4.2 KA seizure did not affect RGS14 expression in the immature CA2 structure.

4.4.3 KA-induced early life seizures impaired social behavior in immature mice.
4.4.4 KA-induced early life seizures selectively increased AMPAR function in CA2 neurons.................................................................96

4.4.5 Chemogenetic suppression of seizure-sensitive neurons reverses early KA seizure-induced social deficits in immature mice.........................................................96

5. DISCUSSION.................................................................................................................................100

5.1 Seizures activate a heterogeneous population of mature CA2 PN’s........................................100

5.2 Seizures do not affect mature CA2 function.................................................................................101

5.3 The role of RGS14 and its relation towards seizure resistance.................................................103

5.4 Early life seizures effect CA2 PNs and cause social recognition deficits.................................104

5.5 Functional changes in immature CA2PN AMPARs.................................................................105

5.6 NBQX can rescue ELS induced social behavior deficits.......................................................107

5.7 Selectively suppressing seizure sensitive neurons to rescue social behavior deficits.........108

5.7.1 Seizure sensitive cells in immature CA2.................................................................................108

5.7.2 Suppressing seizure sensitive neurons to rescue social memory deficits.........................109

5.8 Future directions.......................................................................................................................110

6. CONCLUSION.................................................................................................................................111

7. REFERENCE..................................................................................................................................112
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACSF</td>
<td>Artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AED</td>
<td>Antiepileptic drug</td>
</tr>
<tr>
<td>AP</td>
<td>Action potential</td>
</tr>
<tr>
<td>AMPAR</td>
<td>$\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ASD</td>
<td>Autism spectrum disorder</td>
</tr>
<tr>
<td>AVPR1B</td>
<td>Arginine vasopressin 1b receptors</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotropic factor</td>
</tr>
<tr>
<td>CaBP</td>
<td>Calcium-binding proteins</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>DOR</td>
<td>Delta opioid receptor</td>
</tr>
<tr>
<td>EC</td>
<td>Entorhinal cortex</td>
</tr>
<tr>
<td>EEG</td>
<td>Electroencephalogram</td>
</tr>
<tr>
<td>ELP</td>
<td>Early life epilepsy</td>
</tr>
<tr>
<td>ELS</td>
<td>Early Life Seizure</td>
</tr>
<tr>
<td>EPSP</td>
<td>Excitatory Postsynaptic Potential</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GluR2</td>
<td>Glutamate Receptor 2</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>HFS</td>
<td>High-frequency synaptic stimulation</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IPSP</td>
<td>Inhibitory Postsynaptic Potential</td>
</tr>
<tr>
<td>KA</td>
<td>Kainic Acid</td>
</tr>
<tr>
<td>LTD</td>
<td>Long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mammalian target of the rapamycin</td>
</tr>
<tr>
<td>NKCC1</td>
<td>Na(+)-K(+)-Cl(-) cotransporter mechanism 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>NRG1</td>
<td>Neuregulin 1</td>
</tr>
<tr>
<td>OXTR</td>
<td>Oxytocin receptor</td>
</tr>
<tr>
<td>P (ex:P10)</td>
<td>Post-natal day</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBT</td>
<td>Phosphate buffered saline solution with 0.3% triton</td>
</tr>
<tr>
<td>PN</td>
<td>Pyramidal Neuron</td>
</tr>
<tr>
<td>PNN</td>
<td>Perineuronal nets</td>
</tr>
<tr>
<td>PTZ</td>
<td>Pentylenetetrazol</td>
</tr>
<tr>
<td>PV</td>
<td>Parvalbumin-expressing</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus</td>
</tr>
<tr>
<td>p70S6K</td>
<td>Ribosomal S6 protein kinase</td>
</tr>
<tr>
<td>RGS14</td>
<td>Regulator G-protein signalling 14</td>
</tr>
<tr>
<td>sEPSC</td>
<td>Spontaneous excitatory postsynaptic current</td>
</tr>
<tr>
<td>TLE</td>
<td>Temporal lobe epilepsy</td>
</tr>
<tr>
<td>TSC1</td>
<td>Tuberous Sclerosis Complex 1</td>
</tr>
<tr>
<td>TSC2</td>
<td>Tuberous Sclerosis Complex 2</td>
</tr>
<tr>
<td>VGCC</td>
<td>Voltage-gated calcium channels</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
</tr>
</tbody>
</table>
List of Tables and Figures

Table 1. Racine Scale ...........................................................................................................38
Figure 1. RGS14 influence on cellular signaling.................................................................21  
Figure 2. Acute seizures in adult mice did not affect the function of mature hippocampal CA2........54  
Figure 3. Acute seizures in adult mice induced a small but significant increase in the activation of middle and ventral CA2 neurons..........................................................................................56  
Figure 4. PTZ-induced seizure did not diminish dorsal, middle and ventral CA2 function during social behavior..........................................................................................................................57  
Figure 5. PTZ-induced early-life seizures selectively enhanced the excitability of seizure-sensitive CA2 neurons and impaired social recognition memory in immature mice..................................64  
Figure 6. Early life seizures significantly activated dorsal, middle and ventral CA2 pyramidal neurons..................................................................................................................................................66  
Figure 7. PTZ-induced early seizure diminished dorsal, middle and ventral CA2 function during social behavior...........................................................................................................................................67  
Figure 8. Early life seizure induced an enhancement of AMPA receptor function in CA2 pyramidal neurons at P10 and Persistent enhancement at P12..................................................................................73  
Figure 9. NBQX treatment rescued early seizure-induced social recognition memory deficits and reversed enhancement of AMPAR function in CA2 pyramidal neurons..............................................................................78  
Figure 10. NBQX treatment rescued early seizure-induced diminishment of dorsal, middle and ventral CA2 function during social behavior.........................................................................................................80  
Figure 11. Early life seizure induced target specific enhancement of AMPAR function in CA2 seizure sensitive neurons........................................................................................................................................84  
Figure 12. Chemogenetic suppression of the selectively activated seizure-sensitive neurons rescued early seizure-induced social deficits and reversed persistent enhancement of AMPAR function in CA2 pyramidal neurons.................................................................................................................................90  
Figure 13. Chemogenetic suppression of the selectively activated seizure-sensitive neurons rescued early seizure-induced diminishment of dorsal, middle and ventral CA2 function during social behaviors......92  
Figure 14. Chemogenetic inhibition of seizure-sensitive neurons reverses early KA seizure-induced social deficits in immature mice...........................................................................................................98
1. Introduction

1.1 Unprovoked seizure event

The first grand mal seizure has an 8-10% lifetime risk in an individual, with a 3% chance of epilepsy onset (Pohlman-Eden et al., 2006; Hauser and Beghi, 2008). The onset of a provoked seizure event can be based on multiple factors that interrupt normal connectivity between neurons, this includes high fever, low blood sugar or brain injury (Pohlman-Eden et al., 2006; Hauser and Beghi, 2008). This form of seizure tends to have a lower appearance of later life recurrence (3-10%) and cognitive decline, therefore do not require long-term treatment with anti-epileptic drug (AEDs) treatment (Chong, 2010; Pohlman-Eden et al., 2006; Hauser and Beghi, 2008). However, a single unprovoked seizure event estimates a recurrence risk of 34-71%. A retrospective observational study by Maia C. and colleagues identified patients that had a first seizure event and were under antiepileptic drug treatment to have a recurrence rate of 38%. Patients with two or more seizures have a recurrence of 70-80% (Chong, 2010; Pohlman-Eden et al., 2006; Hauser and Beghi, 2008). In animal models and some clinical evidence, it is accepted that recurrent or prolonged seizures can cause long-lasting brain injury, cognitive dysfunction, and acquired epileptogenesis (Ben-Ari and Cossart, 2000; Chong, 2010; Pohlman-Eden et al., 2006; Hauser and Beghi, 2008). However, whether a brief seizure event can cause similar effects is controversial.

1.2 Seizure induced molecular changes

Definition of epilepsy is described as, a disorder of recurrent unprovoked seizures. Seizures are identified as, abnormal, excessive hypersynchronous discharge of a population of neurons (Bonansco and Fuenzalida, 2016; Hanada, 2020). The basic mechanism of a hyper-excitabile state can result from the delicate imbalance of excitation and inhibition in the brain. This imbalance has been associated with
increased synaptic neurotransmission, decreased inhibitory inputs, increased synchronous excitatory stimuli (temporal summation in post-synaptic neurons) or an alteration of ion channels/ion concentrations effect (Gori et al., 2013; Vasilev et. al., 2018). Seizure animal models, such as a single injection of pentylentetrazol (PTZ), an antagonist of gamma-aminobutyric acid (GABA) type A receptor can evoke structural changes in brain tissue and rapid formation of ‘dark neurons’ in the hippocampal pyramidal layer and will last up to 1-week post seizure effect (Gori et al., 2013; Vasilev et. al., 2018). Dark neurons are described as injured neurons that undergo morphological changes such as shrunken soma or dendrites. The increase in dark cells has been documented to affect the normal functioning of cells, influencing short-term synaptic facilitation (Postnikova et al., 2017; Vasilev et. al., 2018) and long-term synaptic potentiation in CA1 hippocampal pyramidal neurons for up to 1-week after seizures.

When investigating at the neuronal level, the major excitatory neurotransmitter that initiates ionotropic receptors such as α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) is the amino acid glutamate. Studies have shown that the NMDA and AMPA receptor agonists can initiate seizure activity and that antagonists towards these receptors can suppress seizure activity (Gori et al., 2013; Vasilev et al., 2018), presenting a major clinical research target.

While there are some presynaptic NMDA receptors, the majority of NMDA receptors exist in the postsynapse (Hanada, 2020). They comprise of two GluN1 subunits with GluN2 subunits or a combination of GluN2 and GluN3 subunits (Zaitsev et al., 2014; Hanada, 2020). NMDA receptors are blocked by Mg2+ at resting membrane potential and are only moved once the cell is depolarized, causing Calcium ions to enter the cell. The kinetics of NMDA is known to be slow based on the machinery controlling the activity of the receptor, however, some studies show that mutations in these receptor subunits may cause epileptic onset in humans (Zaitsev et al., 2014; Hanada, 2020). In recent studies, researchers have found that NMDA receptors seem to be less important in the initiation of synchronized
activity than AMPA receptors. The irregular activity of AMPA receptors appears to have a more prominent role in seizure disorders than NMDA (Zaitsev et al., 2014; Hanada, 2020). AMPA receptors can be influenced by various conditions that can initiate seizure onsets such as, genetically altered receptor trafficking systems or mutated synaptic plasticity events. The turnover rate of AMPA receptors on the synapse is extremely quick, where the number of new AMPA receptors formation in the post-synapse can synthesize and contribute to the change in synaptic potential and plastic changes (Zaitsev et al., 2014; Hanada, 2020). This increased expression of AMPA receptors in the post-synapse is generally seen in epilepsy models but this trend is not observed in NMDA receptors (Zaitsev et al., 2014; Hanada, 2020). Animal models of kainate-induced seizures have indicated an increased AMPA receptor expression in the hippocampal region. The increased expression of AMPA receptors will contribute to the over-activation of AMPA receptors and could initiate the development of temporal lobe epilepsy.

1.3 Epilepsy

Dr. Berger first described epilepsy in the 1920s, a condition that has remained very prevalent and complex while affecting populations of all ages. Epilepsy affects more than 65 million people worldwide and most commonly starts before the age of 1 year (Tracey et al., 2021). The definition and treatments of epilepsy have changed dramatically over time, owing to the complexity and heterogeneity of the disease, and thus, many of the underlying mechanisms remain unknown. According to the International League Against Epilepsy (ILAE), epilepsy seizures are characterized as “transient symptoms caused by abnormal excessive or synchronous brain activity” (Falco-Walter et al., 2018). In a clinical setting, electroencephalogram (EEG) monitoring can detect the beginning and end of an episode of seizures (Bromfield et al., 2006; Falco-Walter et al., 2018). A patient is diagnosed with epilepsy if having at least one unprovoked epileptic seizure (unrelated to drugs, alcohol, hyponatremia, or glucose
abnormality) separated by more than 24 hours. Strikingly, a single seizure will increase the risk of another seizure by more than 60% (Fisher et al., 2018).

According to the current understanding of epilepsy, it is defined as a disease beyond seizures, based on the comorbidities and cognitive deficits associated with it (Fisher et al., 2018; Salpekar and Mula, 2018). In recent years, several studies have identified the long-term adverse effects of recurrent or single seizures on neuronal networks (Katsarou et al., 2017; Lee et al., 2000; Minjarez et al., 2017; Oh et al., 2017). These effects predispose the generation of epileptic seizures, and neuropsychosocial comorbidities (Salpekar and Mula, 2018; Tracey et al., 2021).

Recent evidence shows that approximately 70% of patients may become seizure-free with appropriate treatments, however, the remaining 30% of patients show drug-resistant epilepsy. This is due to the failure of adequate trials and antiepileptic drugs (AED) to achieve sustained seizure absence (Tracey et al., 2021). Overall, while epilepsy management is getting better owing to improved diagnostic tools, further research is urgently needed to determine the pathophysiology and mechanisms of the disease so more effective treatments can be developed.

1.3.1 Outcomes of Temporal Lobe Epilepsy

Temporal lobe epilepsy (TLE) is one of the most common and severe types of refractory focal acquired epilepsy (Ben-Ari, 1985; Lopes et al., 2016). This form of epilepsy has been identified to severely affect the hippocampus and its process in learning and memory consolidation. The molecular basis of learning and memory is dependent on synaptic plasticity (Lopes et al., 2016). These plastic changes are depended on network activation to produce strengthening of the synapse (high-frequency stimulation) through long-term potentiation (LTP) or decreased synaptic efficacy, long-term depression (LTD). Studies have indicated that the hippocampus after being influenced by TLE show diminished
LTP in the CA1 which can attribute to learning and memory dysfunction (Lopes et al., 2016; Schubert et al., 2005).

Animal models of epilepsy have helped understand the relationship between epilepsy and behavioural abnormalities (Lopes et al., 2016; Schubert et al., 2005). A model that mimics the progression of epileptogenesis is the pilocarpine model of TLE. The TLE development is split into two phases, the maturation of phase and the chronic condition phase. During the maturation phase, a variety of brain alterations occur, including hyperexcitability of neurons, alterations of ions and their function, neuronal cell death, mossy fibre sprouting and increased inflammatory processes (Lopes et al., 2016; Schubert et al., 2005). During the chronic phase, recurrent seizures take place. These two events will contribute to altering the functionality of circuits and induce neurobehavioral impairments (Lopes et al., 2016; Schubert et al., 2005). A study by Lopes et al. 2016, demonstrated that rats with i.p. administration of pilocarpine-induced TLE showed impairment in visual-spatial learning, anxiety-like behaviour, and social recognition memory processes in both maturation and chronic phases of the model. Interestingly, there is very limited understanding of whether an acute seizure event may contribute to neurobehavioral deficits, even though studies have demonstrated that a single seizure event can cause brief structural, molecular and functional alterations in brain circuits (Lopes et al., 2016; Schubert et al., 2005).

1.4 Seizure effect on CA2 hippocampus

1.4.1 CA2 region of the hippocampus

Sitting between CA3 and CA1, hippocampal CA2 has recently emerged as a critical region in processing hippocampal-dependent memory, including social recognition memory (Dudek et al., 2016; Tzakis and Holahan, 2019). Recent evidence shows that the CA2 acts as a hub where it receives inputs from the CA3 through the Schaffer collateral pathway onto the proximal CA2 neuronal axis (stratum
radiatum area) and the L2/L3 entorhinal cortex through the perforant pathway onto the distal CA2 neuronal axis (Dudek et al., 2016; Tzakis and Holahan, 2019). Newborn dentate gyrus (DG) neurons can also make connections to CA2 pyramidal neurons. The major target of projection of the CA2PNs is the basal dendrites of the CA1 in stratum oriens. CA2 also projects back to the CA3 creating a network for receiving and delivering inputs, making it an important region for hippocampal circuitry and functioning (Carstens and Dudek, 2019; Dudek et al., 2016; Kohara et al., 2014). Several unique characteristics differentiate the CA2 from other hippocampal regions, one aspect is its ability to form social recognition memory, the ability to recognize a novel from a familiar conspecific. Other aspects involve resistance toward neuronal cell death, hyperactivation and resistance to long-term potentiation (LTP) during the mature adult period (Laham et al., 2021).

1.4.2 CA2 and social recognition memory

Tools to manipulate genetically specific populations of neurons have made addressing the behavioural function of the CA2 possible. Using the CA2-specific Cre-expressing mouse strain, where CA2 synaptic output is silenced to inhibit neurotransmission, Cre-dependent constructs resulted in selective impairment of social recognition memory but did not impair other hippocampus-dependent memory spatial tasks, like novel object recognition (Hitti, and Siegelbaum, 2014). These mice were unable to differentiate between the novel and familiar mice, which demonstrates that synaptic transmission from CA2 pyramidal neurons is essential for encoding social information into memories.

1.4.2a Oxytocin and vasopressin influence on CA2 neuronal firing

Studies have also demonstrated how social stimuli such as exposure to either novel or familiar animals would influence CA2 neuronal firing. One study demonstrates that place fields shift significantly in response to social stimulation, suggesting that neuronal firing in area CA2 is sensitive to novel local
cues and social stimuli. Furthermore, CA2 pyramidal neurons express high levels of social neuropeptides such as vasopressin and oxytocin. The oxytocin neuromodulator is produced in the paraventricular nucleus (PVN) hypothalamus and binds to oxytocin receptors (Oxtr) throughout the brain (Cilz et al., 2018; Marsh et al., 2020). These receptors have been identified to be located within the hippocampal region, strictly localizing in most pyramidal neurons of the CA2 and CA3. Oxtr in the hippocampus has been recognized to form social recognition processes (Cilz et al., 2018; Marsh et al., 2020; Raam et al., 2017). Arginine vasopressin 1b receptors (Avpr1b) receptors are also densely expressed in the CA2 region of the hippocampus and have been found to initiate excitatory postsynaptic potentials (ESPSs) within the CA2 (Chafai et al., 2012). These receptors can contribute toward LTP and synaptic plasticity which is seen in the perforant L2/L3 entorhinal cortex projection to CA2 pyramidal neurons. Mice lacking AVPr1b and mice lacking Oxtr have deficits in social recognition memory and social aggression behaviours. AVPr1b is largely expressed in CA2 pyramidal neurons, when AVPr1b was re-expressed in the dorsal CA2 AVPr1b knockout mice, normal aggressive behaviours were recovered. Interestingly, spatial memory, as shown using the morris water maze task was not affected in the knockout mice. The findings from this study demonstrate that area CA2 has a critical role in processing social recognition memory (Cilz et al., 2018; Marsh et al., 2020; Raam et al., 2017).

1.4.2b Parvalbumin-expressing interneurons in CA2 aid social memory

The CA2 region has a dense population of parvalbumin-expressing (PV+) interneurons when compared to other hippocampal sub-regions, such as the CA1 and CA3 (Botcher et al., 2014). These interneurons play a vital role in the function of the CA2, causing long-term depression (iLTD) in the pyramidal neurons of the CA2. Studies have shown that a loss of the PV+ interneurons in area CA2 will decrease synaptic transmission and plasticity in CA2 PNs and form social recognition memory deficits.
(Piskorowski et al., 2016). Providing evidence that plasticity at inhibitory synapses can modulate the excitability of CA2 PNs (Botcher et al., 2014; Piskorowski et al., 2016).

Along with PV+ interneurons, perineuronal nets (PNN) encase PV+ interneurons and CA2 PNs while regulating their signalling (Carstens et al., 2016). Within PNNs, there are extracellular and transmembrane molecules that regulate neurotransmitter release, such as neuregulin 1 (NRG1) and its receptor ErbB4. These units play a role in regulating inhibitory transmission, migration and synaptic plasticity in the CA2 region. The activation of ErbB4 by NRG1 on PV+ interneurons can regulate GABA release in the CA2 (Vulhorst et al., 2009). Furthermore, ErbB4 is only expressed on gabaergic interneurons and not on pyramidal neurons (Vulhorst et al., 2009). This demonstrates that PNNs along with PV+ interneurons create a harmonized link to modulate iLTD induction in the CA2 region which plays a role in social recognition memory processes.

1.4.3 CA2 resistance to seizures

Within the mature hippocampus, seizures are known to induce neuronal cell death, however, sparing CA2 pyramidal neurons. These effects are determined by a process towards neuronal excitotoxicity, where membrane depolarization ensures the activation of voltage-gated calcium channels (VGCCs) through excessive glutamate release followed by an influx of calcium. Intracellular calcium overload can trigger the apoptotic signalling pathway leading to cell death (Tymianski, 2011). Adult patients suffering from TLE with hippocampal sclerosis and prolonged seizures demonstrate significant neuronal loss in the CA1 and CA3 while showing little to no neuronal death in the CA2 (Gary Mathern et al., 1995; Wittner et al., 2009). Hippocampal neurons that tend to be extremely vulnerable to epileptic neuronal death show a pattern of heterogeneity where epileptic processes including onset, propagation and generalized seizures involve a specific set of neurons (Wittner et al., 2009; Yang et al., 2020). However, CA2 pyramidal neurons (PNs) have been reported to be resistant to damage in adult humans.
and animal models of epilepsy. A study by Pourzaki et al. introduced convulsive seizures induced by GABA inhibitor pentylenetetrazole (PTZ) in adult male rats to study pyramidal neurons releasing excessive glutamate “dark neurons” in the CA regions. The CA2 showed the least damage where apoptotic cells population were drastically less when compared to the CA1 and CA3 after PTZ-induced seizures.

1.4.3a NKCC1 defense mechanism

There are several mechanisms associated with CA2 PN resistance against cell death. During epileptogenesis, CA2 PNs show homeostatic regulation through the NKCC1 (Na(+)-K(+)-Cl(-) cotransporter) mechanism of regulating receptor and transporter-mediated GABA function (Kang et al., 2002). In a healthy brain, NKCC1 transports three ions (sodium, potassium and chloride) across the neuronal plasma membrane while regulating internal chloride levels. When the amount of NKCC1 transporters decreases, internal chloride levels decrease simultaneously. The interneurons that transmit inhibitory neurotransmitters onto pyramidal neurons to evoke inhibitory postsynaptic potentials (IPSPs) rely upon the conductive movement of chloride, particularly low internal chloride levels (Kang et al., 2002). Only when internal chloride levels are low can inhibitory neurotransmitter GABA hyperpolarize the membrane (Kang et. al., 2002). A study by Kang et. al. introduces the relationship between seizure susceptible adult gerbils after the onset of seizures and NKCC1 immunoreactivity in the CA regions. NKCC1 is shown to be upregulated in the CA1 immediately after seizure as a mean of restoring intracellular homeostatic chloride levels (Kang et al., 2002). 30 min after seizure onset, CA2 PNs NKCC1 immunoreactivity was significantly decreased and returned to the pre-seizure levels at 24 hr. The altered expression of NKCC1 can be associated with the attenuation of seizure activity. Furthermore, studies have demonstrated that during the 30 min period after the onset of seizures, the expression of GABA increases and GABA transaminase (GABA-T), an enzyme involved in GABA degradation, decreases,
serving as the mechanism of regulating neuronal excitability in the CA2 region in place of the NKCC1 system (Kang et al., 2001a; Kang et al., 2001b; Kang et al. 2002).

