Modulation Exerted by an Inhibitor of Chloride Co-Transporters on 4-Aminopyridine Induced Epileptiform Discharges in the Juvenile Entorhinal Cortex *In Vitro*.

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Abstract

Mesial temporal lobe epilepsy (MTLE) is one of the most prevalent types of focal epilepsy and it is characterized by hypersynchronous neuronal activity originating from limbic structures such as the entorhinal cortex (EC) that leads to spontaneous seizures. Experimental evidence has shown that epileptiform activity is generated following the blockade of $GABA_A$ receptor signaling. The K^+ channel blocker, 4-aminopyridine (4AP) can also induce interictal and ictal events in both *in vivo* and *in vitro* preparations.

In the adult brain, gamma-aminobutyric acid (GABA) acts as the main inhibitory neurotransmitter, however, in the developing brain, GABA has excitatory actions. The effects resulting from the activation of $GABA_A$ receptors can be hyperpolarization or depolarization, depending on the intracellular chloride [Cl^-] that is regulated by the activity of cation- Cl^-- cotransporters (CCC) such as NKCC1 and KCC2. In neurons, NKCC1 and KCC2 by accumulating and extruding Cl^-, respectively, regulate the functions of GABA receptors by maintaining intracellular Cl^- homeostasis. However, failure of $GABA_A$ receptors' function has been often considered a contributing factor to neuronal hyperexcitability and the generation of epileptic seizures. In juvenile rat brain (10-25 day-old), developmental switching from depolarization to hyperpolarization may not be complete, thus making neuronal networks hyperexcitable due to residual GABA-mediated depolarizations. Based on previous studies, antagonizing the activity of both NKCC1 and KCC2 with bumetanide can inhibit seizure generation. While low doses of bumetanide (10 μM) downregulate the activity of NKCC1, in higher concentrations (50 μM) it blocks the activation of both NKCC1 and KCC2. Therefore, I hypothesized that using low and high doses of bumetanide can differently modify the epileptiform activity recorded in the *in vitro* juvenile rat EC during 4AP application.

I found that blocking NKCC1 activity with low doses of bumetanide (10 μM): (i) significantly decreased the occurrence of interictal discharges without changing their duration; and (ii) increased the duration of ictal events while decreasing their occurrence rate. Blocking both NKCC1 and KCC2 with high concentrations of bumetanide (50 μM), induced a significant reduction in ictal event duration and rate of occurrence.

Résumé

Dans le cerveau adulte, l'acide gamma-aminobutyrique (GABA) agit comme le principal neurotransmetteur inhibiteur, cependant, dans le cerveau en développement, le GABA a des actions excitatrices. Les effets résultant de l'activation des récepteurs GABAA peuvent être une hyperpolarisation ou une dépolarisation, selon le chlorure intracellulaire [Cl-] qui est régulé par l'activité des cotransporteurs de cations-Cl-- (CCC) tels que NKCC1 et KCC2. Dans les neurones, NKCC1 et KCC2, en accumulant et en extrudant respectivement du Cl-, régulent les fonctions des récepteurs GABA en maintenant l'homéostasie intracellulaire du Cl-. Cependant, l'échec de la fonction des récepteurs GABAA a souvent été considéré comme un facteur contribuant à l'hyperexcitabilité neuronale et à la génération de crises d'épilepsie. Dans le cerveau de rat juvénile (âgé de 10 à 25 jours), le passage développemental de la dépolarisation à l'hyperpolarisation peut ne pas être complet, rendant ainsi les réseaux neuronaux hyperexcitables en raison des dépolarisations résiduelles induites par le GABA. Sur la base d'études antérieures, l'antagonisme de l'activité de NKCC1 et de KCC2 avec du bumétanide peut inhiber la génération de crises. Alors que de faibles doses de bumétanide (10 μM) régulent négativement l'activité de NKCC1, à des concentrations plus élevées (50 μM), il bloque l'activation de NKCC1 et de KCC2. Par conséquent, j'ai émis l'hypothèse que l'utilisation de doses faibles et élevées de bumétanide peut modifier différemment l'activité épileptiforme enregistrée dans l'EC de rat juvénile in vitro lors de l'application de 4AP.

L'épilepsie du lobe temporal mésial (MTLE) est l'un des types d'épilepsie focale les plus répandus et se caractérise par une activité neuronale hypersynchrone provenant de structures limbiques telles que le cortex entorhinal (CE) qui entraîne des crises spontanées. Des preuves expérimentales ont montré que l'activité épileptiforme est générée après le blocage de la signalisation des récepteurs GABAA. Le bloqueur des canaux K+, la 4-aminopyridine (4AP) peut également induire des événements interictaux et ictaux dans les préparations in vivo et in vitro.