1.4.3b GABAergic defense mechanism

GABAergic transmission onto CA2 PNs are another form of defense mechanism against excitotoxicity. Inhibition has been known to regulate plasticity and strength of excitatory synapses in the hippocampus, however, the CA2 exhibits extensive control over inhibition through a high density of interneurons compared to other CA regions. Axons from the CA3 pyramidal neurons project to the CA2 through the Schaffer collateral pathway, where depolarizing EPSPs are downregulated by large hyperpolarizing currents from feedforward inhibition (Botcher et al., 2014; Chevaleyre and Piskorowski, 2014; Nasrallah et al., 2019; Nasrallah et al., 2015). This feedforward inhibition originates from parvalbumin-expressing (PV) interneurons in area CA2, which prevents CA3 excitatory inputs from evoking action potentials (APs) in the CA2 (Chevaleyre and Piskorowski, 2014). A study by Nasrallah et. al. investigates the disinhibitory drive by evoking long-term depression on inhibitory synapses (iLTD) mediated by delta opioid receptor (DOR) activation in PV interneurons of the CA2 region. The results demonstrate that inhibiting PV interneurons sufficiently increases the excitatory drive from the CA3 PNs to the CA2 PNs through Schaffer collateral inputs driving action potential firing in CA2 PNs (Nasrallah et al., 2019). Although decreasing the inhibitory transmission in the CA2 region influences the excitatory drive between the CA3 and CA2 areas, studies have demonstrated that even after blocking inhibition through GABA_A receptor antagonist, picrotoxin, lack of LTP remains in CA2 PNs (Nasrallah et al., 2015; Zhao et al, 2007). This indicates that the mature CA2 region may have different types of cellular defenses against neuronal alteration through synaptic plasticity.
1.4.3c Protein based defense mechanism

There are several other mechanisms that underlie the CA2 PN resistance against excitotoxic injury after epileptogenesis. For instance, an enriched scaffolding protein in the CA2, regulator G-protein signalling 14 (RGS14), is crucial for regulating receptor-mediated signalling and LTP. RGS14 limits neurotransmitter receptor and G-protein linked signalling by presenting as a GTPase activating protein (GAPs) once the neuron receives excitatory inputs. This interference suppresses calcium influx, neuronal excitability and synaptic plasticity (Harbin et al., 2021; Evans et al., 2018; Lee et al., 2010). A study by Lee et al. investigated RGS14 in the CA2 by using wild type (WT) C57BL/6J and RGS14 knockout (RGS14 KO) adult mice. The electrophysiological recordings of the CA2 PNs demonstrated an increase in EPSC in RGS14 KO mice when compared to WT mice after high-frequency synaptic stimulation (HFS), 100 Hz at 20-s intervals, to induce LTP (Lee et al., 2010). Furthermore, this study administered an inhibitor for ERK/MAP kinase (synaptic plasticity signalling pathway) in RGS14 KO mice, which attenuated LTP, demonstrating that RGS14 acts as a natural suppressor of ERK1/2 signalling pathway, suppressing LTP and neuronal activity (Lee et al., 2010). Based on the characteristics of RGS14, it can act as a factor for resisting seizure excitotoxicity. RGS14 demonstrates regulating properties of seizure pathological responses without influencing the threshold of the seizure (Harbin et al., 2021a; Harbin et al., 2021b). Although RGS14 shows some resistant properties to the effects of seizures, a systematic administration of 330mg/kg pilocarpine (influencing the muscarinic receptor and activation of the cholinergic system) induced chronic seizures in RGS14 KO adult mice, which appeared to be resistant while taking significantly longer to reach tonic seizures and demonstrating the greatest damage in the CA1 region while sparing CA2 (Lee et al., 2010; Vezzani, 2009).
**Figure 1: RGS14 influence on cellular signalling.** Demonstrating the effects of RGS14 on G-protein-coupled receptor and LTP

### 1.4.3d PNN defense mechanism

Additional mechanisms that modulate CA2 PN resistance towards seizures are perineuronal nets (PNNs). PNNs are a specialized form of extracellular matrix found around interneurons and are programmed to inhibit plasticity (Carstens et al., 2016; Carulli et al., 2021; Noguchi et al., 2017). When compared to other CA regions, dense population of PNNs surround excitatory pyramidal neurons in the CA2. The mechanism by which PNNs suppress synaptic potentiation at excitatory synapses in the CA2 may come from PNN peptidoglycans, which are negatively-charged molecules functioning to restrict the diffusion of cations like calcium (Bruckner et al., 1993; Carstens et. al., 2016). PNNs may then limit AMPAR insertion after potentiation and stabilize the postsynaptic density in PNs (Carstens et al., 2016; Frischknecht et al., 2009; Noguchi et al., 2017). A study by Carstens et. al. investigated the effects of disrupted PNNs in the CA2 region. Their results revealed that CA2 PNs exhibit synaptic potentiation.
while recruiting excitatory synapses. Based on PNNs mode of action to limit calcium influx and neuronal excitation, loss of PNNs around CA2 PNs will lead to increased susceptibility to damage, rendering it vulnerable as other CA regions.

1.4.3e Calcium-binding protein defense mechanism

With RGS14 and PNNs serving to protect the mature CA2 from excitotoxicity through calcium buffering, raising external calcium levels to high concentrations (10 mM) permits the induction of LTP in the CA2 (Simons et al., 2009). Simons et. al. carried a series of experiments increasing and prolonging the rise of postsynaptic calcium levels which augment LTP in the CA2. Using a dual indicator 2-photon laser microscopy imaging for calcium-dependent fluorescence transients, they identified the normal calcium concentration in the dendritic spines of the CA1, CA2 and CA3 regions. The CA2 presented with much lower concentrations of calcium when compared to the CA1 and CA3, higher endogenous calcium-buffering capacity and significantly higher rates of calcium extrusion (Simons et al., 2009). Furthermore, based on the relationship of calcium buffering and synaptic dysplasticity in the CA2, studies have reported a large density of calcium-binding proteins (CaBPs) in the CA2 when compared to other CA regions (Leranth and Ribak, 1991; Simons et al., 2009). CaBPs such as calbindin, efhcbp2, and parvalbumin account for the resistance against epileptic consequences by diminishing excitotoxicity from hyperstimulation, which results in neuronal death (Leranth and Ribak, 1991; Simons et al., 2009). A study by Scharfman and Schwartzkroin demonstrated that injecting calcium chelators to hyperstimulated cells, specifically hyperstimulated mossy cells in the hilus of the dentate gyrus, allows those cells to become resistant to cell death.
1.4.4 CA2 vulnerability to TLE

Although neuronal cell death may be limited in the mature CA2 after epileptic-like events, CA2 neurons still receive strong excitatory inputs from L2/L3 entorhinal cortex onto the distal CA2 axis, forming a circuit link between the cortical inputs and CA1 (Chevaleyre, 2010). This pathway undergoes robust LTP, efficiently drives the CA2 firing and can influence hippocampal output by mediating the CA1 pyramidal neuron firing. However, inputs from hippocampal CA3 neurons through the Schaffer collateral pathway onto the proximal (stratum radiatum area) CA2 neurons do not support activity-dependent LTP (Chevaleyre et al., 2010; Zhao et al., 2007). This demonstrates that the CA2 can regulate the effect of pathway-specific stimulation and heterogeneity on the expression of specific events. This unique characteristic of manifesting neuro-modulatory influence is not seen in other CA regions and therefore defines the CA2 as being protected against excitatory inputs in the tri-synaptic hippocampal circuit. Despite the CA2’s defensive properties, abnormal activities were identified by Wittner et al. The study investigated the effects of TLE on CA2 PNs in adult humans using field potential recordings. The results demonstrate CA2 PNs as having interictal-like spike activity in vitro while exhibiting dramatically less cell loss when compared to the CA1 and CA3 regions (Houser et. al., 1990; Wittner et al., 2009). Another study also claims to find mossy fibre projections to the CA2 while undergoing sprouting during epileptogenesis in a kainate adult mouse model of mesial temporal lobe epilepsy (MTLE) (Haussler et al., 2016). CA2 PNs were preserved compared to the CA1 and CA3 regions, although their cell somata were surrounded by synaptoporin-expressing mossy fibre boutons post MTLE. These functional and morphological changes caused by TLE can attribute to normal functioning and therefore disrupt the phenotypic property of the CA2, disrupting social recognition memory processes.
1.5 Early life seizure

Studies have identified the early life period as having maximal synaptogenesis and plasticity, which is modulated by a developmentally heightened ratio of excitation to inhibition in the immature brain (Sanchez and Jensen, 2001). The glutamate receptors, N-methyl-D-aspartate receptor (NDMAR) and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) are transiently overexpressed in the immature brain and are thought to contribute to the high level of synaptic plasticity in the developing neuronal circuits (Silverstein and Jensen, 2007). The subunit composition of these receptors varies between the mature and immature brain. NMDAR expression in the hippocampus peaks in the first postnatal week. Specifically, NMDAR with NR2B, NR2C, NR2D and NR3A subunits are increased and are found to facilitate a higher potential for depolarization and excitability while reducing sensitivity to magnesium blockade (Sanderson et al., 2016; Silverstein and Jensen, 2007). Along with NMDAR, AMPAR expression peaks in the second postnatal week (Hanley et al., 2018; Wang et al., 2010). In adults, AMPAR contain GluR2 subunits which are calcium impermeable, whereas calcium permeable GluR2-lacking AMPARs are immensely expressed in the immature brain. GluR2-lacking AMPARs mediate large postsynaptic potentials than GluR2-containing (Sanchez and Jensen, 2001). Based on the developmentally regulated excitatory ionotropic receptors, the immature brain demonstrates a vulnerable period towards enhanced excitability in the neuronal network, increasing the immature brain’s susceptibility to seizures either spontaneously or triggered by different insults, such as fever, hypoxia or early life trauma (Jensen and Baram, 2000; Rigas et al., 2018). Clinical observations have suggested that seizure disorders during early-life are associated with severe neurological and behavioral impairments in adult life (Rigas et al., 2018; Rosch et al., 2019). Statistically, more than half of children with early-life seizures will suffer from cognitive and neurological impairments such as attention deficit disorders, autistic-like social impairments, epilepsy or premature death (Rigas et al., 2018). Although, the immature brain seems to be less vulnerable to seizure-induced neuronal death, recurrent and even
acute seizures can permanently alter the neuronal network. These changes can be differentiated into three categories: 1) dendritic and neuronal alterations 2) molecular and receptor reorganizations and 3) network connectivity and plasticity changes (Botterill et al., 2017; Lawrence and Inder, 2010; Minjarez et al., 2017; Swann, 2002).

Early life risks can cause the onset of seizures such as, hypoxic-ischemic encephalopathy (HIE), which is likely to occur during complications at birth (Kasahaha et al., 2018; Ronen et al., 1999). Other common causes of early life seizure (ELS) include infections of the central nervous system, maternal drug abuse and rare epilepsy syndromes (Volpe, 2001). Preterm infants have a much higher prevalence of seizures than full-term infants and these seizures are less responsive to conventional AEDs (Spagnoli et al., 2018). Currently, AED treatments that are relatively effective for adults have much lower success rates in treating early life patients with seizures (Silverstein and Jensen, 2007; Van Rooji et al., 2013). This is based on the physiological and functional differences between the immature and mature brain.

Much of what is revealed about the long-term effects of early-life seizures has come from animal models in which the investigator has control over the etiology and treatments of the seizures. It is difficult to conduct studies on human children with seizure pathology due to their differing effects in terms of duration, types, EEG abnormalities and antiepileptic drug therapies (Bromfield et al., 2006). Animal studies have identified that early-life seizures in rodents do not result in cell loss, however, they significantly influence the neural network connectivity, temporal coding and the excitatory and inhibitory balance (Bromfield et al., 2006).

1.6 Theories of altered neuronal network after ELS

Seizures may result from pathological processes that disrupt the balance of excitation and inhibition. Based on previous rodent studies, the developing temporal lobe seems highly susceptible to seizure activity (Abdelmalik et al., 2005). The results from Abdelmalik and colleagues show that the
developing mesial temporal lobe structure, specifically the hippocampus, demonstrates recurrent ictogenesis: the generation of seizures. Based on these findings, the investigations of the limbic system have led to two theories regarding the hippocampus and its susceptibility to cellular network changes due to ELS (Bromfield et al., 2006). The first theory proposes that selective changes in interneurons influences decreased feedback inhibition to principal neurons. The second theory suggests that recurrent excitatory connections may take place through axonal sprouting between neighbouring principal neurons. Both theories indicate hyperexcitability, which increases the firing rate in the neuronal network of the hippocampus (Bromfield et al., 2006).

Studies have identified that acute ELS can alter AMPA receptor subunit compositions (Cornejo et al., 2007). A study conducted by Rakhade et. al. identified ELS to induce changes in synaptic potency of CA1 hippocampal pyramidal neurons and was associated with increased phosphorylation in AMPAR subunits (Rakhade et al., 2008) and the functional conversion of silent synapses (Zhou et al., 2011). Phosphorylation of GluR2 S845/S831 and GluR2 S880 sites is due to the increased activity of various protein kinases that mediate the phosphorylation (Rakhade et al., 2008). Studies also claim ELS to induce long lasting changes that may lower the seizure threshold and increase the likelihood of epileptogenesis. However, the research surrounding the mechanisms of ELS remains incomplete and further research is needed to clarify the exact mechanisms mediating its effects on the immature brain.

1.6.1 Heterogeneous population of seizure sensitive cells

It has become clear that seizures involve an intricate play of different neurons and circuits (Bui et al., 2015). A study conducted by Muldoon and colleagues demonstrated that there is a high degree of variability in cell populations that are influenced during a seizure event, both in animal models and human patients with epilepsy. Determining which set of selective neurons fire synchronously during a seizure event has been uncertain, however there are studies illustrating a heterogenous population of
seizure-sensitive cells during a seizure event (Bui et al., 2015; Keller et al., 2010; Muldoon et al., 2013). These cells can be described to have a unique neuronal spike activity during seizure initiation (Bui et al., 2015; Keller et al., 2010; Muldoon et al., 2013). Epileptic animal models have shown significant heterogeneity between macroscopically recurrent epileptic events at the microcircuits (Muldoon et al., 2013). However, the heterogeneity of cells during seizure events is not limited to laboratory animal models. Patients have also shown nonuniform recruitment of neuronal populations during the initiation and progression of epileptiform events (Bui et al., 2015; Keller et al., 2010). Furthermore, between subsequent seizures, the variability of active neurons was high, with different populations of neurons participating during a seizure, evident for both interneurons and principal neurons (Keller et al., 2010; Bui et al., 2015). During the early life period, the heterogeneity of seizure sensitive cells has also been identified with numerous theories pertaining to developmental changes. The neonatal period poses a diverse progression of cells developing at different rates and at different timepoints, leaving a more vulnerable period for specific neurons to be seizure sensitive.

1.7 ELS influence autistic-like social behavior deficits

The timepoint with the highest incidences of epilepsy and associated neuropsychiatric comorbidities is during the early neonatal period (Bernard and Benke, 2014; Haas et. al., 2001; Trevisan et al., 2020). Early life epilepsy (ELP) is commonly associated with learning disorders and autism (Bernard and Benke, 2014; Haas et. al., 2001; Trevisan et al., 2020), where 46% of patients with autism have epilepsy and correlated with a lower IQ (Bernard and Benke, 2014; Haas et. al., 2001). This creates a correlation between ELP and autism. The onset of ELP arises from ELS, where previous animal studies on ELS have shown consequences of epilepsy development and altered synaptic plasticity (Bernard and Benke, 2014; Haas et. al., 2001). For decades, it was thought that ELS in animal models does not produce long-term consequences when compared to similar seizure models during adulthood. This was due to the
widespread hippocampal cell loss observed in adult rodents but not in pups, which were spared (Bernard and Benke, 2014; Haas et. al., 2001). Gross morphological changes were not observed following ELS, making them appear comparatively benign (Bernard and Benke, 2014; Haas et. al., 2001). However, recent evidence shows a causal relationship between ELS and later life social and cognitive deficits. ELS animal models demonstrate increased hippocampal and cortical neuronal sensitivity to excitation, later life seizure susceptibility, mossy fibre sprouting, cognitive deficits, and spontaneous seizures (Bernard and Benke, 2014; Haas et. al., 2001). Studies have also demonstrated an early change in neuronal functioning, where early modification of AMPARs take place (Bernard and Benke, 2014; Haas et. al., 2001). Interestingly, seizures unrelated to epilepsy during adulthood may result in some cognitive and/or behavioural deficits, however, only through ELS can cause the onset of social behavioural deficits manifestation (Bernard and Benke, 2014; Rigas et. al., 2018; Sayin et. al., 2015).

Immature neurons have an elevated excitation/inhibition ratio during a critical period. Therefore, ELS poses a significant threat to developing neurons in the hippocampus and contributes to epileptic networks and autistic-like behaviours later in life (Rigas et. al., 2018; Sayin et. al., 2015). In some epileptic cases, children who have ELS in the first year of life are more likely to develop autism spectrum disorder (ASD) and have some degree of learning difficulty (Lugo et. al., 2014). Furthermore, ELS is often refractory to traditional antiepileptic medications and may result in long-term cognitive deficits varying from disabilities in learning, memory and social behaviours. Children with epilepsy demonstrate high incidences of later-life psychiatric disorders and ASD. There is a critical period during development that is involved in influencing the child’s behaviour outcome with ASD and cognitive dysfunction. Patients with ASD have a spectrum of symptoms however some common symptoms are impaired emotional recognition and impaired social memory (Trevisan et al., 2020). Patient-based studies have shown that ASD patients’ abilities on memory tasks, perform functional verbal and logical working memory, but demonstrate impaired recognition of faces and social scenes (Williams et al., 2005). In
animal models, a study by Lugo et. al. (2014), examines the effect of early life flurothyl-induced seizure during postnatal days 7 to 11 in mice. Using social partition and social chamber tests, they identified that adult mice that had undergone ELS demonstrated impaired social behaviour. Their results indicate that ELS causes deficits in hippocampal-dependent memory tasks and produces long-term disruptions in social behaviour (Bernard and Benke, 2014; Lugo et. al., 2014). Based on previous human and animal research studies, there is new interest between hippocampal CA2’s ability in social recognition memory processes and its connection to social memory impairments in ASD. Knowing that ELS may provoke ASD onset, the CA2 region may serve as an area of interest that might be involved in the social behaviour consequences. Therefore, there’s an urgent need to identify novel mechanisms and specific treatment strategies for ELS and associated cognitive deficits.

1.7.1 ELS dysregulation of mTORC1 pathway

The mammalian target of the rapamycin (mTORC1) pathway is known to modulate neuronal modulation and plasticity in the developing brain by regulating protein translation through the activation of ribosomal S6 protein kinase (p70S6K) (Talos et al., 2012). Furthermore, it regulates neuronal growth, synaptic plasticity and memory consolidation through the activation of ionotropic glutamate receptors, brain-derived neurotropic factor (BDNF) and activity-dependent extracellular signal-regulated kinase (ERK) pathway. The pathway is generally controlled and suppressed by the Tuberous Sclerosis Complex TSC1 and TSC2 complex (Talos et al., 2012). Studies have related the Tsc1 or Tsc2 mutations with an upregulation of the mTORC1 pathway to cause abnormal neuronal functioning, unregulated synaptic plasticity and disruption of protein synthesis (Talos et al., 2012). This dysregulation has been associated with seizures, epilepsy, cognitive disorders and autistic-like behaviour (Talos et al., 2012). A study by Talos et al., 2012, demonstrated that the mTORC1 pathway was upregulated after ELS, along with AMPA receptor function. These results are correlated with epileptogenesis and autistic-like social
deficits. The study also demonstrated that by administrating rapamycin (mTORC1 inhibitor) in ELS animals, they can prevent later life seizure susceptibility, epileptogenesis and autistic-like behavioural deficits. This introduces a new question of whether ELS can influence CA2 PN function and mTORC1 pathway, leading to later life social recognition memory deficits associated with CA2 function.

1.8 Seizure susceptibility in immature CA2 pyramidal neurons

Hippocampal neurons during early life are hyperexcitable due to the elevated excitatory synaptic strength, demonstrating a ‘critical period’ where neurons present with enhanced NMDA and AMPA excitatory glutamate receptors as well as prominent depolarizing excitatory GABA. The neonatal period represents the highest incidence of seizures, defining this period as vulnerable to insults (Rigas et. al., 2018; Sayin et. al., 2015). Furthermore, ELS may adversely affect developing hippocampal neurons and contribute to epileptic networks and social behaviour deficits later in life.

Although there are several properties underlying the defensive mechanisms against neuronal excitation in the CA2 region of the hippocampus in adults, these properties are underdeveloped during the early postnatal period. For instance, during development, PNNs regulate neuronal plasticity where sensory inputs either drive synaptogenesis in an activity-dependent manner or cause synaptic pruning due to the lack of sufficient activity (Carulli and Verhaagen et. al., 2021; Carstens et. al., 2016; Chaunsali et. al., 2021). Once PNNs are formed in a specific region, they forcefully end the critical period and stop all synaptic contacts. However, before PNNs are expressed, changes at cellular and molecular levels can take place to influence synaptic plasticity. Studies demonstrate that PNN depletion promotes synaptic plasticity by modulating ion channel/receptor expression and changing dendritic spine dynamics (Carulli and Verhaagen et. al., 2021; Chaunsali et. al., 2021; Fawcett et. al., 2019). In mouse CA2 region, PNNs start to express after postnatal day (PD) 14 (Carstens et. al., 2016), marking the timepoint preceding PD
vulnerable, where maladaptive plasticity may influence extensive rewiring and shape epileptogenesis by disrupting the balance of excitation and inhibition (Chaunsali et. al., 2021; Zhou et. al., 2011).

CA2 protein-based defense mechanisms gradually appear during development, including RGS14, Purkinje cell protein 4 (PCP4) and striatal-enriched protein tyrosine phosphatase (STEP) (Boulanger et. al., 1995; Cases et. al., 2018; Evans et. al., 2014; San Antonio et. al., 2014). RGS14, PCP4 and STEP protein immunoreactivities are present in animal models (mouse and rat), undetectable at birth (P 0) and first detected at P 7 while gradually increasing in expression throughout life and into adulthood (Evans et. al., 2014; San Antonio et. al., 2014). During the first postnatal week, due to the immature presence of RGS14, PCP4 and STEP, the CA2 region may be vulnerable as its adult defense mechanisms are absent and excitatory synapses are strengthened. The natural suppressor RGS14 normally suppresses the MAP kinase signaling pathway, thereby blocking CA2 LTP, however, when RGS14 is not present, CA2 PNs are able to display nascent and robust LTP (Evans et. al., 2014; Evans et. al., 2018; Lee et. al., 2010). PCP4, also known as PEP-19, has been shown to indirectly influence calcium buffering by mediating calmodulin, which can contribute to the variability in synaptic plasticity between the CA2 and other CA regions (Simons et. al., 2009). STEP, particularly STEP61 is enriched in the CA2 of the hippocampus and serves to regulate the ERK1/2 pathway through dephosphorylation and thereby modulation of neuronal activity (Cases et. al., 2018; Won and Roche, 2020). These three proteins function as the cellular defenses against neuronal excitation in the CA2, however seizure activity generated during the early postnatal period at a time when they are not completely developed may influence CA2 PNs, leading to later life learning and behavioral deficits.

Early in life, the hippocampus undergoes a critical period of synaptic development where incidences of seizures most commonly occur (Zhou et. al., 2011). Although the neonatal hippocampus is resistant to neuronal cell death during glutamate hyperexcitation, ELS can give rise to later-life epilepsy with an association to learning disabilities and social behavioral deficits (Zhou et. al., 2011). Animal
studies have demonstrated that the hippocampal CA2 PNs play an important role in learning and memory as well as social memory. Lesions and silencing of CA2 PNs have resulted in impaired social recognition memory (Hitti and Siegelbaum, 2014; Stevenson et. al., 2014). This creates a link between the CA2 and the long-term social effects of ELS, establishing an area for intervention.

1.9 Animal models of early life seizure

Animal models of epilepsy are used to understand the neurobiology of disease while investigating novel anti-epileptic targets.

Kindling model of seizures:

Kindling models of seizures use repeated sub-convulsive chemical or electrical stimuli to initiate seizures. The repeated electrical or chemical stimulation will gradually lower the threshold of seizures and eventually cause brain regions such as the hippocampus to exhibit electrical discharges. This long-lasting change in neural network excitability during early-life involves long-lasting structural bioc and hemical changes in the neural circuits involved. The kindling models are often used to identify the heterogeneity of neurons which may cause long-term lasting effects (Gaito, 1979).

Pentylenetetrazol and Kainic Acid models

Administration of chemical convulsant substances such as, pentylenetetrazol or kainic acid in rodents are commonly used to induce early life seizures. Different early life seizure models may represent different forms of seizures through unique mechanistic qualities. The well-validated Pentylenetetrazol (PTZ) model use PTZ as a GABA\textsubscript{A} receptor antagonist to reduce the inhibition and evoke seizures (Dhir, 2012). The kainic acid (Kainate, KA)-induced seizure model is another well-established model of temporal lobe epilepsy. KA induces the onset of SRSs for an extended period when compared to other
models to study the long-lasting effects of early-life seizures on the developing brain. ELS evoked using chemoconvulsant models have been shown to induce long-term cognitive deficits, including autistic behaviours (Bernard and Benke, 2015). Other models with low seizure frequency or low susceptibility to developing spontaneous seizures are not an adequate representation of ELS and their long-lasting effects (Bertoglio et al., 2017). Therefore, to study ELS and their effects on the heterogeneity of cells, the induction of PTZ or kainate will account for the appropriate representation of SRSs without leading to death while sustaining repetitive seizures along with the long-lasting effects (Dudek and Staley, 2017).

2. Rationale and objectives

In the mature brain, hippocampal CA2 has been known to be resistant to seizure-induced cell death when compared to the CA1 and CA3 regions (Botterill et al., 2017; Lawrence and Inder, 2010; Minjarez et al., 2017; Swann, 2002). However, whether adult seizures affect the function of CA2 neurons and social recognition memory is completely unknown. In the immature brain studies have shown that a single seizure event during the neonatal period can influence later life autistic-like social recognition deficits (Talos et al., 2012), leading us to believe that the immature CA2 may be vulnerable to a single seizure event.

Here we hypothesize that early life presents as a vulnerable period where a seizure can influence CA2 function and therefore impair associated social recognition memory function.

To test this hypothesis, we have three aims:

Aim 1: Identify an age-dependent seizure vulnerability of the CA2
c-fos, an immediate early gene, has been widely used as a marker for neuronal activation. Using pentylenetetrazol (55 mg/kg, i.p.) in P10 and (40 mg/kg i.p.) P60 c-Fos-GFP based transgenic mice, we are able to identify and monitor ELS selectively activated neurons by examining the expression of GFP. We hypothesize that the level of GFP+ cells in the CA2 will show an increase after ELS during P10 when compared to control saline injected mice. However, P60 animals will show no difference in the level of activated GFP+ cells between the seizure and control saline groups.

CA2 neurons have been shown to be essential for social recognition memory (Hitti, and Siegelbaum, 2014). We will use social recognition memory tests to examine the functionality of the CA2 after PTZ in both P10 and P60 WT C57B6 mice. To further confirm the direct involvement of CA2 in the social recognition memory test. We will measure the number of c-Fos+ cells in the CA2 at 1 hour after the social behavior testing. The level of c-Fos+ cells will indicate whether CA2 is functional during the social behavior testing. We expect that PTZ injected P60 animals will not hinder social performance and such will demonstrate increased level of activated c-Fos+ cells once they undergo behavior testing compared to PTZ injected mice without behavior. However, P10 animals with PTZ-induced social behavior deficits will demonstrate a decreased level of c-Fos+ cells compared to saline injected animals that go through behavior testing. Hypothesizing that a single seizure event during early life will change CA2 PN functionality and cause social recognition memory deficits.

**Aim 2: To identify and investigate seizure sensitive neurons in immature CA2 PNs**

Given that ELS could cause social recognition memory deficits as well as an impairment in CA2 neuronal function during the behavior testing, we will then determine whether these deficits are associated with changes in the synaptic function of CA2 neurons.
Through whole-cell patch clamp recordings of spontaneous AMPAR-mediated postsynaptic currents (sEPSCs) we will determine if ELS induces an acute change in AMPAR function in randomly selected CA2 PNs, considering the critical role of AMPARs in the brain development during this period. Here, an enhanced AMPAR activity in CA2 PNs after PTZ in p10 pups can be associated with a postsynaptic or presynaptic change. To monitor the contribution of postsynaptic changes, silent synapses will be measured in CA2 PNs while stimulating the Schaffer collateral pathway or Perforant pathway. To investigate the presynaptic changes, paired pulse facilitation in CA2 PNs will be measured while stimulating the Schaffer collateral or Perforant pathway. Next, we will verify if the change in AMPAR-mediated sEPSC frequency observed in P10 mice after PTZ seizure remains at P12 when the social behavior testing takes place. We will induce PTZ seizure in P10 pups and record spontaneous AMPAR sEPSCs in CA2 pyramidal neurons at P12.

If our results demonstrate an acute initiation and persistent enhancement of AMPAR function in CA2 PNs after PTZ induced seizures in P10, we will then determine a pharmacological suppression of AMPAR function in vivo by an AMPA receptor antagonist NBQX to rescue early seizure-induced social behavior deficits. Immediately after a PTZ seizure at P10, the mouse pups will be treated with a single dose of NBQX (20mg/kg) or vehicle and then tested for social behavior two days after treatment at P12. To further confirm the direct involvement of CA2 in the social recognition memory test, we will measure the number of c-Fos+ cells in the CA2 1 hour after the social behavior testing. The level of c-Fos+ cells will indicate whether CA2 is functional during the social behavior testing in animals that received PTZ and NBQX. If we identify a significant increase in number of c-Fos+ cells after behavior testing in the CA2, we can then conclude that the CA2 is functional due to NBQX treatment. Next, we will determine whether NBQX treatment could reverse PTZ seizure-induced persistent enhancement of AMPAR function in P12 CA2 PNs.
NBQX treatment blocks AMPA receptors on all neurons and is not specific to the specific set of neurons that are selectively activated during a seizure. Previous studies have found that early life seizures activate a selective group of neurons referred as seizure-sensitive neurons (Bui et al., 2015; Keller et al., 2010; Muldoon et al., 2013). As such, we will then focus on investigating seizure-sensitive cells using our c-Fos-GFP based transgenic mice, where we can visually target GFP+ cells during a seizure event. Using whole-cell patch clamp recordings we can determine whether PTZ seizures-induced AMPAR function enhancement in selective GFP+ seizure sensitive neurons in immature CA2. Here, we hypothesize that early life seizure induces target specific functional changes in these seizure-sensitive neurons which mediate early seizure-induced social recognition memory deficits.

**Aim 3: Examine whether chemogenetic suppression of seizure-sensitive neurons can rescue social recognition deficits.**

If our results indicate that early life seizure-induced AMPAR function enhancement occurs in selective GFP+ cells, this can suggest a critical role of the GFP+ cells in early seizure-induced network changes and social behavior deficits. We will then determine whether precisely suppressing selectively activated seizure-sensitive neurons could reverse early seizure-induced social behavior deficits. To precisely suppress the selectively activated seizure-sensitive neurons, we will incorporate a unique activity-dependent tagging and manipulation system, the c-Fos-GFP/c-Fos-tTA/TRE-hM4Di mouse model. This model has three co-injected transgenes, c-fos-tTA, c-fos-eGFP, and TRE-hM4Di, that drive expression of GFP, tetracycline transcriptional activator (tTA) and Tet-off construct controlling inhibitory chemogenetic hM4Di expression directed to activated neurons by the c-fos promoter in the absence of Doxycycline (DOX). Mouse pups will be raised on DOX-containing food from P0 to P6 and then taken off DOX until P10 when early life seizure will be induced. Seizures will be able to induce both temporary GFP (green) and persistent hM4Di in activated neurons. Mice will be put back on DOX
immediately following seizures and returned to home cage. After seeing the first seizure event we will then inject the animal with clozapine N-oxide (CNO) twice a day until behavior testing day to actively suppress the activity of GFP+ cells. We then will test the social behavior using the pre-weaning two choice social recognition memory test at P12. To further confirm the direct involvement of CA2 in the social recognition memory test, we will measure the number of c-Fos+ cells in the CA2 1 hour after the social behavior testing. The level of c-Fos+ cells will indicate whether CA2 is functional during the social behavior testing in animals that received PTZ and CNO. If we identify a significant increase in number of c-Fos+ cells after behavior testing in the CA2, we can then conclude that precise suppression of the seizure activated sensitive neurons can rescue social deficits caused by PTZ seizures. Next, we will determine whether chemogenetic suppression of the selectively activated seizure-sensitive neurons could reverse PTZ seizure-induced persistent enhancement of AMPAR function in P12 CA2 pyramidal neurons.

3. Methods

3.1 Experimental Animals

Male and female P10-12 and P60-62 wild type C57B6, c-Fos-GFP, c-Fos-GFP/c-Fos-tTA, and c-Fos-GFP/c-Fos-tTA/TRE-hM4Di mice were used for experiments in this study. Triple transgenic c-Fos-GFP/c-Fos-tTA/TRE-hM4Di were generated by crossing heterozygous double transgenic c-Fos-GFP/c-Fos-tTA mice with heterozygous transgenic TRE-hM4Di mice (Jax #024114). All mice were provided with food and water while being housed under controlled room temperature (22 °C) with 12 hours of the light-dark cycle. All experimental procedures were in accordance with the guidelines set by the Canadian Council for the Use and animal handling procedures, and were approved by Carleton
University Animal Care, and Veterinary Services (ACVS). All efforts were carefully made to minimize animal suffering and the number of animals used throughout the study.

3.2 Early life seizure and adult seizure induction

Early-life seizures were reliably induced in P10 mouse pups by i.p. injection of a low-dose (2.0 mg/kg) kainate or (55 mg/kg) PTZ while assuring optimal animal survival (Dudek and Staley, 2017). Adult seizures were induced in P60 mice by injecting PTZ (i.p. 40mg/kg). The Racine scale was used as a scoring system to monitor the different stages and severity of behavioral seizure activity (Table 1, IHARA et. al., 2016). Stages 4 and 5 seizures were used as the criteria for determining when the perfusion step should take place while the animal is monitored by video recordings. The mice were perfused 1 hour after the first seizure event.

<table>
<thead>
<tr>
<th>Seizure Stage</th>
<th>Behavioural stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No change in behaviour</td>
</tr>
<tr>
<td>1</td>
<td>Sudden behavioral arrest and shivering</td>
</tr>
<tr>
<td></td>
<td>Head nodding</td>
</tr>
<tr>
<td>2</td>
<td>Forelimb clonus</td>
</tr>
<tr>
<td>3</td>
<td>Rearing with forelimb clonus and falling</td>
</tr>
<tr>
<td>4</td>
<td>Tonic-clonic activity, rearing and falling</td>
</tr>
<tr>
<td>5</td>
<td>Death</td>
</tr>
</tbody>
</table>

Table 1: Racine scale.
3.3 **NBQX and CNO treatment**

A selective and competitive AMPA and kainate receptor antagonist 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline (NBQX) (20mg/kg i.p.), or The DREADD agonist clozapine N-oxide (CNO) was administered immediately after identifying the first behavioral seizure phenotype in P10 pups. The social cognitive functions were examined at P12.

Triple transgenic c-Fos-GFP/c-Fos-tTA/TRE-hM4Di mice were used to rescue phenotype post seizure event. Triple transgenic mice were on doxycyclin (DOX) from P0 to P6 and then taken off DOX until P10 when PTZ or KA was injected. From P10 to behavior test day, P12, the animals were put back on DOX. Clozapine N-oxide (CNO) (10mg/kg, S.C) injections were conducted after identifying the first seizure event post PTZ injection in P10, followed by 2 doses (8 hours apart) on P11 and one dose on P12. 8 hours later, the behavior test was conducted in P12 pups.

3.4 **Immunohistochemistry**

3.4.1 **Perfusion**

Mice were anesthetized (sodium pentobarbital, 200mg/kg) and then perfused with 20ml of 0.9% saline followed by 20ml of 4% paraformaldehyde (PFA) solution (40 g Paraformaldehyde powder, ddH₂O, 1N HCl, 1N NaOH and 800ml PBS) in 0.1M Phosphate buffer Saline (PBS) (0.1g KCl, 4.0g NaCl, 0.72g Na₂HPO₄, 0.12g KH₂PO₄). Brains were carefully removed from the skull and post-fixed in ice-cold paraformaldehyde for 90 min and then stored in PFA mixture overnight at 4°C. In the next three days, the brain underwent sucrose washes: 0.1M PB pH 7.4 with a 10% sucrose solution for the first day and 0.1M PB pH 7.4, 20% sucrose for the second day and 0.1M PB pH 7.4 with 30% sucrose
on the third day. The brains were then ready to be sliced into 40 \( \mu \text{m} \) slices for immunohistochemical staining.

### 3.4.2 Immunohistochemistry staining

The washed brains were sliced coronally into 40 \( \mu \text{m} \) slices and immediately placed in a well plate with 0.08 PB + Azide. The brain slices were then immediately stored in 4\(^{\circ}\)C until the day of staining. During the staining procedure, the brain slices were washed three times for 5 minutes in well plates filled with phosphate-buffered saline (PBS) (pH 7.2). Sections were then placed in blocker; 5% normal goat serum (NGS) and 0.3% Triton-X (Triton-X in PBS) with rotation for 1 hour. Next, sections were incubated with a diluted primary antibody overnight in 4\(^{\circ}\)C. To visualize the GFP antibody, anti-GFP primary antibody (AB 2307313, Aves Labs Inc.) at 1:5000 and raised in chicken was used as well as mouse anti-RGS14 (1:1000, UC Davis/NIH NeuroMab #75–170). The next day, sections were rinsed in PBS 3x5 minutes and later incubated for 2 hours in biotinylated anti-chicken and anti-mouse IgG secondary at a dilution of 1:1000. The following secondary solutions were used: anti-chicken (647) (1:1000) and anti-mouse (488) (1:1000). Sections were washed again in PBS 3x5 minutes, followed by adding DAPI+PBS in 1:5000 concentration for 5 minutes and later re-washing in PBS 3x5 minutes. Slices were then mounted on positively charged slides and cover-slipped with Fluoromount and placed away in a cloased dark slide holder to dry overnight. All incubations during the staining procedure occurred at room temperature.

The signal was visualized with immunofluorescence microscopy using the micro-brightfield image acquisition software on Zeiss Axioimager 2 microscope. All slices were compared between experimental and control groups. DAPI, GFP and RGS14 stained slices were analyzed using the optical
fractionator workflow on the stereoinvestigator (MBF, Williston, VT, USA). The coverage of GFP fluorescent intensity was quantified using ImageJ version 1.52 (National Institute of Health, USA).

3.5 Behavioral testing

All tests were conducted during the light cycle. One day before behavior testing, post-weaning mice were habituated to the testing room for 1 hr before being placed in the testing apparatus for the duration of the specific test. On the day of testing, post-weaning mice were habituated to the testing room for 1hr before the initiation of the test. Behavioral tests were video-recorded and a trained observer scored behavior testing. Testing apparatuses were cleaned with 70% ethanol after each trial.

3.6 Preweaning two-choice social recognition memory test

At P12, mouse pups had undergone behavior testing to study their memory of the dam using a modified two-choice social recognition test (Laham et al., 2021, Moy et al., 2004). Mouse pups were placed in a 12” × 12” opaque plexiglass testing apparatus with two wire mesh containers enclosing the caregiving mother and a novel mother. The testing apparatus was placed on a heating pad to maintain pup body temperature comparable to that of pups in the home cage. The novel mother was a lactating mother with a litter of age-matched pups. Because P12 mouse pups have limited locomotion capabilities, and specifically rely on olfaction, we scored investigation of one or the other social stimuli whenever the test pup’s nose was oriented toward a stimulus animal and investigation was characterized as sniffing within one inch of the wire mesh container holding the social stimulus. Since the social stimulus mice were confined within wire mesh containers, no attempt was made to quantify their behaviors. Before each testing period, pups were separated for 1hr. The duration of each test was 10 minutes. At the start of each test, the pup was placed in a neutral corner equidistant from the two social stimuli with the head directed toward the center of the apparatus. The location of the mother and novel mother in different
corners was counterbalanced in each test. Time spent investigating was recorded when the pup’s snout moved in the direction of one or the other social stimuli. Testing periods only occurred for 20 minutes at a time for each caregiving mother/novel mother pair, after which dams were returned to their respective home cages to allow adequate nursing of their litters. Mothers remained with their litters for 20 minutes before beginning the next round of behavior.

3.7 Three Chamber social recognition memory test

At P60, control and PTZ mice underwent three-chamber sociability testing (Moy et al., 2004; Tal;os et al., 2012). The three-chamber apparatus consisted of a neutral center chamber with two adjacent chambers, each containing a single wire mesh container. All chambers were equal in size. In the first trial, mice were allowed to explore all three chambers of the apparatus for 5 minutes for habituation. Immediately following the first trial, test mice were placed in the center neutral chamber while a novel female or male (same sex as the testing mice) was placed in one of the wire mesh containers in an adjacent chamber. The adjacent chamber in which the mouse was placed was counterbalanced between trials. In the second trial, mice were once again given 10 minutes to explore the three chambers now consisting of a neutral center chamber, a non-social chamber, and a social chamber containing the novel female or male, sociability. Sniffing within 1” of a wire mesh container was counted toward total investigation time. 1 hour later inter-session, the third trial was social novelty where the testing mouse either interacts with the novel mouse, placed in the other chamber, or the familiar mouse for 10 min.

3.8 Caregiving mother/novel mother exposure for immediate early gene analysis

To determine the involvement of CA2 cell activation during social recognition behaviors, P12 mouse pups were placed in a 12” × 12” opaque plexiglass testing apparatus containing the caregiving mother or the novel mother and allowed to investigate for 10-minutes. Pups were exposed to a stimulus
mouse located in a wire mesh container. Before each testing period, pups were separated for 1 hr. At the conclusion of the 10-minute trial, pups were transferred to a clean cage without the caregiving mother, and perfused one hour later. The brains were processed as previously described for immunohistochemistry and stained using rabbit anti-c-Fos (1:3000, Cell Signaling Technology #2250S) combined with mouse anti-RGS14 (1:1000) to identify the CA2 region. Researchers were blinded from the experimental conditions of each slide before conducting c-Fos staining. Counts were conducted using micro- brightfield image acquisition software on Zeiss Axioimager 2 microscope. c-Fos cell counts taken from a one in three series of coronal sections containing CA2. One hemisphere was randomly selected and all counts for a given region were made from the chosen hemisphere. Only c-Fos+ cells located within RGS14 labeling that passed a stringent intensity threshold using Stereo Investigator (Microbrightfield) were included in CA2 analyses. Within the CA2 region DAPI was counted to determine the percentage of activated c-Fos+ cells.

Positive cell counts were taken from equal numbers of age-matched controls that remained in the home cage and did not undergo social interaction along with the social interaction and PTZ groups that received the chemoconvulsant 2 days prior to testing day, p10. The timeline starts with P10 mice being injected with PTZ two days before their behavior test. The pre-weaning two choice social recognition memory test was used. The process consisted of three steps where the pup is separated for 60 min and then placed in the behavior test for five minutes to choose between their caregiving mother or novel mother. Followed by perfusing the animals 1 hr after the behavior test.

3.9 Hippocampal slice preparation

Hippocampal slices containing CA2 were taken from P10 and P12 pups. Mice pups were weighted and rapidly decapitated. The brain was removed from the skull and placed in ice-cold cutting solution containing (mM): 119 choline chloride, 5 KCl, 4 MgSO4, 1.25 NaH2PO4, 0.8 CaCl2, 26 NaHCO3, 18
glucose, and 5 sucrose bubbled with 95%O₂/5%CO₂. Coronal slices of the middle hippocampus were sectioned at 300µm using a vibratome (Leica Microsystems VT1000S) while oxygenated in cutting solution. Slices were then incubated in oxygenated artificial cerebral spinal fluid (ASCF) chamber containing (mM): 124 NaCl, 5 KCl, 1.2 MgSO₄, 1.25 NaH₂PO₄, 2 CaCl₂, 26 NaHCO₃, 10 glucose, in recovery for 15 minutes at 35 °C in a water bath. The slices were then kept at room temperature for at least 1 hour before any electrophysiological recordings. Electrophysiological recordings were performed using a Zeiss Axio Scope A1 microscope in a 2.5mL electrophysiology recording chamber, where slices were placed and perfused with oxygenated ACSF at room temperature. The chamber was attached to an infra-red differential interference contrast imagining system.

### 3.10 Whole-Cell Patch Clamp Recording

Whole-cell patch-clamp recordings were taken from hippocampal CA2 pyramidal neurons from P10 and p12 pups. When recording GFP+ and GFP- neurons, blue light LED was switched on to identify the endogenous fluorescence of GFP+ cells. The recording micropipettes were made using a pipettes puller with a resistance of 8-12 MΩ with internal solution containing (mM): 110 Cs-methanesulfate, 10 TEA-Cl, 4 NaCl, 2 MgCl₂, 10 EGTA, 10 HEPES, 4 ATP-Mg(Na₂), 0.3 GTP-Na₃, 5 QX-314 chloride, and 7 phosphocreatine-Na₂ with pH 7.25. AMPAR mediated spontaneous EPSCs (sEPSCs) were recorded at -60mV holding potential to isolate them from NMDAR responses. Furthermore, 100 uM picrotoxin (PTX) was added into the perfused ACSF to block the activity of GABAₐ receptors. sEPSCs consisted of a minimum of 60 sweeps with 10.3 seconds each, and analysed using Clampfit 10.2 (Molecular Devices). Clampfit 10.2 automatically detected the events, which were used to construct the frequency and amplitude histograms. Event detection threshold was set at 4-5pA, based on the noise level. The rise and decay times were used to visually confirm an event before final analysis. Event frequency and amplitude were obtained for all AMPAR mediated sEPSCs. All data was collected using
the Axopatch 200A amplifier (Molecular Devices) and pClamp 9.2 software with a 2k HZ filtration. Data was digitized at 20kHz with a Digidata 1320A interface.

To measure silent synapses, minimally evoked EPSCs (eEPSCs) in CA2 pyramidal neurons were elicited by stimulating the Schaffer collateral pathway in P10 mice. The percentage of silent synapses was calculated using the failure rate method. The failure rate method refers to the percentage of trials when no response is elicited compared to the percentage of trials when a response is elicited at a specific stimulation intensity. Firstly, the stimulation intensity required to elicit a response is reached. Next, stimulation intensity was slowly increased until the 50% failure rate was attained, then recorded for a minimum of 20 trials at the same stimulation intensity. To find the proportion of silent synapses, the membrane potential of a cell was first held at -60mV, and 50% failure rate was reached, then, at the same intensity, the cell was stimulated at +40mV holding potential. Failure responses were visually confirmed. The fraction of silent synapses was calculated using the established formula: \(1 - \frac{\ln(F_{-60})}{\ln(F_{+40})}\) (Liao et al., 1995). The paired-pulse ratio was calculated by comparing the amplitude of evoked EPSC at the second pulse to the amplitude of evoked EPSC at the first pulse with 50ms pulse interval.

3.11 **Statistical data analysis**

All experimental data are presented as mean ± SEM and analyzed using GraphPad Prism (Version 8 for windows, GraphPad Software, La Jolla, California USA). The Shapiro-Wilk normality test was used to screen all data. For normally distributed data, the two-tailed unpaired, paired t tests were performed for two-group comparisons. For non-normally distributed data, the Mann-Whitney rank test was utilized. Statistical significance was established at \(p<0.05\).
3.11.1 Analysis of GFP, RGS14 and c-Fos staining

All statistical tests were performed using Prism 8 (GraphPad, San Diego, CA). A two-way analysis of variance (ANOVA) was used to reveal differences in the vulnerabilities of the CA2 or other hippocampal areas studied in P60, P62, P10 and P12 animals followed by a Tukey’s test for multiple comparison of the experimental and control groups. In other cases, a one-way ANOVA, followed by Tukey’s post hoc test or an unpaired t-test. An alpha level of 0.05 was used as a significance criterion in all the tests. The data are given as the mean ± S.E.M. Colocalization of RGS14 and GFP or c-FOS was examined using 40x lens and analyzed towards the distribution to total RGS14 counts or GFP/c-Fos counts.

3.11.2 Analysis of behavior testing

During the first phase and second phase of the three-chamber social recognition test, zone preference indexes for the conspecific and object zones or familiar mouse and novel mouse will be recorded and a 2×2 Between-Factor ANOVA (control saline vs PTZ × object vs mouse) or (control saline vs PTZ × familiar mouse vs novel mouse) followed by Sidaks’s post-hoc test. For the post-weaning two choice social recognition memory test, zone preference for the caregiving mother and novel mother will be recorded and a 2x2 Between-Factor ANOVA (control vs experiment x caregiving mother vs novel mother) followed by a Sidak’s post-hoc test. Time difference in seconds between the caregiving mother a novel mother in each experimental group will be analyzed, unpaired t-test (control vs PTZ). Total time of interaction in seconds will be accounted for along with an unpaired t-test between experimental groups (control vs PTZ).
4. Results

4.1 Acute seizures in adult mice did not affect the function of mature hippocampal CA2

In mature brain, hippocampal CA2 has been known to be resistant to seizure-induced cell death (Botterill et al., 2017; Lawrence and Inder, 2010; Minjarez et al., 2017; Swann, 2002). However, whether adult seizures affect the function of CA2 neurons is completely unknown. To investigate the effects of acute seizures on the mature hippocampal CA2 neurons, we induced acute PTZ seizures (single dose, 40mg/kg, i.p.) in P60 mice. Each mouse included had reached stages 4 and 5 seizures on the Racine scale.

4.1.1 Acute seizures in adult mice induced a small but significant increase in the activation of CA2 neurons

Using the GFP antibody we were able to identify the selectively activated hippocampal neurons in mice following PTZ-induced seizures (Figure 2 A-O). We found that PTZ-induced acute seizures significantly increased the activation of hippocampal neurons showing significant increases in the number of GFP+ cells in the CA1 (18.567±0.470%, n=6) and CA3 (26.740±1.233%, n=6) compared to the control groups (CA1: 3.970±0.637%, Sidak HSD, n=5, p<0.0001; CA3: 8.800±0.095%, Sidak HSD, n=5, p<0.0001, Figure 2 C). RGS14 known to be enriched in CA2 neurons was used here to define the CA2 area. Interestingly, at P60 PTZ-induced acute seizures evoked a small but significant increase in the number of GFP+ cells in the CA2 (Middle CA2: 5.614±0.190%, n=8) compared to those in the littermate controls (3.446±0.297%, n=11, Sidak HSD, p<0.0001, Figure 2 C). In addition, no sex difference was identified in PTZ seizure-induced CA2 neuron activation (2-way ANOVA: F(1,15)=0.6764, n=19, p=0.4237; F(1,15)=0.2287, n=19, p=0.6394, Figure 2 G). We therefore pooled in the following data from male and female mice. Importantly, the numbers of PTZ seizure-induced activated GFP+ cells in CA1
and CA3 were significantly higher than CA2 (one-way ANOVA: F(2,16)=229.9, n=19, p<0.0001) (CA1: 234.6±17.89% of CA2 GFP+ cells, Tukey HSD, n=13, p<0.0001; CA3: 143.0±18.57% of CA2 GFP+ cells, Tukey HSD, n=12, p<0.0001, Figure 2 D). These results strongly support that the mature CA1 and CA3 is affected more by the acute seizure than the CA2.

Next, we further analyzed the distribution of CA2 GFP+ cells along the hippocampal dorsal-ventral axis in both control saline and PTZ groups (Figure 3 A-G). There is a main effect when comparing the level of GFP+ cells between the dorsal, middle and ventral regions (2-way ANOVA: F(2, 35)=26.47, n=41, p<0.0001, Figure 3 D). There is also a main effect when comparing the control saline and PTZ groups in the dorsal, middle and ventral regions (2-way ANOVA: F(1,35)=54.79, n=41, p<0.0001, Figure 3 D). Interestingly, the dorsal CA2 is resistant to PTZ-induced acute seizures (P60 control - dorsal: 2.686±0.385%, n=7; P60 PTZ – dorsal: 3.586±0.724%, n=5) showing no significant difference in the level of GFP+ cells following PTZ seizures (2-way ANOVA followed by Sidak’s post doc test: 1.490±1.100, n=12, p=0.2085, Figure 3 D). However, similar to the middle section of the CA2, the ventral CA2 demonstrated significant increases in the level of GFP+ cells in the PTZ group compared to control saline group (P60 control – ventral: 4.540±0.734%, n=5; P60 PTZ – ventral: 8.754±0.848%, n=5) (2-way ANOVA followed by Sidak’s post doc test: 4.070±1.192, n=10, p=0.0112, Figure 3 D). These results confirm the region-specific effects of PTZ seizures within mature CA2 structure.

4.1.2 Acute seizures in adult mice did not influence social recognition memory

CA2 neurons have been shown to be essential for social recognition memory (Hitti, and Siegelbaum, 2014; Laham et al., 2021). Given that our above results demonstrate that a small but significant increase in the activation of mature CA2 neurons by PTZ induced seizures, we next aimed to determine whether acute seizures in adult mice could affect CA2- social recognition memory. Standard
three-chamber sociability test was used to measure the social recognition memory in mice at P62 two days after seizures at P60.

Our results from the behaviour testing demonstrate that both control and post-PTZ seizure mice showed a significant preference for sociability, indicated by spending much more time with a mouse (control mice: 195.875±72.371 sec, n=8; PTZ seizure mice: 109.5±19.593 sec, n=6) than the object (control mice: 98.375±41.559 sec, n=8, 2-way ANOVA followed by Sidak’s post doc test: p<0.001; post-PTZ seizure mice: 45.167±11.339 sec, n=6, 2-way ANOVA followed by Sidak’s post doc test: p=0.0474, Figure 2 L). In addition, both control and post-PTZ seizure mice showed a significant preference for interacting with a novel mouse (control mice: 113.375±10.690 sec, n=8; post-PTZ seizure mice: 118.833±14.630, n=6) than a familiar mouse (Control mice: 52.25±5.143 sec, n=8, 2-way ANOVA followed by Sidak’s post doc test: p<0.001; post-PTZ seizure mice: 64.5±12.481, n=6, 2-way ANOVA followed by Sidak’s post doc test: p<0.01, Figure 2 M). These results indicate that acute seizures in adult mice did not impair social recognition memory.

To further confirm the direct involvement of CA2 in the social recognition memory. We measured the number of c-Fos+ cells in the CA2 at 1 hour after the social behavior testing. The level of c-Fos+ cells indicates whether CA2 is functional during the social behavior testing. Our results demonstrated that P62 control mice that went through behavior testing had a significant increase in level of c-Fos+ cells (middle CA2: 21.648±1.865%, n=5) when compared to control P62 animals (no behavior) (middle CA2: 2.767±0.211%, n=6, 2-way ANOVA followed by Sidak’s post doc test: p<0.0001, Figure 2 O), strongly supporting the direct involvement of CA2 neurons during the social behavior test. Consistent with our behavioral results, post-PTZ seizure mice also showed a significant increase in level of c-Fos+ cells in the CA2 after social behavioral testing (middle CA2: 25.060±3.261%, n=5) when compared to mice that received PTZ 2 days before and didn’t go through behaviour (2.943±0.326%, n=7, 2-way ANOVA followed by Sidak’s post doc test: p<0.0001, Figure 2 O), indicating a functional CA2 in post-
PTZ seizure mice. Furthermore, we examined the number of CA2 c-Fos+ cells along the hippocampal dorsal-ventral axis in both control saline and PTZ groups (Figure 4 A-F). We found that the dorsal and ventral CA2 structures demonstrate a similar pattern as the results from the middle structure of the CA2. The dorsal CA2 showed a significant increase in the level of c-Fos+ cells in the control mice with behavior group (dorsal: 27.670±4.081, n=5) when compared to control mice with no behavior group (dorsal: 1.616±0.313, n=5) (one-way ANOVA followed by Tukey’s post doc test: p<0.001, Figure 4 D1). The dorsal CA2 also showed an increase in P62 PTZ 2 days before with behavior group (dorsal: 23.925±4.777, n=4) when compared to P62 PTZ injected 2 days before without behavior (dorsal: 1.013±0.154, n=4) (one-way ANOVA followed by Tukey’s post doc test: p=0.003, Figure 4 D1). The ventral CA2 showed a significant increase in the level of c-Fos+ cells in the control mice with behavior group (ventral: 22.100±1.484, n=4) when compared to control saline no behavior group (ventral: 1.938±0.367, n=4) (one-way ANOVA followed by Tukey’s post doc test: p<0.0001, Figure 4 F1). The ventral CA2 also showed an increase in c-Fos+ cells in animals that received PTZ 2 days before with behavior group (ventral: 29.383±3.908, n=6) when compared to PTZ injected 2 days before without behavior (ventral: 2.850±0.857, n=4) (one-way ANOVA followed by Tukey’s post doc test: p<0.001, Figure 4 F1). Interestingly, it is worthwhile mentioning that post PTZ seizure P62 mice did not show a significant increase in the number of baseline c-Fos+ cells when compared to control P62 mice (one-way ANOVA followed by Tukey’s post doc test: dorsal: n=9, p=0.1561; middle: n=13, p=0.6714; ventral: n=8, p=0.3653, Figure 4 D1, E1 and F1), further supporting that PTZ seizures in adult did not change the excitability of CA2 neurons.

Overall, these data suggest whether the adult mouse has had seizures or not, social recognition memory is not impaired.
4.2 RGS14 contributes to the seizure resistance in CA2 neurons

Multifunctional signaling protein RGS14 is a critical factor suppressing synaptic plasticity in CA2 neurons and is thought to contribute to the resistance of CA2 pyramidal neurons to epilepsy-induced cell death (Lee et al., 2010; Evans et al., 2018; Harbin et al., 2021). We therefore examined the potential effects of adult PTZ seizures on the expression of RGS14 in the CA2 using immunohistochemistry.

4.2.1 Adult PTZ seizures did not alter RGS14 expression in CA2

We first quantified the number of RGS14+ cells in CA2 and found that consistent with previous study, there was a high level of RGS14+ cells within the mature CA2 region in control mice (P60 middle control: 68.299±1.968%, n=11) and adult PTZ seizures did not alter the expression of RGS14 (P60 middle PTZ: 71.184±2.242%, n=8, unpaired t test, p=0.3475, Figure 2 H). Interestingly, there was little to no colocalization of RGS14+ and GFP+ cells in both PTZ and control groups (colocalization percentage to RGS14: middle control: 0±0, n=11; middle PTZ: 0.236±0.076, n=8, unpaired t test, p<0.01; colocalization percentage to GFP: middle control: 0±0, n=11; middle PTZ: 2.850±0.923, n=8, unpaired t test, p<0.01, Figure 2 I-J). These results support the possible role of RGS14 in suppressing PTZ seizure-induced neuronal activation in the mature CA2.

We next examined the expression levels of RGS14 along the dorsal-ventral CA2 axis and found that there is a slight decrease in the level of RGS14+ cells from the dorsal CA2 to ventral CA2 structure in P60 control mice (dorsal control: 82.514±1.406, n=7; ventral control: 53.372±2.659, n=5, Figure 3 E). The distribution of RGS14+ cells between the dorsal, middle and ventral has a main effect (2-way ANOVA: F(2, 35)=81.10, n=41, p<0.0001, Figure 3 E). There is no effect of PTZ on RGS14 expression in all three areas, dorsal, middle and ventral (dorsal PTZ: 83±1.049, n=5; ventral PTZ: 52.572±1.975, n=5, 2-way ANOVA: F(1,35)=0.2337, n=41, p=0.6319, Figure 3 E). In addition, the colocalization between RGS14 and GFP remains to be low to none in the dorsal and ventral CA2 of P60 control and
PTZ groups (colocalization percentage to RGS14: dorsal control: 0±0, n=7; ventral control: 0±0, n=5; dorsal PTZ: 0.05±0.05, n=5; ventral PTZ: 0.426±0.215, n=5, Figure 3 G) (colocalization percentage to GFP: dorsal control: 0±0, n=7; ventral control: 0±0, n=5; dorsal PTZ: 0.9±0.9, n=5; ventral PTZ: 2.538±1.271, n=5, Figure 3 F). There is a change in the level of colocalization between P60 PTZ and P60 control groups in middle section of the CA2 only (2-way ANOVA followed by Sidak’s post doc test: colocalization percentage to RGS14: dorsal: n=12, p=0.2549; middle: n=19, p<0.01; ventral: n=10, p=0.0830, Figure 3 G) (2-way ANOVA followed by Sidak’s post doc test: colocalization percentage to GFP: dorsal: n=12, p=0.2549; middle: n=19, p<0.01; ventral: n=10, p=0.0808, Figure 3 F).

We also assessed the expression of RGS14 after the social recognition memory testing. The social behavior at P62 did not significantly alter the expression of RGS14 in both control and post-PTZ seizure mice (P62 control - dorsal: 81.920±0.540, n=5; middle: 69.167±0.909, n=6; ventral: 51.433±1.742, n=4 vs P62 behavior - dorsal: 83.8±0.515, n=5; middle: 70.280±2.075, n=5; ventral: 48.475±2.170, n=4, Figure 4 D2, E2 and F2) (P62 PTZ injected at P60 - dorsal: 87.275±1.324, n=4; middle: 74.043±1.553, n=7; ventral: 57.675±0.923, n=4 vs P62 PTZ injected at P60 and behavior - dorsal: 83.55±1.099, n=4; middle: 67.62±1.994, n=5; ventral: 51.988±1.076, n=6, Figure 4 D2, E2 and F2). Importantly, little colocalization between RGS14 and c-Fos was detected following the social behavioral testing (colocalization percentage to RGS14: P62 control - dorsal: 0.0±0.0, n=5; middle: 0.0±0.0, n=6; ventral: 0.0±0.0, n=4 - P62 behavior - dorsal: 0.268±0.191, n=5; middle: 0.746±0.325, n=5; ventral: 1.400±0.223, n=4 - P62 PTZ injected at P60 - dorsal: 0.0±0.0, n=4; middle: 0.0±0.0, n=7; ventral: 0.0±0.0, n=4 - P62 PTZ injected at P60 and behavior - dorsal: 0.390±0.190, n=4; middle: 0.490±0.218, n=5; ventral: 1.732±0.381, n=6, Figure 4 D4, E4 and F4) (colocalization percentage to c-Fos: P62 control - dorsal: 0.0±0.0, n=5; middle: 0.0±0.0, n=6; ventral: 0.0±0.0, n=4 - P62 behavior - dorsal: 1.014±0.702, n=5; middle: 2.752±1.195, n=5; ventral: 3.303±0.620, n=4 - P62 PTZ injected at P60 - dorsal: 0.0±0.0, n=4; middle: 0.0±0.0, n=7; ventral: 0.0±0.0, n=4 - P62 PTZ injected at P60 and behavior - dorsal: 1.618±0.678,
n=4; middle: 1.808±0.830, n=5; ventral: 2.988±0.537, n=6, Figure 4 D3, E3 and F3). These results indicate the potential of RGS14 negative cells in the social recognition memory.
Figure 2.
Figure 2. Acute seizures in adult mice did not affect the function of mature hippocampal CA2. Representative immunofluorescence microscope 2.5x magnification images showing GFP counterstained with DAPI following Saline (A) or PTZ (B) injected P60 mice. The percentage of GFP+ cells within the CA1, CA2 and CA3 regions of the control saline and PTZ seizure P60 groups (CA1 control: n=5; CA2 control: n=11; CA3 control: n=5) (CA1 PTZ: n=6; CA2 control: n=8; CA3 control: n=6) 2-way ANOVA ***p<0.05 (C). along with normalized GFP+ cell percentage to CA2 PTZ group within the CA1, CA2 and CA3 PTZ groups, 2-way ANOVA ***p<0.05 (D). Representative immunofluorescence microscope 10x magnification image showing RGS14 and GFP counterstained with DAPI following Saline (E1) with 40x magnification split RGB channel, RGS14 (E2), GFP (E3), DAPI (E4) and merged channel with RGS14, GFP and DAPI (E5). Representative immunofluorescence microscope 10x magnification image showing RGS14 and GFP counterstained with DAPI following PTZ (F1) with 40x magnification split RGB channel, RGS14 (F2), GFP (F3), DAPI (F4) and merged channel with RGS14, GFP and DAPI (F5). Male and Female distinction of GFP+ cell percentage between control saline and PTZ P60 groups (2-way ANOVA (F(1,15)=0.6764, n=19, p>0.05; F(1,15)=0.2287, n=19, p>0.05) (G). RGS14+ cell percentage between saline control (n=11) and PTZ (n=8) groups, unpaired t-test p>0.05 (H). Investigation of colocalization of GFP+ cells and RGS14+ cells against GFP+ cell percentage in control saline (n=11) and PTZ (n=8) groups, unpaired t-test **p<0.05 (I) along with colocalization of GFP+ cells and RGS14+ cells against RGS14+ cell percentage in control saline (n=11) and PTZ (n=8) groups, unpaired t-test P>0.05 (J). Diagram illustrating the timeline of PTZ administration along with behavior followed by immunohistochemistry procedure and three chamber social recognition memory test paradigm (K). Sociability result on average time interaction (sec) in control saline (n=8) and PTZ group (n=6), 2-way ANOVA-Sidak HSD ***p<0.05 (L). Social novelty result average time interaction (sec) in control saline (n=8), Sidak HSD ***p<0.05 and PTZ group (n=6), 2-way ANOVA-Sidak HSD *p<0.05 (M). Representative immunofluorescence microscope 40x magnification image showing RGS14, c-Fos, DAPI and overlay RGS14/ c-Fos/DAPI in P62 control, P62 behavior, P62 PTZ injected without behavior and P62 PTZ injected with behavior groups (N). c-Fos cell percentage in P62 control, P62 behavior, P62 PTZ injected without behavior and P62 PTZ injected with behavior groups (P62 control and P62 behavior, Sidak HSD, n=11, ***p<0.05) (P62 PTZ injected without behavior and P62 PTZ injected with behavior, 2-way ANOVA-Sidak HSD, n=12, ***p<0.05) (O).
Figure 3. Acute seizures in adult mice induced a small but significant increase in the activation of middle and ventral CA2 neurons. Illustrations representing dorsal (A1), middle (B1) and ventral (C1) hippocampal coronal slices. Representative immunofluorescence microscope 20x magnification images showing RGS14, GFP counterstained with DAPI in dorsal (A2), middle (B2) and ventral (C2) P60 control saline group. Representative immunofluorescence microscope 20x magnification images showing RGS14, GFP counterstained with DAPI in dorsal (A3), middle (B3) and ventral (C3) P60 PTZ group. GFP+ cell percentage in dorsal, middle, and ventral P60 control saline and PTZ groups (2-way ANOVA: F(2, 35)=26.47, n=41, ***p<0.05) (P60 dorsal: Sidak HSD, n=11, p>0.05) (P60 middle: Sidak HSD, n=19, ***p<0.05) (P60 ventral: Sidak HSD, n=10, *p<0.05) (D). RGS14+ cell percentage in dorsal, middle, and ventral P60 control and saline and PTZ groups (dorsal, middle and ventral – 2-way ANOVA: F(2, 35)=81.10, n=41, ***p<0.05) (control and PTZ – 2-way ANOVA: F(1,35)=0.2337, n=41, p>0.05) (E). Colocalization percentage of RGS14 and GFP when compared to GFP in dorsal (n=12), middle (n=19) and ventral (n=10) sections, 2-way ANOVA-Sidak HSD, **p<0.05 (F). Colocalization percentage of RGS14 and GFP when compared to RGS14 in dorsal (n=12), middle (n=19) and ventral (n=10) sections, 2-way ANOVA-Sidak HSD, p>0.05 (G).
Figure 4.
Figure 4. PTZ-induced seizure did not diminish dorsal, middle and ventral CA2 function during social behavior. Illustrations representing dorsal (A1), middle (B1) and ventral (C1) hippocampal coronal slices. Representative immunofluorescence microscope 20x magnification images showing RGS14, c-Fos counterstained with DAPI in dorsal (A2), middle (B2) and ventral (C2) P62 control saline group. Dorsal (A3), middle (B3) and ventral (C3) P62 behavior group. Dorsal (A4), middle (B4) and ventral (C4) P62 PTZ group. Dorsal (A5), middle (B5) and ventral (C5) P62 PTZ+ behavior group. C-Fos+ cell percentage in dorsal P62 control saline (n=5), P62 behavior (n=5), P62 PTZ (n=4) and P62 PTZ+behavior (n=4) groups, one-way ANOVA-Tukey HSD, ***p<0.05 (D1). RGS14+ cell percentage in dorsal P62 control saline (n=5), P62 behavior (n=5), P62 PTZ (n=4) and P62 PTZ+behavior (n=4) groups, one-way ANOVA-Tukey HSD, p>0.05 (D2). Colocalization percentage of RGS14 and c-Fos when compared to c-Fos in dorsal P62 control saline (n=5), P62 behavior (n=5), P62 PTZ (n=4) and P62 PTZ+behavior (n=4) groups, one-way ANOVA-Tukey HSD, p>0.05 (D3). Colocalization percentage of RGS14 and c-Fos when compared to RGS14 in dorsal P62 control saline (n=5), P62 behavior (n=5), P62 PTZ (n=4) and P62 PTZ+behavior (n=4) groups, one-way ANOVA-Tukey HSD, p>0.05 (D4). C-Fos+ cell percentage in middle P62 control saline (n=6), P62 behavior (n=5), P62 PTZ (n=7) and P62 PTZ+behavior (n=5) groups, one-way ANOVA-Tukey HSD, ***p<0.05 (E1). RGS14+ cell percentage in middle P62 control saline (n=6), P62 behavior (n=5), P62 PTZ (n=7) and P62 PTZ+behavior (n=5) groups, one-way ANOVA-Tukey HSD, p>0.05 (E2). Colocalization percentage of RGS14 and c-Fos when compared to c-Fos in middle P62 control saline (n=6), P62 behavior (n=5), P62 PTZ(n=7) and P62 PTZ+behavior (n=5) groups, one-way ANOVA-Tukey HSD, *p<0.05 (E3). Colocalization percentage of RGS14 and c-Fos when compared to RGS14 in middle P62 control saline (n=6), P62 behavior (n=5), P62 PTZ (n=7) and P62 PTZ+behavior (n=5) groups, one-way ANOVA-Tukey HSD, *p<0.05 (E4). C-Fos+ cell percentage in ventral P62 control saline (n=4), P62 behavior (n=4), P62 PTZ (n=4) and P62 PTZ+behavior (n=6) groups, one-way ANOVA-Tukey HSD, ***p<0.05 (F1). RGS14+ cell percentage in ventral P62 control saline (n=4), P62 behavior (n=4), P62 PTZ (n=4) and P62 PTZ+behavior (n=6) groups, one-way ANOVA-Tukey HSD, p>0.05 (F2). Colocalization percentage of RGS14 and c-Fos when compared to c-Fos in ventral P62 control saline (n=4), P62 behavior (n=4), P62 PTZ (n=4) and P62 PTZ+behavior (n=6) groups, one-way ANOVA-Tukey HSD, **p<0.05 (F3). Colocalization percentage of RGS14 and c-Fos when compared to RGS14 in ventral P62 control saline (n=4), P62 behavior (n=4), P62 PTZ (n=4) and P62 PTZ+behavior (n=6) groups, one-way ANOVA-Tukey HSD, **p<0.05 (F4).
4.2.2 Expression of RGS14 in CA2 neurons is developmentally regulated

Interestingly, we found that the expression of RGS14 is developmentally regulated as indicated by a lower level of RGS14+ cells in the immature CA2 at P10 (middle CA2: 34.954±1.030%, n=5) compared to the mature CA2 at P60 (middle CA2: 66.088±1.988%, n=8, unpaired t test, p<0.0001, Figure 5 C). Given the critical role of RGS14 in inhibiting synaptic plasticity, we then hypothesized that the immature CA2 is vulnerable to seizures when compared to the mature CA2.

4.3 PTZ-induced early-life seizures selectively enhanced the excitability of seizure-sensitive CA2 neurons and impaired social recognition memory in immature mice

To investigate the effects of early life seizures on the immature CA2, we induced seizures in P10 c-Fos-GFP based transgenic mice using a single dose of PTZ (55mg/kg, i.p.). Each mouse included for further analysis had reached stages 4 and 5 seizures on the Racine scale (Table 1).

4.3.1 Early life seizures significantly activated CA2 pyramidal neurons

Using the GFP antibody we were able to identify selectively activated CA2 cells. We found that the level of GFP+ cells are significantly greater in post-PTZ seizure mice (middle: 21.108±0.784%, n=10) than the littermate saline control mice (middle CA2: 2.969±0.307, n=8, unpaired t test, p<0.0001, Figure 5 G). No significant sex difference was identified in PTZ seizure induced CA2 neuron activation (Sidak HSD: p=0.5600). We therefore pooled the following data from male and female mice (Figure 5 G). In addition to the middle CA2 structure, the dorsal and ventral CA2 structures also showed an increased level of GFP+ cells in the PTZ group (dorsal: 15.386±0.408, n=7; ventral: 23.062±2.100, n=5, Figure 6 D) compared to control saline group (dorsal: 2.325±0.694, n=4, one-way ANOVA followed my Tukey’s post doc test: p<0.0001; ventral: 8.560±1.139, n=5, one-way ANOVA followed my Tukey’s
post doc test: p<0.001, Figure 6 D). These results support that the immature CA2 is vulnerable to early seizures.

4.3.2 Level of RGS14 is negatively correlated to early seizure-induced neuronal activation in the immature CA2

We next investigated the level of RGS14+ cells within the dorsal, middle and ventral CA2 structures in P10 control and PTZ groups. We found a decrease in RGS14 expression along the CA2 dorsal-ventral axis (P10 control - dorsal: 52.525±1.009, n=4; middle: 35.852±0.953, n=9; ventral: 12.102±1.084, n=5, Figure 6 E), which is not significantly affected by PTZ seizures in dorsal, middle and ventral CA2 (P10 PTZ - dorsal: 52.023±1.711, n=7, one-way ANOVA followed my Tukey’s post doc test: p=0.9932; middle: 34.239±1.333, n=9, one-way ANOVA followed my Tukey’s post doc test: p=0.6701; ventral: 16.064±0.710, n=5, p=0.1189, Figure 6 E). Interestingly, the decrease in RGS14 expression along the CA2 dorsal-ventral axis was associated with the increase in the level of CA2 neuronal activation (Figure 6 D and E) as confirmed by a negative correlation between the level of RGS14 expression and the number of GFP+ cells (Figure 5 F).

We next examined the colocalization of GFP+ and RGS14+ in both PTZ and control groups and found little colocalization of RGS14 and GFP (colocalization percentage to RGS14 - dorsal control: 0±0, n=4; middle control: 0±0, n=8; ventral control: 0±0, n=5; dorsal PTZ: 0.064±0.064, n=7; middle PTZ: 0.698±0.380, n=10; ventral PTZ: 0.820±0.508, n=5, Figure 6 G) (colocalization percentage to GFP - dorsal control: 0±0, n=4; middle control: 0±0, n=8; ventral control: 0±0, n=5; dorsal PTZ: 0.2±0.2, n=7; middle PTZ: 1.387±0.650, n=10; ventral PTZ: 0.720±0.480, n=5, Figure 6 F). There is no significant difference in the level of colocalization between PTZ and control in P10 animals (colocalization percentage to RGS14 – Tukey HSD: dorsal: p=0.4789; middle: p=0.1218; ventral: p=0.1453, Figure 6
G) (colocalization percentage to GFP – Tukey HSD: dorsal: p=0.4789; middle: p=0.0765; ventral: p=0.1720, Figure 6 F).

4.3.3 Early seizures impaired social behaviors in immature mice

Given early seizures significantly activated CA2 pyramidal neurons and CA2 neurons are essential for social recognition memory, we next investigate whether early seizures impair social behaviors in the immature mice. To achieve this aim, we used an established the preweaning two-choice social recognition memory test in P12 post-seizure pups that received a PTZ injection at P10 or P12 control mice that received a saline injection at P10. We found that P12 control pups showed a significant preference for the caregiving mother (345.053±50.727 sec, n=19) over a novel mother (72.789±36.099 sec, n=19, 2-way ANOVA followed by Sidak’s post doc test: p<0.0001, Figure 5 M). In contrast, P12 post-seizure pups lost the preference for the caregiving mother (102.727±38.810 sec, n=11) over a novel mother (143.727±56.548 sec, n=11, 2-way ANOVA followed by Sidak’s post doc test: p=0.8386, Figure 5 M). In addition, the total time socializing was significantly reduced in P12 post-seizure pups (246.455±67.528 sec, n=11, unpaired t test: p=0.036, Figure 5 N) compared with P12 control mice (417.842±44.764 sec, n=19). The social time difference between the caregiving mother and novel mother in the control group was (272.263±75.822, n=19) and the PTZ group was (41±69.625, n=11), there was also a significant difference between the control and PTZ group when comparing their social time difference (unpaired t-test: n=30, p<0.01, Figure 5 O).

After the behavior testing the P12 pups in both groups were perfused an hour later for c-Fos staining to determine the involvement of CA2 neurons in the social behavior. First, we found that middle CA2 from P12 post seizure mice at baseline (without behavior) showed significantly higher number of c-Fos+ cells than P12 control mice without behaviors (one-way ANOVA followed by Tukey’s post doc test: p<0.01, Figure 7 E1), while no significant differences were identified in both the dorsal (one-way
ANOVA followed by Tukey’s post doc test: p=0.2232, Figure 7 D1) and ventral CA2 (one-way ANOVA followed by Tukey’s post doc test: p=0.4076, Figure 7 F1).

We next identified that P12 control pups that have gone through the behavior test showed a significant increase in c-Fos+ cells (P12 control post-behavior middle: 15.001±1.181%, n=10), compared to control pups that have not gone through behavior (P12 control middle: 1.868±0.338%, n=4, two-way ANOVA followed by Sidak post doc test: p<0.0001, Figure 7 E1), indicating a direct involvement of CA2 in the social behavior. In contrast, P12 post-seizure pups that have gone through behavior testing showed no significant changes in the level of c-Fos+ cells (P12 PTZ+behavior middle: 4.433±0.391%, n=6) when compared to P12 post-seizure pups with no behavior testing (P12 PTZ only middle: 5.640±0.659%, n=5, two-way ANOVA followed by Sidak post doc test: p=0.1353, Figure 7 E1).

Assessment of c-Fos+ cells after behavior testing demonstrated a similar pattern of c-Fos+ cells in the dorsal CA2 (P12 control pups with behavior testing 13.599±1.478%, n=10 vs P12 control pups without behavior testing 2.060±0.633%, n=4, one-way ANOVA followed by Tukey’s post doc test: p<0.001; P12 post-PTZ seizure pups with behavior testing 3.600±0.698%, n=4 vs P12 post-PTZ seizure pups without behavior testing 3.625±0.963%, n=4, one-way ANOVA followed by Tukey’s post doc test: p=0.9839, Figure 7 D1), and ventral CA2 (P12 control pups with behavior testing 17.849±1.949%, n=9 vs P12 control pups without behavior testing 1.943±0.676%, n=4, one-way ANOVA followed by Tukey’s post doc test: p<0.001; P12 post-PTZ seizure pups with behavior testing 3.220±0.971%, n=9; and P12 post seizure - dorsal: 67.035±0.998%, n=4; middle: 56.020±2.196%, n=5; ventral:
39.125±1.951%, n=4 vs P12 post seizure with behavior - dorsal: 66.913±2.357, n=4; middle: 52.560±0.679, n=6; ventral: 37.120±2.723, n=5, one-way ANOVA, p>0.05, Figure 7 D2, E2 and F2), and the little colocalization of RGS14 and c-Fos at P12 following behavioral testing (colocalization percentage to RGS14: P12 control – dorsal: 0±0, n=4; middle: 0±0, n=4; ventral control: 0±0, n=4; P12 behavior-dorsal: 0.498±0.203, n=10; middle:1.789±1.289, n=10; ventral PTZ: 1.106±0.437, n=9; P12 PTZ injected at P10 - dorsal: 0.0±0.0, n=4; middle: 0.0±0.0, n=5; ventral: 0.0±0.0, n=4; P12 PTZ injected at P10 and behavior - dorsal: 0.0±0.0, n=4; middle: 0.158±0.158, n=6; ventral: 0.0±0.0, n=4, Figure 7 D4, E4 and F4) (colocalization percentage to c-Fos: P12 control – dorsal: 0±0, n=4; middle: 0±0, n=4; ventral control: 0±0, n=4; P12 behavior-dorsal: 2.279±0.834, n=10; middle: 2.169±0.707, n=10; ventral: 2.429±0.878, n=9; P12 PTZ injected at P10 - dorsal: 0.0±0.0, n=4; middle: 0.0±0.0, n=5; ventral: 0.0±0.0, n=4; P12 PTZ injected at P10 and behavior - dorsal: 0.0±0.0, n=4; middle: 1.5±1.5, n=6; ventral: 0.0±0.0, n=4, Figure 7 D3, E3 and F3). There is no change in the level of colocalization between P12 PTZ injected at P10, and P12 PTZ injected at P10 with behavior (colocalization percentage to RGS14 – one-way ANOVA followed by Tukey’s post doc test: dorsal: n=8, p>0.05; middle: n=11, p=0.3892; ventral: n=8, p>0.05) (colocalization percentage to c-Fos - one-way ANOVA followed by Tukey’s post doc test: dorsal: n=8, p>0.05; middle: n=11, p=0.4468; ventral: n=8, p>0.05). (Figure 7). These data highly suggest that the function of CA2 neurons that are critically involved in social behaviors is impaired after early life seizures.
Figure 5.
Figure 5. PTZ-induced early-life seizures selectively enhanced the excitability of seizure-sensitive CA2 neurons and impaired social recognition memory in immature mice. Representative immunofluorescence microscope 20x magnification images showing RGS14, GFP counterstained with DAPI in P10 hippocampal mouse slice (A) and P60 hippocampal mouse slice (B). Percentage of RGS14+ cells between P10 (n=5) and P60 (n=8) animal groups, unpaired t-test, ***p<0.05 (C). Representative immunofluorescence microscope 20x magnification images in P10 control group showing RGS14 (D1), GFP (D2), DAPI (D3) and overlay RGS14/GFP/DAPI (D4) along with 40x magnification image overlay RGS14/GFP/DAPI in the CA2 region (D5). Representative immunofluorescence microscope 20x magnification images in P10 PTZ seizure group showing RGS14 (E1), GFP (E2), DAPI (E3) and overlay RGS14/GFP/DAPI (E4) along with 40x magnification image overlay RGS14/GFP/DAPI in the CA2 region (E5). Distribution of GFP percentage to RGS14 percentage in P10 (n=7) and P60 (n=8) animals, linear regression, unpaired t-test, ***p<0.05 (F). Male and Female distinction of GFP+ cell percentage between control saline and PTZ P10 groups (2-way ANOVA: F(1,14)=0.034, n=19, p<0.05; F(1,14)=337.5, n=19, ***p<0.05) (G). RGS14+ cell percentage between saline control (n=9) and PTZ (n=9) groups, unpaired t-test, p>0.05 (H). Investigation of colocalization of GFP+ cells and RGS14+ cells against GFP+ cell percentage in control saline (n=8) and PTZ (n=10) groups, unpaired t-test, p>0.05 (I) along with colocalization of GFP+ cells and RGS14+ cells against RGS14+ cell percentage in control saline (n=8) and PTZ (n=10) groups, unpaired t-test, p>0.05 (J). Diagram illustrating the timeline of PTZ administration along with behavior followed by immunohistochemistry procedure (K) and two-choice social recognition paradigm (L). Time spent socializing between the caregiving mother and novel mother in P12 control saline (n=19) and P12 PTZ seizure (n=11) groups, 2-way ANOVA – Sidak HSD, ***p<0.05 (M). Total social time between P12 control saline and P12 PTZ seizure groups, unpaired t-test, *p<0.05 (N). Time difference socializing between caregiving mother and novel mother in P12 control saline and P12 PTZ seizure groups, unpaired t-test, ***p<0.05 (O). Representative immunofluorescence microscope 40x magnification image showing RGS14, c-Fos, DAPI and overlay RGS14/ c-Fos/DAPI in P12 control, P12 behavior, P12 PTZ injected without behavior and P12 PTZ injected with behavior groups (P). c-Fos cell percentage in P12 control (n=4), P12 behavior (n=10), P12 PTZ injected without behavior (n=5) and P12 PTZ injected with behavior (n=6) groups, 2-way ANOVA-Sidak HSD, ***p<0.05 (Q). RGS14 cell percentage in P12 control, P12 behavior, P12 PTZ injected without behavior and P12 PTZ injected with behavior groups, 2-way ANOVA-Sidak HSD, p>0.05 (R).
Figure 6. Early life seizures significantly activated dorsal, middle and ventral CA2 pyramidal neurons. Illustrations representing dorsal (A1), middle (B1) and ventral (C1) hippocampal coronal slices. Representative immunofluorescence microscope 20x magnification images showing RGS14, GFP counterstained with DAPI in dorsal (A2), middle (B2) and ventral (C2) P10 control saline group. Representative immunofluorescence microscope 20x magnification images showing RGS14, GFP counterstained with DAPI in dorsal (A3), middle (B3) and ventral (C3) P10 PTZ group. GFP+ cell percentage in dorsal (n=4,7), middle (n=8,10), and ventral (n=5,5) P10 control saline and PTZ groups, 2-way ANOVA-Sidak HSD, ***p<0.05 (D). RGS14+ cell percentage in dorsal (n=4,7), middle, and ventral P10 control saline and PTZ groups, 2-way ANOVA-Sidak HSD, p>0.05 (E). Colocalization percentage of RGS14 and GFP when compared to GFP in dorsal (n=4,7), middle (n=8,10), and ventral (n=5,5) P10 control saline and PTZ groups, 2-way ANOVA-Sidak HSD, *p<0.05 (F). Colocalization percentage of RGS14 and GFP when compared to RGS14 in dorsal (n=4,7), middle (n=8,10), and ventral (n=5,5) P10 control saline and PTZ groups, 2-way ANOVA-Sidak HSD, p>0.05 (G).
Figure 7.

[Image of cellular and histological sections with graphs showing c-Fos expression and DAPI staining across different brain regions and conditions.]
Figure 7. PTZ-induced early seizure diminished dorsal, middle and ventral CA2 function during social behavior. Illustrations representing dorsal (A1), middle (B1) and ventral (C1) hippocampal coronal slices. Representative immunofluorescence microscope 20x magnification images showing RGS14, c-Fos counterstained with DAPI in dorsal (A2), middle (B2) and ventral (C2) P12 control saline group. Dorsal (A3), middle (B3) and ventral (C3) P12 behavior group. Dorsal (A4), middle (B4) and ventral (C4) P61 PTZ group. Dorsal (A5), middle (B5) and ventral (C5) P12 PTZ+ behavior group. c-Fos+ cell percentage in dorsal P12 control saline, P12 behavior, P12 PTZ and P12 PTZ+behavior groups, one-way ANOVA-Tukey HSD, ***p<0.05 (D1). RGS14+ cell percentage in dorsal P12 control saline, P12 behavior, P12 PTZ and P12 PTZ+behavior groups, one-way ANOVA-Tukey HSD, p>0.05 (D2). Colocalization percentage of RGS14 and c-Fos when compared to c-Fos in dorsal P12 control saline, P12 behavior, P12 PTZ and P12 PTZ+behavior groups, one-way ANOVA-Tukey HSD, p>0.05 (D3). Colocalization percentage of RGS14 and c-Fos when compared to RGS14 in dorsal P12 control saline, P12 behavior, P12 PTZ and P12 PTZ+behavior groups, one-way ANOVA-Tukey HSD, p>0.05 (D4). C-Fos+ cell percentage in middle P12 control saline, P12 behavior, P12 PTZ and P12 PTZ+behavior groups, one-way ANOVA-Tukey HSD, ***p<0.05 (E1). RGS14+ cell percentage in middle P12 control saline, P12 behavior, P12 PTZ and P12 PTZ+behavior groups, one-way ANOVA-Tukey HSD, p>0.05 (E2). Colocalization percentage of RGS14 and c-Fos when compared to c-Fos in middle P12 control saline, P12 behavior, P12 PTZ and P12 PTZ+behavior groups, one-way ANOVA-Tukey HSD, p>0.05 (E3). Colocalization percentage of RGS14 and c-Fos when compared to RGS14 in middle P12 control saline, P12 behavior, P12 PTZ and P12 PTZ+behavior groups, one-way ANOVA-Tukey HSD, p>0.05 (E4). C-Fos+ cell percentage in ventral P12 control saline, P12 behavior, P12 PTZ and P12 PTZ+behavior groups, one-way ANOVA-Tukey HSD, ***p<0.05 (F1). RGS14+ cell percentage in ventral P12 control saline, P12 behavior, P12 PTZ and P12 PTZ+behavior groups, one-way ANOVA-Tukey HSD, p>0.05 (F2). Colocalization percentage of RGS14 and c-Fos when compared to c-Fos in ventral P12 control saline, P12 behavior, P12 PTZ and P12 PTZ+behavior groups, one-way ANOVA-Tukey HSD, p>0.05 (F3). Colocalization percentage of RGS14 and c-Fos when compared to RGS14 in ventral P12 control saline, P12 behavior, P12 PTZ and P12 PTZ+behavior groups, one-way ANOVA-Tukey HSD, p>0.05 (F4).
4.3.4 Early life seizures cause functional changes in immature CA2 pyramidal cells

Given that early life seizures can cause social recognition memory deficits as well as an impairment in CA2 neuronal function during the behavior testing, we next set out to determine whether these behavioural deficits in social recognition are associated with changes in the synaptic function of CA2 neurons.

4.3.4.1 Early life seizure induced an enhancement of AMPA receptor function in CA2 pyramidal neurons at P10

Whole-cell patch clamp recordings of spontaneous AMPAR-mediated EPSCs in randomly selected CA2 pyramidal neurons were made in P10 mouse hippocampal slices removed 1h after PTZ seizures and control mouse pups. We found that slices from PTZ pups showed a significant increase in the frequency of AMPAR sEPSCs (0.0614 ± 0.0113 Hz; n = 18; unpaired t test, two-tailed, equal variance, p=0.0242) compared with hippocampal slices from control pups (0.0323 ± 0.0066 Hz; n = 14; Figure 8 B). However, we found no significant differences of AMPAR sEPSC amplitude, rise time and decay time in PTZ mice compared to control mice (AMPAR sEPSC amplitude: 13.43 ± 1.61 pA, n=18 vs 12.93 ± 1.15 pA, n=14, unpaired t test, two-tailed, equal variance, p = 0.920, Figure 8 C; rising time: 4.29 ± 0.48ms, n=18, vs 4.08 ± 0.50ms, n=14, unpaired t test, two-tailed, equal variance, p = 0.848; decay time: 11.41 ± 0.98ms, n=18 vs 12.40 ± 1.74ms, n=14, unpaired t test, two-tailed, equal variance, p = 0.789, Figure 8 D-E). These data suggest early life seizures induced acute enhancement of AMPAR function in CA2 pyramidal neurons.
4.3.4.2 Early-life seizures induced AMPAR function enhancement was through a presynaptic mechanism

The increases in the frequency of AMPAR sEPSCs in CA2 pyramidal neurons in P10 mice could be due to a presynaptic or postsynaptic change.

To determine if postsynaptic changes occurred in CA2 neurons following early life seizures, we first investigated the amount of silent synapses in CA2 neurons. At this age, most synapses are silent, meaning that they are inactive or immature because they lack functional AMPARs. As the brain matures, AMPARs are integrated at the synapse marking these synapses active and mature. To calculate the number of silent synapses, the stimulation intensity is adjusted until AMPAR-mediated eEPSCs are evoked 50% of the time at -60mV holding. The membrane potential is then increased to +40mV holding and with the same stimulation intensity, the cell is stimulated to evoke responses from both AMPA and NMDA receptors. The fraction of silent synapse was calculated using the difference of failure rates at -60 mV and +40mV holding potentials (Liao et al., 1995). Here, we aimed to investigate if the enhanced AMPAR activity in the CA2 after PTZ in p10 pups is associated with a postsynaptic change by measuring silent synapses in the hippocampal CA2.

We first investigated the Schaffer collateral pathway of the CA2. P10 control mice showed a failure rate of 35.88 ± 5.68% at -60mV and 16.38± 5.36 % at +40mV (n=7, paired t test, p<0.01; Figure 8 L), generating a failure rate difference of 19.50 ± 4.78 % (n=7; Figure 8 L) in hippocampal CA2 pyramidal cells. Hippocampal slices from PTZ pup mice in CA2 pyramidal cells exhibited a failure rate of 42.25 ± 3.80% at -60mV and 33.07 ± 3.53% at +40mV (n=9, unpaired t test, p=0.066; Figure 8 M), resulting in a smaller difference of 10.74 ± 3.44 % (n=9, unpaired t test, two-tailed, equal variance, p=0.176) compared with slices form control mice (Figure 8 N). Furthermore, the fraction of NMDAR-only silent synapses in slices from PTZ mice was lower (23.51 ± 6.30%, n=9, unpaired t test, two-tailed, equal variance, p=0.054) compared with that of slices from control mice (48.64 ± 9.75%, n=7, Figure 8...
As we didn’t observe a significant change in the fraction of silent synapses in the Schaffer Collateral pathway, we next determine if these changes are observed in the Perforant pathway. P10 control mice showed a failure rate of 56.46 ± 13.95% at -60mV and 45.00 ± 11.77% at +40mV (n=4, unpaired t test, p=0.173; Figure 8 Q), generating a failure rate difference of 11.61 ± 4.87% (n=4; Figure 8 Q) in hippocampal CA2 pyramidal cells. Hippocampal slices from PTZ pup mice in CA2 pyramidal cells exhibited a failure rate of 54.52 ± 5.43% at -60mV and 46.97 ± 4.36% at +40mV (n = 8, paired t test, p=0.066; Figure 8 R), resulting in a difference of 8.33 ± 2.89 % (n=8, unpaired t test, two-tailed, equal variance, p=0.597) compared with slices form control mice (Figure 8 S). Finally, the fraction of NMDAR-only silent synapses in slices from PTZ mice was (21.22 ± 6.87%, n=8, unpaired t test, two-tailed, equal variance, p=0.753) compared with that of slices from control mice (25.47 ± 9.79%, n=4, Figure 8 T). These data suggest that early seizures did not change the postsynaptic silent synapses in either the Schaffer collateral or Perforant pathways.

We next proceeded with investigating whether a presynaptic change occurred at either of either the Schaffer collateral or Perforant pathways. To investigate presynaptic changes, we first measured the paired pulse facilitation in CA2 pyramidal neurons while stimulating the Schaffer collateral pathway. Here, we observed no change in the paired pulse ratio in slice from PTZ mice (1.89 ± 0.18; n = 9; unpaired t test, two-tailed, equal variance, p=0.057) compared with hippocampal slices from control pups (1.29±0.10; n = 11; Figure 8 H). We then investigated the Perforant pathway-mediated presynaptic changes, as the Perforant pathway has been shown to be implicated in social recognition (Chevaleyre et al., 2010; Ding and Van Hoesen, 2010; Kohara et al., 2014; Zhao et al., 2007). Indeed, we observed a significant decrease in the paired pulse ratio in hippocampal slices from PTZ mice (1.70 ± 0.08; n = 24; unpaired t test, two-tailed, equal variance, p <0.05) compared with control pups (1.99 ± 0.10; n = 19; Figure 8 J). These results indicate that the increases in the frequency of AMPAR-sEPSCs in the immature CA2 is mediated by the presynaptic changes in the Perforant pathway.
4.3.4.3 **Persistent early seizure-induced AMPAR function enhancement in CA2 neurons was observed in P12 mice**

Next, we wanted to verify if the change in AMPAR-mediated sEPSC frequency observed in P10 mice after PTZ seizures remains at P12. We induced PTZ seizures in P10 pups and recorded spontaneous AMPAR sEPSCs in CA2 pyramidal neurons at P12. Here, we found that P12 PTZ pups showed a significant increase in the frequency of AMPAR sEPSCs (0.097 ± 0.008 Hz; n = 8; unpaired t test, two-tailed, equal variance, p<0.001) compared with hippocampal slices from control P12 pups (0.040 ± 0.006 Hz; n = 8; Figure 8 V). Again, we found no significant differences of AMPAR sEPSC amplitude, rise time and decay time in P12 PTZ mice compared to control pups (AMPAR sEPSC amplitude: 18.66 ± 2.26pA, n=8 vs 16.68 ± 1.21pA, n=8, unpaired t test, two-tailed, equal variance, p = 0.482, Figure 8 W; rising time: 3.75 ± 0.24ms, n=8, vs 4.03 ± 0.42ms, n=8, unpaired t test, two-tailed, equal variance, p = 0.589; decay time: 11.75 ± 1.42ms, n=8 vs 10.28 ± 1.41ms, n=8, unpaired t test, two-tailed, equal variance, p = 0.50, Figure 8 X-Y). Therefore, these results indicate a persistent hyperexcitability of CA2 neurons following early life seizures at P10, which might account for the early seizure-induced social behavior deficits.
Figure 8.
Figure 8. Early life seizure induced an enhancement of AMPA receptor function in CA2 pyramidal neurons at P10 and Persistent enhancement at P12. Representative AMPAR-mediated sEPSCs in CA2 pyramidal neuron from P10 Control (upper traces) and PTZ (lower traces) mice (A). Group data of AMPAR-mediated sEPSC frequency in CA2 pyramidal neurons from Control and PTZ mice. n=14, 18, unpaired t test, *p<0.05. Error bars indicate S.E.M (B). Group data of AMPAR-mediated sEPSC amplitude in CA2 pyramidal neurons from Control and PTZ mice. n=14, 18, unpaired t test, p>0.05. Error bars indicate S.E.M (C). Group data of AMPAR-mediated sEPSC. (D) rise time and (E) decay time in CA2 pyramidal neurons from Control and PTZ mice. n=14, 18, unpaired t test, p>0.05. Error bars indicate S.E.M (D-E). Representative traces of paired pulse evoked AMPAR EPSCs at a holding potential of -60 mV in CA2 pyramidal neurons by stimulating Schaffer collateral pathway in hippocampal slices from control and PTZ mice. The pulse interval was 50ms (F-G). Group data of paired-pulse facilitation in CA2 pyramidal neurons from control and PTZ mice; n 11, 8; unpaired t test was used. unpaired t test, p=0.05. Error bars indicate S.E.M. (H). Representative traces of paired pulse evoked AMPAR EPSCs at a holding potential of -60 mV in CA2 pyramidal neurons by stimulating Perforant pathway in hippocampal slices from control and PTZ mice. The pulse interval was 50ms (I). Group data of paired-pulse facilitation in CA2 pyramidal neurons from control and PTZ mice; n 24, 19; unpaired t test was used. unpaired t test, *p<0.05. Error bars indicate S.E.M. (J). Representative evoked EPSC recordings at holding potentials of +40 (upper trace) and -60mV (bottom trace) in CA2 pyramidal neurons from Control p10 pups in the Schaffer Collateral Pathway. Representative evoked EPSC recordings at holding potentials of +40 (upper trace) and -60mV (bottom trace) in CA2 pyramidal neurons from PTZ pups at p10 pups in the Schaffer Collateral Pathway (K). Group data of eEPSC failure rates at -60 and +40mV holding potentials in CA2 pyramidal neurons in the Schaffer Collateral Pathway from control mice. n=7, paired t test, *p<0.05, Error bars indicate S.E.M. (L). Group data of eEPSC failure rates at -60 and +40mV holding potentials in CA2 pyramidal neurons in the Schaffer Collateral Pathway from PTZ pup mice. n=9, paired t test, p>0.05, Error bars indicate S.E.M. (M). Group data of eEPSC failure rate difference between -60 and +40mV holding potentials in CA2 pyramidal neurons in the Schaffer Collateral Pathway from control and PTZ pup mice. n=7, 9, unpaired t test, p>0.05, Error bars indicate S.E.M. (N). Group data of the calculated fraction of silent synapses in CA2 pyramidal neurons in the Schaffer Collateral Pathway from control and PTZ pup mice. n=7, 9, unpaired t test, p>0.05, Error bars indicate S.E.M. (O). Representative evoked EPSC recordings at holding potentials of +40 (upper trace) and -60mV (bottom trace) in CA2 pyramidal neurons from Control p10 pups in the Perforant Pathway. Representative evoked EPSC recordings at holding potentials of +40 (upper trace) and -60mV (bottom trace) in CA2 pyramidal neurons from PTZ pups at p10 in the Perforant Pathway (P). Group data of eEPSC failure rates at -60 and +40mV holding potentials in CA2 pyramidal neurons in the Perforant Pathway from control mice. n=4, paired t test, p>0.05, Error bars indicate S.E.M. (Q). Group data of eEPSC failure rates at -60 and +40mV holding potentials in CA2 pyramidal neurons in the Perforant Pathway from PTZ mouse. n=8, paired t test, p>0.05, Error bars indicate S.E.M. (R). Group data of eEPSC failure rate difference between -60 and +40mV holding potentials in CA2 pyramidal neurons in the Perforant Pathway from control and PTZ pup mice. n=4, 8, unpaired t test, p>0.05, Error bars indicate S.E.M. (S). Group data of the calculated fraction of silent synapses in CA2 pyramidal neurons in the Perforant Pathway from control and PTZ pup mice. n=4, 8, unpaired t test, p>0.05, Error bars indicate S.E.M. (T). Representative AMPAR-mediated sEPSCs in CA2 pyramidal neuron from P12 Control (upper traces) and PTZ (lower traces) mice (U). Group data of AMPAR-mediated sEPSC frequency in CA2 pyramidal neurons from Control and PTZ mice. n=8, 8, unpaired t test, ***p<0.001. Error bars indicate S.E.M. (V). Group data of AMPAR-mediated sEPSC amplitude in CA2 pyramidal neurons from Control and PTZ mice. n=8, 8, unpaired t test, p>0.05. Error bars indicate S.E.M. (W). Group data of AMPAR-mediated sEPSC rise time and decay time in CA2 pyramidal neurons from Control and PTZ mice. n=8, 8, unpaired t test, p>0.05. Error bars indicate S.E.M. (X-Y).
4.3.5 **NBQX treatment reverses early seizure-induced social recognition memory deficits**

So far, our results have demonstrated an acutely initiation and persistent enhancement of AMPAR function in CA2 neurons after PTZ induced seizures in P10. We then determine whether pharmacological suppression of AMPAR function in vivo by an AMPA receptor antagonist NBQX could rescue early seizure-induced social behavior deficits.

**4.3.5.1 NBQX treatment rescued early seizure-induced social recognition memory deficits**

Immediately after PTZ seizures at P10, the mouse pups were treated with a single dose of NBQX (20mg/kg) or vehicle. We then tested the social behavior two days after treatment at P12. Our results showed that mice that received PTZ and then NBQX were able to identify and show a significant preference for the caregiving mother (446.143±50.241 sec, n=14) over a novel mother (33.357±13.742 sec, n=14, 2-way ANOVA followed by Sidak’s post doc test: p<0.0001, Figure 9 G). In addition, mice that received PTZ and then NBQX showed a significantly greater socializing time (479.5±40.469, n=14) than mice that received PTZ only (246.455±67.528, n=11, unpaired t-test: p=0.005, Figure 9 H). The social time difference between the caregiving mother and novel mother was significantly longer in the PTZ+NBQX group (414.429±61.683, n=14) than the PTZ only group (41±69.625, n=11, p<0.0001, Figure 9 I). Therefore, NBQX treatment rescued the social deficits caused by PTZ seizures.

**4.3.5.2 NBQX treatment reversed early seizure-induced enhancement of AMPAR function in CA2 pyramidal neurons**

We next determined whether NBQX treatment could reverse PTZ seizure-induced persistent enhancement of AMPAR function in CA2 pyramidal neurons. We found that hippocampal slices from the P12 PTZ+NBQX mice showed a significant decrease in the frequency of AMPAR sEPSCs (0.023±0.004 Hz; n =10) compared with P12 PTZ only mice (0.097±0.008 Hz; n =8; unpaired t test, two-
tailed, equal variance, p<0.001, Figure 9 C). We found no significant differences of AMPAR sEPSC amplitude, rise time and decay time in p12 PTZ mice compared to PTZ+NBQX pups (AMPAR sEPSC amplitude: 18.66±2.26pA, n=8 vs 13.59±0.66pA, n=8, unpaired t test, two-tailed, equal variance, p<0.05, Figure 9; rising time: 3.74±0.24ms, n=8, vs 3.86±0.35ms, n=10, unpaired t test, two-tailed, equal variance, p=0.819; decay time: 11.75±1.42ms, n=8 vs 8.25±1.00ms, n=10, unpaired t test, two-tailed, equal variance, p=0.069, Figure 9 D-F). These results strongly support NBQX-mediated rescue of PTZ seizure-induced AMPAR function enhancement.

4.3.5.3 NBQX treatment rescued early seizure-induced diminishment of CA2 function during social behaviors

Using immunohistochemistry, we were able to identify the functionality of the CA2 neurons during the behavior test in P12 pups. Mice from the PTZ and NBQX group showed significantly higher levels of c-Fos+ cells after the preweaning two-choice social recognition test (middle: 12.804±0.670, n=5) when compared to mice from the PTZ and NBQX group without behavior testing (middle: 2.588±0.703, n=5, 2-way ANOVA followed by Sidak’s post doc test: p<0.0001, Figure 9 K). In addition, NBQX treatment also induced a rescue of a similar pattern of c-Fos+ cells in the dorsal (PTZ and NBQX group: 13.090±1.221, n=4: The PTZ only group: 1.694±0.272, n=5, one-way ANOVA followed by Tukey’s post doc test: p<0.0001, Figure 10 D1) and ventral CA2 (the PTZ and NBQX group: 17.070±1.802, n=4: The PTZ only group: 1.980±0.317, n=5, one-way ANOVA followed by Tukey’s post doc test: p<0.0001, Figure 10 F1).

Moreover, NBQX treatment did not induce any significant changes in the RGS14 expression (P12 PTZ+NBQX only - dorsal: 64.588±1.397, n=5; middle: 54.020±1.583, n=5; ventral: 43.940±1.224, n=5) (P12 PTZ+NBQX+behavior - dorsal: 66.093±1.441, n=4; middle: 60.160±1.115, n=5; ventral: 45.518±1.803, n=4) (one-way ANOVA followed by Tukey’s post doc test: dorsal: n=9, p=0.4822;
middle: n=10, p=0.2850; ventral: n=9, p=0.4781, Figure 10 D2, E2 and F2) and colocalization of c-Fos/RGS14 (colocalization percentage to RGS14: P12 PTZ+NBQX only - dorsal: 0±0, n=5; middle: 0.104±0.104, n=5; ventral: 0.180±0.180, n=5; P12 PTZ+NBQX+behavior - dorsal: 0.345±0.128, n=4; middle: 0.602±0.202, n=5; ventral: 1.045±0.559, n=4) (colocalization percentage to c-Fos: P12 PTZ+NBQX only - dorsal: 0±0, n=5; middle: 1.660±1.660, n=5; ventral: 2.840±2.840, n=5; P12 PTZ+NBQX+behavior - dorsal: 1.900±0.892, n=4; middle: 2.844±1.041, n=5; ventral: 2.448±1.226, n=4) (colocalization percentage to RGS14 - one-way ANOVA followed by Tukey’s post doc test: dorsal: n=9, p=0.018; middle: n=10, p=0.0596; ventral: n=9, p=0.1479, Figure 10 D4, E4 and F4) (colocalization percentage to c-Fos - one-way ANOVA followed by Tukey’s post doc test: dorsal: n=9, p=0.045; middle: n=10, p=0.5625; ventral: n=9, p=0.9112, Figure 10 D3, E3 and F3). These results strongly support that NBQX treatment can restore CA2 neuronal function and therefore rescue social behavior deficits.
Figure 9.
Figure 9. NBQX treatment rescued early seizure-induced social recognition memory deficits and reversed enhancement of AMPAR function in CA2 pyramidal neurons. Diagram illustrating the timeline of PTZ and NBQX administration along with behavior followed by immunohistochemistry procedure and two-choice social recognition paradigm (A). Representative AMPAR-mediated sEPSCs in CA2 pyramidal neuron from P12 PTZ+Vehicle (upper traces) and PTZ+NBQX (lower traces) mice (B). Group data of AMPAR-mediated sEPSC frequency in CA2 pyramidal neurons from PTZ+Vehicle and PTZ+NBQX mice. n=8,10, unpaired t test, ***p<0.001. Error bars indicate S.E.M (C). Group data of AMPAR-mediated sEPSC amplitude in CA2 pyramidal neurons from Vehicle and PTZ+NBQX mice. n=8,10, unpaired t test, *p<0.05. Error bars indicate S.E.M (D). Group data of AMPAR-mediated sEPSC (D) rise time and (E) decay time in CA2 pyramidal neurons from PTZ+Vehicle and PTZ+NBQX mice. n=8,10, unpaired t test, p>0.05. Error bars indicate S.E.M (E-F). Time spent socializing between the caregiving mother and novel mother in P12 PTZ seizure (n=11) and P12 PTZ+NBQX (n=14) groups, 2-way ANOVA – Sidak HSD, ***p<0.05 (G). Total social time between P12 PTZ (n=11) and P12 PTZ+NBQX (n=14) groups, unpaired t-test, **p<0.05 (H). Time difference socializing between caregiving mother and novel mother in P12 PTZ (n=11) and P12 PTZ+NBQX (n=14) groups, unpaired t-test, **p<0.05 (I). Representative immunofluorescence microscope 40x magnification image showing RGS14, c-Fos, DAPI and overlay RGS14/ c-Fos/DAPI in P12 PTZ+NBQX and P12 PTZ+NBQX with behavior groups (J). c-Fos cell percentage in P12 PTZ (n=5), P12 PTZ with behavior (n=6), P12 PTZ+NBQX without behavior (n=5) and P12 PTZ+NBQX with behavior (n=5) groups, 2-way ANOVA-Sidak HSD, ***p<0.05 (K).
Figure 10.
Figure 10. NBQX treatment rescued early seizure-induced diminishment of dorsal, middle and ventral CA2 function during social behavior. Illustrations representing dorsal (A1), middle (B1) and ventral (C1) hippocampal coronal slices. Representative immunofluorescence microscope 40x magnification images showing RGS14, GFP counterstained with DAPI in dorsal (A2), middle (B2) and ventral (C2) P12 PTZ+NBQX. Representative immunofluorescence microscope 40x magnification images showing RGS14, GFP counterstained with DAPI in dorsal (A3), middle (B3) and ventral (C3) P12 PTZ+NBQX+behavior group. C-Fos+ cell percentage in dorsal P12 PTZ (n=4), P12 PTZ+behavior (n=4), P12 PTZ+NBQX (n=5) and P12 PTZ+NBQX+behavior groups (n=4), one-way ANOVA – Tukey HSD, ***p<0.05 (D1). RGS14+ cell percentage in dorsal P12 PTZ (n=4), P12 PTZ+behavior (n=4), P12 PTZ+NBQX (n=5) and P12 PTZ+NBQX+behavior (n=4) groups, one-way ANOVA – Tukey HSD, ***p<0.05 (D2). Colocalization percentage of RGS14 and c-Fos when compared to c-Fos in dorsal P12 PTZ (n=4), P12 PTZ+behavior (n=4), P12 PTZ+NBQX (n=5) and P12 PTZ+NBQX+behavior (n=4) groups, one-way ANOVA – Tukey HSD, *p<0.05 (D3). Colocalization percentage of RGS14 and c-Fos when compared to RGS14 in dorsal P12 PTZ (n=4), P12 PTZ+behavior (n=4), P12 PTZ+NBQX (n=5) and P12 PTZ+NBQX+behavior (n=4) groups, one-way ANOVA – Tukey HSD, ***p<0.05 (D4). C-Fos+ cell percentage in middle P12 PTZ (n=6), P12 PTZ+behavior (n=6), P12 PTZ+NBQX (n=5) and P12 PTZ+NBQX+behavior groups (n=5), one-way ANOVA – Tukey HSD, ***p<0.05 (E1). RGS14+ cell percentage in middle P12 PTZ (n=5), P12 PTZ+behavior (n=6), P12 PTZ+NBQX (n=5) and P12 PTZ+NBQX+behavior (n=5) groups, one-way ANOVA – Tukey HSD, p<0.05 (E2). Colocalization percentage of RGS14 and c-Fos when compared to c-Fos in middle P12 PTZ (n=5), P12 PTZ+behavior (n=6), P12 PTZ+NBQX (n=5) and P12 PTZ+NBQX+behavior (n=5) groups, one-way ANOVA – Tukey HSD, p>0.05 (E3). Colocalization percentage of RGS14 and c-Fos when compared to RGS14 in middle P12 PTZ (n=5), P12 PTZ+behavior (n=6), P12 PTZ+NBQX (n=5) and P12 PTZ+NBQX+behavior (n=5) groups, one-way ANOVA – Tukey HSD, *p<0.05 (E4). C-Fos+ cell percentage in ventral P12 PTZ (n=4), P12 PTZ+behavior (n=5), P12 PTZ+NBQX (n=5) and P12 PTZ+NBQX+behavior (n=4) groups, one-way ANOVA – Tukey HSD, ***p<0.05 (F1). RGS14+ cell percentage in ventral P12 PTZ (n=4), P12 PTZ+behavior (n=5), P12 PTZ+NBQX (n=5) and P12 PTZ+NBQX+behavior (n=4) groups, one-way ANOVA – Tukey HSD, p>0.05 (F2). Colocalization percentage of RGS14 and c-Fos when compared to c-Fos in ventral P12 PTZ (n=4), P12 PTZ+behavior (n=5), P12 PTZ+NBQX (n=5) and P12 PTZ+NBQX+behavior (n=4) groups, one-way ANOVA – Tukey HSD, p>0.05 (F3). Colocalization percentage of RGS14 and c-Fos when compared to RGS14 in ventral P12 PTZ (n=4), P12 PTZ+behavior (n=5), P12 PTZ+NBQX (n=5) and P12 PTZ+NBQX+behavior groups (N=4), one-way ANOVA – Tukey HSD, p>0.05 (F4).
4.3.6 Early life seizure induced target specific enhancement of AMPAR function in CA2 seizure sensitive neurons

Thus far, we were able to recue early seizure-induced social behavior deficits by blocking AMPA-mediated synaptic transmission with NBQX. However, the NBQX treatment blocks AMPA receptors on all neurons and is not specific to the specific set of neurons that were selectively activated during a seizure. As shown in Figure 5, we found that early life seizures only activated a selectively group of neurons in CA2 which are referred as seizure-sensitive neurons. We hypothesize that early life seizure induced target specific functional changes in these seizure-sensitive neurons which mediated early seizure-induced social recognition memory deficits.

Using whole-cell patch clamp recordings in c-Fos-GFP mice, our first set of experiments was set to determine whether PTZ seizures-induced AMPAR function enhancement selectively in GFP+ seizure sensitive neurons in CA2. As shown in Figure 11, we found that AMPAR-mediated sEPSCs showed significantly higher frequency in seizure sensitive GFP+ neurons (0.071 ± 0.009 Hz; n = 24; Tukey’s HSD, two-tailed, equal variance, p<0.05) compared with both control pups (0.034 ± 0.007 Hz; n = 15; Figure 11 C) and non-activated GFP- cells (0.042 ± 0.009 Hz; n = 20; Fig. 11C). Importantly, AMPA sEPSC frequency in non-activated GFP- neurons were not different from controls (Tukey’s HSD, two-tailed, equal variance, p<0.05). In addition, we found no significant differences of AMPAR sEPSC amplitude, rise time and decay time between any of GFP+, GFP- and control cells (AMPAR sEPSC amplitude: 14.67 ± 0.97pA, n=24 vs 15.47 ± 0.93pA, n=20 vs 12.93 ± 1.61pA, One-way ANOVA, equal variance, F(2,56)=1.136, p = 0.3284, Fig 4D; rising time: 3.90 ± 0.30ms, n=24, vs 3.96 ± 0.65ms, n=20, vs 3.84± 0.81ms, n=15, One-way ANOVA, equal variance, F(2,56)=0.005, p = 0.995, Fig 4E; decay time:10.69 ± 1.14ms, n=24, vs 9.97 ± 0.88ms, n=20, vs 12.74 ± 1.72ms, n=15, One-way ANOVA, equal variance, F(2,56)=1.182, p = 0.314, Figure 11 D-F). These results suggest selective enhancement of AMPAR function only in the activated GFP+ neurons following PTZ seizures.
We next determined whether the presynaptic contribution to the increases in AMPAR sEPSC frequency in GFP+ cells by measuring the paired pulses ratio in GFP+, GFP- and control cells in both Schaffer collateral pathway and Perforant pathway. When stimulating the Schaffer collateral pathway, we observed no significant differences in the paired pulse ratio between GFP+, GFP- and control cells (1.70 ± 0.14; n = 7 vs 1.43 ± 0.177, n=8 vs 1.29 ± 0.10; n = 11, One-way ANOVA, equal variance, F(2,56)=1.053, p = 0.365, Figure 11 H). In contrast, when stimulating the Perforant pathway, we found that GFP+ cells exhibit significantly lower paired pulse ratio (1.63 ± 0.09; n = 19; Tukey’s HSD, equal variance, p<0.05) compared with control pups (1.99 ± 0.10; n = 19; Figure 11 J), but no significant differences (1.63 ± 0.09; n = 19; Tukey’s HSD, equal variance, p=0.179) compared with GFP- cells (1.90 ± 0.11; n = 12; Figure 11 J), and no change observed between GFP- cells (1.90 ± 0.11; n = 12; Tukey’s HSD, equal variance, p=0.838) compared with control cells (1.99 ± 0.10; n = 19; Figure 11 J).

These results indicate that early life seizure-induced AMPAR function enhancement occurred selectively in GFP+ cells through a presynaptic mechanism in the Perforant pathway, suggesting a critical role of the GFP+ cells in early seizure-induced network changes and social behavior deficits.
Figure 11.
Figure 11. Early life seizure induced target specific enhancement of AMPAR function in CA2 seizure sensitive neurons. Representative AMPAR-mediated sEPSCs in CA2 pyramidal neuron from P10 Control (upper traces), GFP- (middle traces) and GFP+ (lower traces) mice (A). Group data of AMPAR-mediated sEPSC frequency in CA2 pyramidal neurons from Control, GFP- and GFP+ mice; n=15, 24, 20, One-way Anova, $F(2,56)=4.97$, *$p<0.05$. Error bars indicate S.E.M. Control and GFP+, Tukey’s HSD, *$p<0.05$, GFP- and GFP+, Tukey’s HSD, *$p<0.05$, Control and GFP-, Tukey’s HSD, p>0.05 (B). Group data of AMPAR-mediated sEPSC amplitude in CA2 pyramidal neurons from Control, GFP- and GFP+ mice; n=15, 24, 20, One-way Anova, $F(2,56)=1.136$, p>0.05. Error bars indicate S.E.M (C). Group data of AMPAR-mediated sEPSC rise time in CA2 pyramidal neurons from Control, GFP- and GFP+ mice; n=15, 24, 20, One-way Anova, $F(2,56)=0.005$, p>0.05. Error bars indicate S.E.M (D). Group data of AMPAR-mediated sEPSC decay time in CA2 pyramidal neurons from Control, GFP- and GFP+ mice; n=15, 24, 20, One-way Anova, $F(2,56)=1.182$, p>0.05. Error bars indicate S.E.M (E). Representative traces of paired pulse evoked AMPAR EPSCs at a holding potential of -60 mV in CA2 pyramidal neurons by stimulating Schaffer collateral pathway in hippocampal slices from control, GFP- and GFP+ mice. The pulse interval was 50ms (F-G). Group data of paired-pulse facilitation in CA2 pyramidal neurons from control GFP- and GFP+ mice; n=15, 24, 20, One-way Anova, $F(2,56)=1.053$, p>0.05. Error bars indicate S.E.M (H). Representative traces of paired pulse evoked AMPAR EPSCs at a holding potential of -60 mV in CA2 pyramidal neurons by stimulating Perforant collateral pathway in hippocampal slices from control, GFP- and GFP+ mice. The pulse interval was 50ms (I). Group data of paired-pulse facilitation in CA2 pyramidal neurons from control GFP- and GFP+ mice; n=15, 24, 20, One-way Anova, $F(2,56)=3.874$, p>0.05. Error bars indicate S.E.M. Control and GFP+, Tukey’s HSD, *$p<0.05$, GFP- and GFP+, Tukey’s HSD, p>0.05, Control and GFP-, Tukey’s HSD, p>0.05 (J).
4.3.7 **Target-specific chemogenetic inhibition of seizure-sensitive neurons reverses early seizure-induced social recognition memory deficits in immature mice**

Given early life seizures induce target specific modifications only in the selectively activated neurons following seizures, we then determine whether precisely suppression of the selectively activated seizure-sensitive neurons could reverse early seizure-induced social behavior deficits.

4.3.7.1 **Chemogenetic suppression of the selectively activated seizure-sensitive neurons rescued early seizure-induced social deficits**

To precisely suppress the selectively activated seizure-sensitive neurons, we used a unique activity-dependent tagging and manipulation system, the c-Fos-GFP/c-Fos-tTA/TRE-hM4Di mouse model. This model has three co-injected transgenes, c-fos-tTA, c-fos-eGFP, and TRE-hM4Di, that drive expression of GFP, tetracycline transcriptional activator (tTA) and Tet-off construct controlling inhibitory chemogenetic hM4Di expression directed to activated neurons by the c-fos promoter in the absence of Doxycycline (DOX). Mouse pups were raised on DOX-containing food from P0 to P6 and then taken off DOX until P10 when early life seizures were induced. Seizures were able to induce both temporary GFP (green) and persistent hM4Di in activated neurons. Mice were put back on DOX immediately following seizures and returned to home cage. After seeing the first seizure event we injected the animal with clozapine N-oxide (CNO) twice a day until behavior testing day to actively suppresses the activity of GFP+ cells. We then tested the social behavior using the pre-weaning two choice social recognition memory test at P12. Our results showed that CNO treated post PTZ seizure mice were able to identify and show a significant preference for the caregiving mother (379.50±75.756 sec, n=10) than the novel mother (10.90±4.388 sec, n=10, p<0.0001, Figure 12 G), demonstrating normal social behavior. In addition, the social time difference between the caregiving mother and novel mother was significantly longer in the PTZ+CNO group (368.60±78.048 sec, n=10) than the PTZ only group.
Mice that received PTZ and then CNO showed a similar socializing time (395.40±71.636 sec, n=10) as mice that received PTZ only (246.455±67.528 sec, n=11, p=0.1465, Figure 12 H). These results demonstrate that precise suppression of the seizure activated sensitive neurons rescued the social deficits caused by PTZ seizures.

### 4.3.7.2 Chemogenetic suppression of the selectively activated seizure-sensitive neurons reversed early seizure-induced persistent enhancement of AMPAR function in CA2 pyramidal neurons

We next determined whether chemogenetic suppression of the selectively activated seizure-sensitive neurons could reverse PTZ seizure-induced persistent enhancement of AMPAR function in CA2 pyramidal neurons. We found that hippocampal slices from the P12 PTZ+CNO mice showed a significant decrease in the frequency of AMPAR sEPSCs (0.027 ± 0.004 Hz; n = 10) compared with P12 PTZ only mice (0.097 ± 0.008 Hz; n = 8; unpaired t test, two-tailed, equal variance, p <0.001, Figure 12 C). We found no significant differences of AMPAR sEPSC amplitude, rise time and decay time in p12 PTZ mice compared to PTZ+CNO pups (AMPAR sEPSC amplitude: 18.66 ± 2.26pA, n=8 vs 17.19±1.71pA, n=10, unpaired t test, two-tailed, equal variance, p=0.625, Figure 12 D; rising time: 3.74 ± 0.24ms, n=8, vs 3.42 ± 0.31ms, n=10, unpaired t test, two-tailed, equal variance, p = 0.463; decay time: 11.75 ± 1.42ms, n=8 vs 9.15± 0.92ms, n=10, unpaired t test, two-tailed, equal variance, p = 0.154, Figure E-F). These results strongly support chemogenetic suppression of the selectively activated seizure-sensitive neurons reversed PTZ seizure-induced AMPAR function enhancement.
4.3.7.3 **Chemogenetic suppression of the selectively activated seizure-sensitive neurons rescued early seizure-induced diminishment of CA2 function during social behaviors**

Using immunohistochemistry, we were able to identify the functionality of the CA2 during the behavior test in P12 pups. Mice that were injected with PTZ and CNO, and undergone through the preweaning two-choice social recognition memory test had significantly higher levels of c-Fos+ cells (middle: 26.113±2.298, n=5) when compared to mice that were injected with PTZ and CNO animals that not gone through behavior (middle: 5.628±0.436, n=4) (2-way ANOVA followed by Sidak’s post doc test: p<0.001, Figure 12 K). In addition, CNO treatment also induced a rescue of a similar pattern of c-Fos+ cells in the dorsal (the PTZ and CNO group with behavior: 31.255±3.442, n=4; without behavior: 5.120±0.541, n=4, p<0.001) and ventral CA2 (the PTZ and CNO group with behavior: 37.755±4.767, n=4; without behavior: 4.900±0.458, n=3, p<0.01, Figure 13 D1 and F1).

Moreover, PTZ with CNO treatment did not induce any significant changes in the RGS14 expression within the dorsal, middle and ventral CA2 structures (P12 PTZ+CNO only - dorsal: 62.480±1.402, n=4; middle: 55.395±2.446, n=4; ventral: 40.333±2.292, n=3) (P12 PTZ+CNO+behavior - dorsal: 64.520±3.399, n=4; middle: 54.680±1.085, n=5; ventral: 38.718±2.143, n=4) (one-way ANOVA followed by Tukey’s post doc test: dorsal: n=8, p=0.5990; middle: n=9, p=0.7810; ventral: n=7, p=0.6328, Figure 13 D2, E2 and F2). Colocalization also showed no changes in the PTZ with CNO treated group (colocalization percentage to RGS14: P12 PTZ+CNO only - dorsal: 0.08±0.08, n=4; middle: 0.125±0.125, n=4; ventral: 0.283±0.283, n=3; P12 PTZ+CNO+behavior - dorsal: 1.465±0.460, n=4; middle: 2.188±0.324, n=5; ventral: 5.408±0.883, n=4) (colocalization percentage to c-Fos: P12 PTZ+CNO only – dorsal: 1.040±1.040, n=4; middle: 1.135±1.135, n=4; ventral: 2.083±2.083, n=3; P12 PTZ+CNO+behavior – dorsal: 2.950±0.684, n=4; middle: 4.384±0.535, n=5; ventral: 5.560±0.682, n=4) (colocalization percentage to RGS14 - one-way ANOVA followed by Tukey’s post doc test: dorsal: n=8, p=0.0250; middle: n=9, p<0.01; ventral: n=7, p<0.01, Figure 13 D4, E4 and F4) (colocalization...
percentage to c-Fos - one-way ANOVA followed by Tukey’s post doc test: dorsal: n=8, p=0.1757; middle: n=9, p=0.0271; ventral: n=7, p=0.1300, Figure 13 D3, E3 and F3). This demonstrates that CA2 neuronal functionality is recued due to selectively inhibiting seizure sensitive neurons immediately after the first seizure event.
Figure 12.
Figure 12. Chemogenetic suppression of the selectively activated seizure-sensitive neurons rescued early seizure-induced social deficits and reversed persistent enhancement of AMPAR function in CA2 pyramidal neurons. Illustration of the c-Fos-GFP/c-Fos-tTA/TRE-hM4Di mouse model along with timeline (A). Representative AMPAR-mediated sEPSCs in CA2 pyramidal neuron from P12 PTZ+Vehicle (upper traces) and PTZ+CNO (lower traces) mice (B). Group data of AMPAR-mediated sEPSC frequency in CA2 pyramidal neurons from PTZ+Vehicle and PTZ+CNO mice. n=8,10, unpaired t test, *p<0.05 Error bars indicate S.E.M (C). Group data of AMPAR-mediated sEPSC amplitude in CA2 pyramidal neurons from PTZ+Vehicle and PTZ+CNO mice. n=8,10, unpaired t test, p>0.05. Error bars indicate S.E.M (D). Group data of AMPAR-mediated sEPSC (E) rise time and (F) decay time in CA2 pyramidal neurons PTZ+Vehicle and PTZ+CNO mice. n=8,10, unpaired t test, p>0.05. Error bars indicate S.E.M. Time spent socializing between the caregiving mother and novel mother in P12 PTZ (n=11) and P12 PTZ+CNO (n=10), 2-way ANOVA – Sidak HSD, **p<0.05 (G). Total social time between P12 PTZ (n=11) and P12 PTZ+CNO (n=10) groups, unpaired t-test, p>0.05 (H). Time difference socializing between caregiving mother and novel mother in P12 PTZ (n=11) and P12 PTZ+CNO (n=10) groups, unpaired t-test, *p<0.05 (I). Representative immunofluorescence microscope 20x magnification images showing RGS14, c-Fos, DAPI and overlay RGS14/c-Fos/DAPI in PTZ+CNO and PTZ+CNO+behavior groups (J). C-Fos+ cell percentage between P12 PTZ injected without behavior (n=5), P12 PTZ injected with behavior (n=6), P12 PTZ+CNO injected without behavior (n=4) and P12 PTZ+CNO injected with behavior (n=5), 2-way ANOVA – Sidak HSD, **p<0.05 (K).
Figure 13.
Figure 13. Chemogenetic suppression of the selectively activated seizure-sensitive neurons rescued early seizure-induced diminishment of dorsal, middle and ventral CA2 function during social behaviors. Illustrations representing dorsal (A1), middle (B1) and ventral (C1) hippocampal coronal slices. Representative immunofluorescence microscope 40x magnification images showing RGS14, GFP counterstained with DAPI in dorsal (A2), middle (B2) and ventral (C2) P12 PTZ+CNO. Representative immunofluorescence microscope 40x magnification images showing RGS14, GFP counterstained with DAPI in dorsal (A3), middle (B3) and ventral (C3) P12 PTZ+CNO+behavior group. C-Fos+ cell percentage in dorsal P12 PTZ (n=4), P12 PTZ+behavior (n=4), P12 PTZ+CNO (n=4) and P12 PTZ+CNO+behavior (n=4) groups, one-way ANOVA – Tukey HSD, ***p<0.05 (D1). RGS14+ cell percentage in dorsal P12 PTZ (n=4), P12 PTZ+behavior (n=4), P12 PTZ+CNO (n=4) and P12 PTZ+CNO+behavior (n=4) groups, one-way ANOVA – Tukey HSD, p>0.05 (D2). Colocalization percentage of RGS14 and c-Fos when compared to c-Fos in dorsal dorsal P12 PTZ (n=4), P12 PTZ+behavior (n=4), P12 PTZ+CNO (n=4) and P12 PTZ+CNO+behavior (n=4) groups, one-way ANOVA – Tukey HSD, *p<0.05 (D3). Colocalization percentage of RGS14 and c-Fos when compared to RGS14 in dorsal P12 PTZ (n=4), P12 PTZ+behavior (n=4), P12 PTZ+CNO (n=4) and P12 PTZ+CNO+behavior (n=4) groups, one-way ANOVA – Tukey HSD, **p<0.05 (D4). C-Fos+ cell percentage in middle P12 PTZ (n=5), P12 PTZ+behavior (n=6), P12 PTZ+CNO (n=4) and P12 PTZ+CNO+behavior (n=5) groups, one-way ANOVA – Tukey HSD, ***p<0.05 (E1). RGS14+ cell percentage in middle P12 PTZ (n=5), P12 PTZ+behavior (n=6), P12 PTZ+CNO (n=4) and P12 PTZ+CNO+behavior groups (n=5), one-way ANOVA – Tukey HSD, p>0.05 (E2). Colocalization percentage of RGS14 and c-Fos when compared to RGS14 in middle P12 PTZ (n=5), P12 PTZ+behavior (n=6), P12 PTZ+CNO (n=4) and P12 PTZ+CNO+behavior (n=5) groups, one-way ANOVA – Tukey HSD, p>0.05 (E3). Colocalization percentage of RGS14 and c-Fos when compared to RGS14 in middle P12 PTZ (n=5), P12 PTZ+behavior (n=6), P12 PTZ+CNO (n=4) and P12 PTZ+CNO+behavior (n=5) groups, one-way ANOVA – Tukey HSD, ***p<0.05 (E4). C-Fos+ cell percentage in ventral P12 PTZ (n=4), P12 PTZ+behavior (n=5), P12 PTZ+CNO (n=3) and P12 PTZ+CNO+behavior (n=4) groups, one-way ANOVA – Tukey HSD, ***p<0.05 (F1). RGS14+ cell percentage in ventral P12 PTZ (n=4), P12 PTZ+behavior (n=5), P12 PTZ+CNO (n=3) and P12 PTZ+CNO+behavior (n=4) groups, one-way ANOVA – Tukey HSD, **p<0.05 (F2). Colocalization percentage of RGS14 and c-Fos when compared to c-Fos in ventral P12 PTZ (n=4), P12 PTZ+behavior (n=5), P12 PTZ+CNO (n=3) and P12 PTZ+CNO+behavior (n=4) groups, one-way ANOVA – Tukey HSD, **p<0.05 (F3). Colocalization percentage of RGS14 and c-Fos when compared to RGS14 in ventral P12 PTZ (n=4), P12 PTZ+behavior (n=5), P12 PTZ+CNO (n=3) and P12 PTZ+CNO+behavior (n=4) groups, one-way ANOVA – Tukey HSD, ***p<0.05 (F4).
4.4 Chemogenetic inhibition of seizure-sensitive neurons reverses early KA seizure-induced social deficits in immature mice

To generalize our above findings discovered in the PTZ seizure model, we next tested the effects of early seizures on immature CA2 neurons in another KA seizure model. Early life seizures were induced by a single dose of KA (2 mg/kg, i.p.) in P10 c-Fos-GFP based transgenic mice. Each mouse had reached stages 4 and 5 seizures on the racine scale and were perfused 1 hour later for immunohistochemistry.

4.4.1 KA-induced early life seizure selectively activated a subgroup of CA2 neurons

Using the GFP antibody we were able to identify a selectively activated CA2 neurons in a KA-early seizure model. The immature CA2 in mice that had KA seizures showed a significant higher level of GFP+ cells (middle: 13.850±1.086%, n=6) than the saline control group (middle: 2.969±0.307%, n=8, unpaired t test, p<0.0001, Figure 14 D). In addition to the middle CA2 structure, the dorsal and ventral CA2 structures demonstrated a significant increase in the level of GFP+ cells in the KA group (dorsal: 12.615±0.815%, n=4; ventral: 16.915±3.715%, n=2) compared to the saline control group (dorsal: 2.325± 0.694%, n=4, p<0.0001; ventral: 8.560± 1.139%, n=5, p<0.05, Figure 14 D). These results support that KA-induced early life seizures selectively activated a subgroup of CA2 neurons.

4.4.2 KA seizure did not affect RGS14 expression in the immature CA2 structure

We also compared the level of RGS14+ cells within the dorsal, middle and ventral CA2 structures in P10 control and KA groups. We found that there was not significant difference in the level of RGS14+ cells between control and KA group in P10 (P10 control - dorsal: 52.525±1.009, n=4; middle: 35.852±0.953, n=9; ventral: 12.102±1.084, n=5 vs P10 KA - dorsal: 49.050±0.903, n=4, Sidak HSD,
Along with investigating the level of GFP+ and RGS14+ cells within the dorsal, middle and ventral CA2 structures, we also measured the colocalization of GFP+ and RGS14+ in both KA and control groups. There was little colocalization of RGS14 and GFP (colocalization percentage to RGS14 - dorsal control: 0±0, n=4; middle control: 0±0, n=8; ventral control: 0±0, n=5; dorsal KA: 0.330±0.330, n=4, p=0.3559; middle KA: 0.515±0.248, n=6, p=0.1357; ventral KA: 0.0±0.0, n=2, p>0.05, Figure 14 G) (colocalization percentage to GFP - dorsal control: 0±0, n=4; middle control: 0±0, n=8; ventral control: 0±0, n=5; dorsal KA: 1.315±1.315, n=4, p=0.3559; middle KA: 1.278±0.631, n=6, p=0.1438; ventral KA: 0.0±0.0, n=2, p>0.05, Figure 14 F).

### 4.4.3 KA-induced early life seizures impaired social behavior in immature mice

To test whether KA-induced early seizures could affect the social behaviors, the preweaning two-choice social recognition memory test was conducted on P12 pups that received a KA injection at P10 or a saline injection at P10. P12 pups that received a saline injection were able to identify their mother from a novel mother, spending more time with the caregiving mother (two-way ANOVA followed by Sidak’s post doc test: n=38, p<0.0001, Figure 14 N). However, P12 pups that received KA at P10 were unable to recognize the caregiving mother (17.33±8.765, n=12) from the novel mother (320.667±65.395, n=12) by spending more time with the novel mother (two-way ANOVA followed by Sidak’s post doc test: n=24, p<0.001, Figure 14 N). There was a significant difference in the social time difference between the caregiving mother and novel mother in the control group was (272.263±75.822 sec, n=19) and the KA group was (-303.333±66.577 sec, n=12) (one-way ANOVA: F(2,33)=17.75, n=36, p<0.0001 followed by Tukey’s post doc test: n=31, p<0.0001, Figure 14 O). The total time socializing between Control (417.842±44.764 sec, n=19) and KA injected (338.833±65.651 sec, n=12) animals showed no
significant difference (one-way ANOVA: F(2,33)=0.5931, n=36, p=0.5584 followed by Tukey’s post doc test: n=31, p=0.5524, Figure 14 P). These results indicate that the KA-induced seizures impaired the mouse’s ability to identify the social stimuli, its own mother, and therefore demonstrating social recognition memory deficits.

4.4.4 KA-induced early life seizures selectively increased AMPAR function in CA2 neurons

As we saw that KA-induced seizure enhances CA2 activation in the CA2 and induces social recognition deficits in pups, we wanted to verify that KA-induced seizures would result in similar effects on the CA2 pyramidal neurons as the PTZ seizure model. Therefore, we recorded AMPAR-mediated sEPSCs in CA2 pyramidal neurons after KA-induced seizure. Here, we found that KA seizure mice show an increase in AMPAR frequency (0.0741 ± 0.0192 Hz; n = 8; unpaired t test, two-tailed, equal variance, p <0.05) compared with hippocampal slices from control pups (0.0323 ± 0.0066 Hz; n = 14; Figure 14 I). However, we found no significant differences of AMPAR sEPSC amplitude, rise time and decay time in Kaiante mice compared to controls (AMPAR sEPSC amplitude: 11.94 ± 1.80 pA, n=8 vs 12.93 ± 1.15 pA, n=14, unpaired t test, two-tailed, equal variance, p = 0.643; rising time: 3.87 ± 0.29ms, n=8, vs 4.08 ± 0.50ms, n=14, unpaired t test, two-tailed, equal variance, p = 0.770; decay time: 9.33 ± 1.43ms, n=8 vs 12.40 ± 1.74ms, n=14, unpaired t test, two-tailed, equal variance, p = 0.249, Figure 14 J-L).

4.4.5 Chemogenetic suppression of seizure-sensitive neurons reverses early KA seizure-induced social deficits in immature mice

We next determined whether precise inhibition of KA-seizure activated neurons could rescue seizure-induced social deficits in immature mice using the pre-weaning two choice social recognition memory test in the c-Fos-GFP/c-Fos-tTA/TRE-hM4Di mice. We found that the P12 mice that received
KA and CNO twice a day spent significantly more time with the caregiving mother (397.2±81.642 sec, n=5) than the novel mother (19.2±11.052 sec, n=5, p=0.001) (2-way ANOVA followed by Sidak post doc test: n=10, p=0.001, Figure 14 N). The social time difference between the caregiving mother and novel mother was significantly longer in the KA+CNO group (378±85.632 sec, n=5) than the KA group (-303.333±66.577 sec, n=12) (one-way ANOVA followed by Tukey’s post doc test: n=17, p=0.0002, Figure 14 O). The total time socializing between KA+CNO (416.4±79.007, n=5) and KA injected (338.833±65.651, n=5) animals showed no significant difference (one-way ANOVA followed by Tukey’s post doc test: n=17, p=0.7577, Figure 14 P). These results strongly support that chemogenetic suppression of seizure-sensitive neurons reverses early KA seizure-induced social deficits in immature mice.
Figure 14.
Figure 14. Chemogenetic inhibition of seizure-sensitive neurons reverses early KA seizure-induced social deficits in immature mice. Illustrations representing dorsal (A1), middle (B1) and ventral (C1) hippocampal coronal slices. Representative immunofluorescence microscope 20x magnification images showing RGS14, GFP counterstained with DAPI in dorsal (A2), middle (B2) and ventral (C2) P10 control saline group. Representative immunofluorescence microscope 20x magnification images showing RGS14, GFP counterstained with DAPI in dorsal (A3), middle (B3) and ventral (C3) P10 KA group. GFP+ cell percentage in dorsal (n=4, 4), middle (n=8, 6), and ventral P10 control saline and KA groups (n=5, 2), 2-way ANOVA-Sidak HSD, ***p<0.05 (D). RGS14+ cell percentage in dorsal (n=4, 4), middle (n=8, 6), and ventral (n=5, 2) P10 control saline and KA groups, 2-way ANOVA-Sidak HSD, p>0.05 (E). Colocalization percentage of RGS14 and GFP when compared to GFP in dorsal (n=4, 4), middle (n=8, 6), and ventral (n=5, 2), 2-way ANOVA-Sidak HSD, p>0.05 (F). Colocalization percentage of RGS14 and GFP when compared to RGS14 in dorsal (n=4, 4), middle (n=8, 6), and ventral (n=5, 2), 2-way ANOVA-Sidak HSD, p>0.05 (G). Representative AMPAR-mediated sEPSCs in CA2 pyramidal neuron from P10 Control (upper traces) and KA (lower traces) mice (H). Group data of AMPAR-mediated sEPSC frequency in CA2 pyramidal neurons from Control and KA seizure mice. n=14, 8, unpaired t test, *p<0.05, Error bars indicate S.E.M. (I). Group data of AMPAR-mediated sEPSC amplitude in CA2 pyramidal neurons from Control and KA seizure mice. n=14, 8, unpaired t test, p>0.05. Error bars indicate S.E.M. (J). Group data of AMPAR-mediated sEPSC (K) rise time and (L) decay time in CA2 pyramidal neurons from Control and KA seizure mice. n=14, 18, unpaired t test, p>0.05. Error bars indicate S.E.M (K-L). Illustration of the c-Fos-GFP/c-Fos-tTA/TRE-hM4Di mouse model along with timeline (M). Time spent socializing between the caregiving mother and novel mother in P12 Control (n=19), P12 KA (n=12) and P12 KA+CNO (n=5), 2-way ANOVA – Sidak HSD, ***p<0.05 (N). Time difference socializing between P12 Control (n=19), P12 KA (n=12) and P12 KA+CNO (n=5) groups, one-way ANOVA, ***p<0.05 (O). Total social time between caregiving mother and novel mother in P12 Control (n=19), P12 KA (n=12) and P12 KA+CNO (n=5) groups, one-way ANOVA, p>0.05 (P).
5. Discussion

Neuronal death and cognitive deficits have been found in adult epileptic animal models caused by prolonged and repeated seizures; however, whether an acute seizure event can produce similar outcomes is still unknown. Although, the CA2 has been shown to be resistant to neuronal death caused by TLE (Botterill et al., 2017; Lawrence and Inder, 2010; Minjarez et al., 2017; Swann, 2002), reorganization of the microcircuitry input and output of CA1 and CA3 can affect normal functioning. Studies have shown a reduction in the density of PV+ interneurons resulting in a complete loss of inhibitory neurotransmission on CA2 PNs (Carstens et al., 2016; Erbs et al., 2012; Haussler et al., 2015; Piskorowski and Chevaleyre, 2013; Reyes-Garcia et al., 2018; Williamson and Spencer, 1994). This outcome of excitatory input reorganization within the CA2 could potentially contribute to the generation of epileptiform activity and cognitive deficits.

Typically, a single episode of clonic-tonic seizure by a single injection of PTZ, an antagonist of GABA type A receptor, will not result in significant neuronal loss during adulthood (Vasilev et al., 2018). However, the PTZ model has been shown to affect the viability of neurons in the hippocampus, causing various morphological alterations of a specific set of neurons in the CA1 and CA3 (Vasilev et al., 2018). Therefore, this suggests that even a single seizure episode is a potent stressor to hippocampal neurons and can cause complex neuroplastic changes in the mature hippocampus. Whether an acute episode of seizures can affect CA2 PNs and its functionality in adulthood is still unknown.

5.1 Seizures activate a heterogeneous population of mature CA2 PN’s

Using the PTZ model, which mimics petit mal convulsions, on our P60 c-Fos-GFP-based transgenic mice, we were able to identify a selective set of seizure-sensitive cells in the CA1, CA2 and
CA3 subregions of the hippocampus. The reasoning behind utilizing the PTZ-induced seizure model was to target the prominent role of inhibitory neurotransmission in the CA2. The inhibitory neurons in the CA2 contain a dense population of fast-spiking PV+ interneurons, which contribute to the excitation/inhibition balance while regulating neuronal activity in the CA2 (Botcher et al., 2014). It has also been documented as a key factor for modulating social recognition memory processes in this region (Carstens et al., 2016; Erbs et al., 2012; Haussler et al., 2015). We believed that by targeting inhibition we will most likely affect CA2 PN activity resulting in social recognition memory deficits.

Using immunohistochemistry, we stained for GFP to identify selectively activated cells, along with RGS14 to mark the CA2 region in our P60 c-Fos-GFP-based transgenic mice. An increase in GFP+ cells in the CA1 (~18%) and CA3 (~28%) regions was found within the PTZ seizure group when compared to controls which is consistent with previous research (Sun et al., 2013; Zhou et al., 2011). Unexpectedly, however, the mature CA2 also showed a significant increase in GFP+ cells (~5%) between the PTZ and control group, demonstrating that an acute seizure event during the mature period can influence neuronal activation in the CA2 region. Previous studies have claimed the mature CA2 to be resistant to neuronal damage and cell death in patients with temporal lobe epilepsy but with the presence of ictal-like activity in pyramidal neurons. Although resistant to neuronal excitotoxicity, CA2 PN’s can still get activated in normal conditions due to strong inputs from the EC2 (Perfornt pathway) and weaker inputs from the CA3 (Schaffer Collateral pathway) (Chevaleyre and Siegelbaum, 2010; Sun et al., 2014). Here, our results demonstrate that the mature CA2 can indeed be affected by an acute seizure event by activating a specific population of CA2 PNs.

5.2 Seizures do not affect mature CA2 function

Since our results demonstrate that the mature CA2 PNs can be influenced by an acute seizure event, we decided to investigate if the phenotypic property of the CA2 is also affected by a single seizure
event. There have been considerable research examining how social stimuli would influence CA2 neuron firing in animal studies (Hitti et al., 2014; Laham et al, 2021). Previous studies have used a CA2-specific Cre-expressing mouse strain to silence CA2 neuronal synaptic output and inhibit neurotransmission (Hitti et al., 2014). The silencing of these specific cells resulted in the selective impairment of social recognition memory, demonstrating that the CA2 region is critical for encoding social recognition memory processes.

Our results from the behaviour testing demonstrated that PTZ-induced seizure in adults do not show social recognition memory deficits, instead, show normal CA2 functioning where we see an increased level of c-Fos+ cells in seizure-induced mice as we do in control mice that went through behavior testing at P62. After behaviour testing, we used c-Fos to identify the pattern of neuronal activation during the social recognition memory test and investigate if the CA2 region is still functional during this period. Immediate early genes (IEG), like c-Fos, are utilized for locating experience-dependent neuronal activity at a single time point (Miyashita et al., 2018; Olga et al., 2021).

Although we saw an immediate increase in neuronal excitation due to PTZ in P60 adults, there could be several compensatory homeostatic responses that reverse the effects of the acute seizure event in CA2 PNs and therefore restore CA2 function and social behaviour. Furthermore, our results identified that CA2 PNs may be resistant to seizure induced neuronal sensitivity, where adult mice that received a single seizure during P60 and were euthanized at P62 demonstrated no change in the level of c-Fos+ cells compared to P62 controls (Figure 4). A previous study by Shimada and Yamagata, 2018 demonstrated that PTZ-induced seizure can decrease neuronal threshold and lead to increased neuronal sensitivity in the CA1 and CA3, along with morphological changes that can last up to 1 week. Where PTZ seizures in adults changed the excitability of CA1 and CA3 neurons, making them more sensitive to excitation. However, this is not the case for the mature CA2 region. Our results provide evidence that the mature CA2 is resistant to acute seizures as its functional property is not affected and social behaviour is intact.
5.3 The role of RGS14 and its relation towards seizure resistance

The mature CA2’s resistance towards seizure induced functional and neuronal sensitivity changes has some relation to the unique protein-based defence mechanism of the CA2, RGS14. While investigating the effects of seizures on the mature brain, we identified the CA2 region through an enriched scaffolding protein, RGS14, as a known marker (Harbin et al., 2021; Evans et al., 2018; Lee et al., 2010), where we saw a dense population of RGS14+ cell (70%). Our results demonstrated that CA2 PN’s that expressed RGS14 had little to no chance to express GFP in the same cell in both P60 control and P60 PTZ animals. The lack of colocalization could suggest that RGS14+ cells may have a higher threshold for activation than RGS14- cells in the CA2 region. This will account for a less likely chance of RGS14+ cells getting activated due to a seizure event.

Although the influence of RGS14 on the c-Fos protein is unknown, RGS14 has been characterized as a key feature in area CA2 to suppress synaptic plasticity which can affect neuronal sensitivity caused by a seizure (Harbin et al., 2021; Evans et al., 2018; Lee et al., 2010). RGS14 controls synaptic signalling by binding to Gα proteins, H-Ras/Rap2 GTPases, 14-3-3, Ca2+/CaM, and CaMKII proteins, which have been shown to affect postsynaptic signalling (Harbin et al., 2021; Evans et al., 2018; Lee et al., 2010), demonstrating an effect on AMPAR membrane insertion due to the RGS14 inhibition of Ca2+ and MAPK signalling (Harbin et al., 2021; Evans et al., 2018; Lee et al., 2010). Based on the dense population of RGS14+ cells in the mature CA2, functional changes are prevented within the CA2, therefore hindering the chance for an excitable neuronal sensitive state. This is precisely what we observed in our immunohistochemically staining in P60 animals injected with PTZ and euthanized at P62 in comparison to control P62 animals.

Interestingly RGS14 expression has been identified to be developmentally regulated (Harbin et al., 2021; Evans et al., 2018; Lee et al., 2010). Supporting previous studies, our results demonstrated a significantly lower percentage of RGS14+ cells within the entire CA2 region in P10 immature pups when
compared to P60 adult mice (Figure 5). Therefore, providing an increased opportunity for cells to be activated by an acute seizure event in the immature brain rather than the mature where RGS14 is extremely dense.

5.4 Early life seizures effect CA2 PNs and cause social recognition deficits

The neonatal period poses as one of the most vulnerable periods of development, prone to seizures and associated long-term deficits (Holmes, 2009; Holmes et al., 1998; Jensen and Baram, 2000; Kang and Kadam, 2015; Sanchez and Jensen, 2006). A single seizure event during the neonatal period can influence later life autistic-like social recognition (Talos et al., 2012), leading us to conclude that the immature CA2 may be vulnerable to a single seizure event. Here, we hypothesize that early life presents as a vulnerable period where seizures can influence CA2 function and therefore impair associated social recognition memory.

Using our P10 c-Fos-GFP-based transgenic mice we were able to identify a selective set of seizure-sensitive cells in the immature CA2 region. Our results showed a significant increase in the level of GFP+ cells in the PTZ injected group when compared to control animals (Figure 5). We also found little to no colocalization of RGS14 and GFP in the P10 PTZ group supporting the same trend seen in our P60 PTZ group. However, there seems to be a negative correlation between the level of RGS14+ cells and GFP+ cells based on age. In P60 animals, the level of RGS14+ cells are high and the level of GFP+ cells are low, whereas, in P10 animals the level of RGS14+ cells are low and GFP+ cells are high. The density of RGS14 expression is developmentally regulated and may be one of the critical factors related to protecting against seizure-induced neuronal activation in the CA2.

After observing the significant influence on the immature CA2 after seizures, we then proceeded to identify if there would be social recognition memory deficits. Our results show that pups that have had seizures two days before their behaviour test demonstrate social recognition memory deficits, where the
pup is unable to identify the social stimuli, the caregiving mother from the novel mother. In addition, the immature CA2 was not functional during behaviour testing in pups that received a seizure, showing significantly low levels of c-Fos+ cells when compared to control animals that went through behavior testing. Impaired social memory has been recognized as one of the common negative symptoms associated with autism spectrum disorder (ASD) (Trevisan et al., 2020). As PTZ injected pups demonstrate autistic-like behaviours, where they are unable to recognize different social stimuli, we can attest that the CA2 function is impaired subsequent to PTZ-induced seizure.

Interestingly, unlike the adult animals, P10 mice that were injected with PTZ and euthanized at P12 showed elevated levels of c-Fos+ cells compared to P12 WT control mice (Figure 7). Demonstrating that PTZ can decrease neuronal threshold and lead to increased neuronal sensitivity in the immature CA2, which may influence PN functionality. Previous studies have noted a change in hippocampal CA1 PN functioning due to ELS, with lasting changes for over 1 week (Postnikova et al., 2017; Talos et al., 2012; Vasilev et al., 2018). The abnormal neuronal functioning was associated with mTORC1 pathway upregulation and AMPAR dysfunction (Talos et al., 2012). Knowing that ELS induced upregulation of the mTORC1 pathway in CA1 PNs and is associated with cognitive disorders and autistic-like behaviours, the immature CA2 PN function may also have some relation to the mTORC1 pathway dysfunction, leading to later life autistic-like social recognition memory deficits. As such, we aimed to investigate if CA2 PN function is altered after ELS.

5.5 Functional changes in immature CA2PN AMPARs

Given that early life seizures can cause social recognition memory deficits as well as an impairment in CA2 neuronal function during the behaviour testing, we next set out to determine whether these behavioural deficits in social recognition are associated with changes in the synaptic function of CA2 neurons. AMPA receptors are gradually integrated at the synapse during early development, critical
for the maturity of these synapses. Trafficked AMPARs are initially non-functional and become functional subsequent to activity and or experience (Hanse et al., 2013). As AMPARs are critical during this early period we used electrophysiology to first study CA2 PNs AMPARs. We recorded spontaneous AMPAR activity while holding the cell at -60mV and blocking GABA receptors in P10 pups during PTZ seizure.

Here, we found an increase in AMPAR frequency and no change in AMPAR amplitude. Interestingly, a neighbouring region, the CA1, important for cognitive function, also shows an increase in PN sEPSC frequency 1-3 hr. subsequent seizure induction in association with cognitive deficits (Sun et al., 2013; Zhou et al., 2011). In the CA2 and at the immature stage of development, the frequency change observed could be due to a presynaptic or postsynaptic change. Therefore, we first used paired-pulse facilitation to study if a presynaptic change has occurred. Paired Pulse facilitation is understood as mediated by the pre-synapse and not changes in the postsynaptic potential (Clark et al., 1994) and as such is the standard method for studying presynaptic alterations. Here, at the 50ms interval, a greater ratio of the second pulse to the first pulse amplitude is expected due to the residual Ca2+ from the first pulse, enhancing the likelihood of neurotransmitter release after the second (Santschi Stanton, 2003). Indeed, after PTZ, we see a decrease in the paired-pulse ratio in comparison to control, supporting that PTZ causes a change in presynaptic AMPAR activity when stimulating the Perforant pathway, which has been shown to be implicated in social recognition memory processes (Chevaleyre et al., 2010; Ding and Van Hoesen, 2010; Kohara et al., 2014; Zhao et al., 2007). This alludes to the critical role of AMPAR in mediating the seizure effects on the CA2 during this period, and present AMPARs as a therapeutic target for alleviating ELS-induced social behaviour deficits. However, to precisely understand what occurs at the presynapse subsequent PTZ induced seizure at p10, further tests are required to study possible changes in short-term plasticity, such as the probability of presynaptic release and the readily releasable pool, in addition to asynchronous release changes.
Finally, to investigate if a postsynaptic change in AMPAR activity has occurred, we investigated the number of silent synapses after PTZ seizure in p10 mice. At this age, most synapses are silent, meaning that they are inactive or immature because they lack functional AMPARs at the postsynapse. And as the brain matures, AMPARs are integrated at the synapse marking these synapses as active and mature. Here, our results show no difference in the % of silent synapses between the two groups, after neither the Schaffer collateral nor the Perforant pathway stimulation, indicating no evidence for a change in the postsynapse. These results present as further evidence that PTZ causes a presynaptic change in AMPAR activity rather than a postsynaptic change in the immature CA2.

5.6 NBQX can rescue ELS induced social behavior deficits

In order to rescue AMPA receptor functioning in immature CA2 PNs after PTZ, we utilized NBQX, an AMPA and kainate receptor antagonist. Previous studies have found that pre-treatment of NBQX reduced the number of seizures, seizure susceptibility and seizure-induced neuronal death during hypoxia (Russel et al., 2006; Sanchez. & Jensen, 2006). Here our results highlight that NBQX-treatment on PTZ injected mice showed rescued social behaviour during the pre-weaning two-choice recognition memory test at P12. Using Immunohistochemistry on P12 mice, we also showed that the CA2 is functional during the behaviour testing in animals that received PTZ and then NBQX in P12 mice. The CA2 region showed an increased level of cfos+ cells when compared to animals that did not go through behaviour testing with the same variable (Figure 9), demonstrating normal CA2 functioning during behavior testing after NBQX treatment. Additionally, we also investigated CA2 PN AMPAR function at p12 after PTZ+NBQX treatment. Here we found no change in AMPAR frequency compared to control animals, indicating normal AMPAR activity after NBQX treatment. Indeed, NBQX treatment after hypoxia induced seizure rescued seizure mediated changes in AMPAR function in p10 animals (Zhou et
al., 2011), attesting to the potential of NBQX as a treatment for autistic-like symptomology subsequent seizures.

5.7 **Selective suppressing seizure sensitive neurons to rescue social behavior deficits**

5.7.1 **Seizure sensitive cells in immature CA2**

Although we were able to rescue social behaviour using NBQX, this form of treatment blocks all AMPA receptors and is not specific to the selective set of neurons observed to be activated after a seizure event. Previous studies and our data have shown that not all cells are activated due to a seizure event in the hippocampus (Feldt, Muldoon, Soltesz, & Cossart, 2013; Lippman-Bell, Zhou, Sun, Feske, & Jensen, 2016; Sparks, Liao, Li, Grosmark, Soltesz, & Losonczy, 2020). As our data clearly illustrates that a specific group of neurons are particularly responsive to seizures, we wanted to understand the function of this set of neurons. No deep investigation of the immature brain and seizure-sensitive neurons has been done yet, especially in regards to the immature CA2. During development, specific immature neurons at this critical period are undergoing synaptogenesis and are hyper-excitabale at different time points. Unsilent synapses are characterized as having functional AMPA receptors whereas, silent synapses, immature synapses, have non-functional or no AMPA receptors. This potentially creates a heterogeneous population of cells, whereby a selective set of cells are more sensitive to a single seizure and some are more resistant.

Using electrophysiology, we investigated CA2 GFP+ and GFP- cells in PTZ animals through AMPAR-mediated spontaneous excitatory post-synaptic recordings. We can confirm that GFP+ cells in the PTZ group show a significant increase in frequency, supporting a presynaptic change, compared to control cells and GFP- cells. Additionally, CA2 GFP+ cells showed a significant decrease in paired pulse ratio after Perforant pathway stimulation in comparison to control cells where as GFP- cells did not. This
establishes that there is a selective set of activated cells, GFP+ cells, which are particularly sensitive to seizures and likely mediate the social recognition memory deficits.

5.7.2 Supressing seizure sensitive neurons to rescue social memory deficits

Given the unique alterations in the immature CA2 of specific seizure-sensitive PNs, we introduced an activity-dependent labelling and manipulating system using the designer receptor exclusively activated by designer drugs (DREADDs) technology. Using our unique triple transgenic c-Fos-GFP/c-Fos-tTA/TRE-hM4Di mouse model, we can identify and control the seizure-sensitive neurons (GFP+ cells) in the developing brain by chemogenetically silencing their activity to reverse the effects of early life seizure and subsequent social recognition memory deficits. This model is controlled using doxycycline, which can uncouple TTA from TRE and therefore inhibit the activity of hM4Di expressing neurons, where hM4Di receptors are linked to GFP and are exclusively expressed in GFP expressing neurons. Therefore, when the seizure takes place, activating a subset of neurons, those GFP+ cells will also now express the hM4Di receptor. To initiate the suppression of GFP+ cells, CNO was administered twice a day till behaviour day. Using the pre-weaning social behaviour test our results demonstrated that our triple transgenic mice that received PTZ and CNO spent significantly more time with the familiar mother than the novel mother, demonstrating normal CA2 function. To confirm normal CA2 PN function after CNO treatment, we investigated AMPAR function in p12 pups after CNO+PTZ treatment. Here we observed a significantly lower AMPAR sEPSC frequency compared to PTZ animals that was no different from a regular control animal. Indeed, hM4DI activity is known to silence synaptic activity by decreasing presynaptic release (Stachniak et al., 2014), explaining the reduction in AMPAR function observed. Our results highlight that the selectively seizure-activated neurons in the CA2 region mediate social recognition deficits and that the phenotypic and functional changes of seizures can be reversed by selectively suppressing these neurons.
5.8 Future directions

Investigating target CA2 neuron functioning through a cre-dependent construct mediated rescue can help determine if the CA2 is the primary area for encoding social recognition memory after ELS event. Unfortunately, viral injections may cause brain injury as it is invasively administered through stereotaxic surgery in the new born mice. Studies have used a cre-dependent construct encoding tetanus toxin light chain to inhibit neurotransmission and to selectively impair adult CA2 neuronal function. This resulted in impairment of social recognition memory but did not impair other hippocampus-dependent memory spatial tasks, like novel object recognition (Hitti and Siegelbaum, 2014). Using the cre-dependent construct to selectively activate CA2 neurons after ELS, can help determine if the CA2 is responsible for the seizure induced social recognition deficits. The limitation of the CA2-specific cre-expressing mouse strain is that it can only be expressed after P21 and therefore limit its use in our study on ELS induced at P10.

In addition, during electrophysiological recordings in the CA2 region, we cannot account for RGS14+ or RGS14- cells, which can affect the neurophysiological properties associated with the proteins function. RGS14 acts as an MEK inhibitor which suppresses LTP in the CA2 region and may have other properties that cause the cell to be less likely active (Lee et al. 2010). Selectively identifying RGS14+ neurons can help us understand the influence of RGS14 on the neuron, and then determine if RGS14+ cells are influenced by a seizure event or not.

Furthermore, electrophysiological recordings on P60 PTZ injected animals can help identify if there are immediate changes in AMPAR functioning in adulthood. This will provide further evidence if the mature CA2 is resistant to a seizure event. Investigating the defense mechanisms associated with the mature CA2 can help understand if these properties inhibit neuronal functional changes due to an acute seizure event and why social behavior is intact.
6. **Conclusion**

In summary, our results discovered a novel cellular target of heterogeneously activated CA2 PNs by which a single acute seizure can significantly influence neuronal network excitability in the immature brain. We show that ELS poses a significant threat on immature CA2 PNs by increasing the frequency of AMPAR mediated sEPSCs one-hour post PTZ-induced seizure in p10 mice. This is mediated by a presynaptic change as illustrated by a decrease in paired pulse ratio of AMPAR eEPSCs via the Perforant pathway in PTZ cells, which is the pathway known to be critical for mediating CA2 PN firing and social recognition memory processing. Furthermore, using the pre-weaning social recognition memory test, we have identified impaired functionality of social recognition memory at p12 post PTZ-induced seizure at p10. These data strongly support ELS-induced dysregulation of hippocampal CA2 pyramidal neurons in the developing hippocampus and associated social recognition memory deficits. Based on the enhanced AMPAR activity, NBQX, an AMPAR antagonist, was administered at p10 immediately after seizure, which rescued social recognition memory of the dam in p12 pups and rescued AMPAR function in CA2 PNs. However, NBQX is designed to block all AMPAR and does not target seizure-sensitive cells. Our data suggests that there are selective group of seizure-sensitive GFP+ cells within the CA2 region and can account for social recognition memory deficits. Using our novel activity-dependent labelling and manipulating system, triple transgenic c-Fos-GFP/c-Fos-tTA/TRE-hM4Di mouse model, we report for the first time that precise suppression of the selectively activated neurons rescued the social recognition memory deficits and CA2 PN function. Our results identify the potential effects of ELS on the function of CA2 neurons and help determine a novel cellular target for intervention for reversing the long-term effects of ELS.
7. References


https://doi.org/10.1093/med/9780199743506.003.0008


https://doi.org/10.1007/BF00228747

https://doi.org/10.1097/wnr.0b013e328333d690

https://doi.org/10.1016/j.celrep.2019.09.044


https://doi.org/10.1134/s0006297917030063

https://doi.org/10.1038/s41467-017-02173-0


Protein 4 (PCP4) immunostaining. *Journal of Comparative Neurology*, 522(6), 1333–1354. https://doi.org/10.1002/cne.23486