J'ai découvert que le blocage de l'activité de NKCC1 avec de faibles doses de bumétanide (10 μM) : (i) diminuait significativement la survenue de décharges intercritiques sans modifier leur durée ; et (ii) augmenté la durée des événements ictaux tout en diminuant leur taux d'occurrence. Le blocage à la fois de NKCC1 et de KCC2 avec des concentrations élevées de bumétanide (50 μM) a induit une réduction significative de la durée et du taux d'occurrence des événements ictaux.

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Contributions of Authors

The current original manuscript as a thesis was written by the candidate, Shabnam Shirdel under the supervision of Dr. Massimo Avoli. The supervisor and Dr. Maxime Lévesque guided this work and revised the final draft of this thesis. All literature reviews, experimental works and data analysis included in this thesis were performed by Shabnam Shirdel for this original research project. Dr. Avoli developed the research idea for this thesis project and established the protocol.

List of Abbreviations

4AP: 4-Aminopyridine

ACSF: Artificial Cerebrospinal Fluid

CCC: Cation Chloride Co-transporters

CNS: Central Nervous System

DG: Dentate Gyrus

EC: Entorhinal Cortex

EEG: Electroencephalographic

GABA: Gamma-Aminobutyric acid

KCC: K^+- Cl^- Co-transporter

LFP: Local Field Potentials

MTLE: Mesial Temporal Lobe Epilepsy

NKCC: Na^+- K^+- Cl^- Co-transporter

SE: Status Epilepticus

TLE: Temporal Lobe Epilepsy

Chapter 1: Introduction

In this chapter, a brief introduction to MTLE which is one of the most common forms of focal epilepsy, and its various experimental models were made, particularly those performed in *in vitro* preparations. This is followed by an overview of pathological neural synchronization. Finally, I investigated the role of NKCC1 and KCC2 co-transporters and their antagonist, bumetanide in ictogenesis.

1.1. Mesial Temporal lobe Epilepsy (MTLE)

Epilepsy is among the most prevalent and complex neurological disorders characterized by the tendency of the brain to generate recurrent seizures due to aberrant or excessive synchronous neuronal activity in the brain which manifests as spontaneous seizures (Fisher et al., 2005; Jiruska et al., 2013). Although seizures are not always localized to a specific region, as epileptic activity propagates to distinct areas, it can have distinctive characteristics depending on the brain regions engaged in their initiation. Based on these characteristics and initiation regions, in the brain, they are divided into generalized or focal seizures (Engel, 2005; Ono & Galanopoulou, 2012).

Temporal lobe epilepsy (TLE) is a form of focal lobe epilepsy which originates from the temporal lobe area of the brain. Approximately 60% of focal epileptic patients suffer from TLE (Sloviter, 2005; Engel, 1996). However, one of the most frequent forms of TLE is MTLE in which recurrent seizures related to abnormal neural activities initiate from the inner part of the temporal lobe, hippocampus proper and para-hippocampal structures such as the amygdala and EC (Quesney, 1986; Wennberg et al., 2002; Behr et al., 2014).

Although the precise cause of MTLE is still uncertain, it usually starts during the early years of life as a consequence of a neurological insult such as birth trauma or complicated febrile seizures (Behr et al., 2014; Fisher et al., 2005; Quesney, 1986; Wennberg, 2002; Harvey et al., 1995). MTLE affects approximately 60% of patients suffering from focal epilepsy, and despite an increasingly growing number of available pharmacological treatments in recent decades, anti-epileptic drugs are still ineffective in approximately 30% of MTLE patients and a majority of

MTLE patients become resistant to medications over time (Semah et al., 1998; Engel et al., 1997; Wieser et al., 2001). Therefore, a significant amount of research has been performed to investigate epileptiform activity generated by limbic structures in MTLE for developing new treatment strategies (Brodie et al., 2014; Löscher & Schmidt, 2011; Pohlen et al., 2017).

In the brain, MTLE is reproduced in rodents using chemoconvulsants (e.g., pilocarpine, kainic acid) (Lévesque et al., 2016; Sharma et al., 2008). Moreover, similar epileptiform activity can be also produced in *in vitro* preparations with chemical manipulations that lead to hyperexcitability in neurons (Lévesque & Avoli, 2021). Several experimental procedures that were previously employed both *in vivo* as a chronic model and *in vitro* as an acute animal model of epilepsy informed us about the mechanisms that underlie epileptic synchronization (Jefferys et al., 2012a). These reports underscore the importance of developing new pharmaceutical treatments for patients with MTLE. Therefore, my experiments aimed to investigate the primary mechanism leading to epileptiform synchronization in the limbic structures involved in MTLE.

1.2. Pathological Neuronal Synchronization

In the brain, synchronized neural activity and their interaction across distinct areas such as between limbic structures and local neuronal networks are the main features of a healthy brain which controls physiological functions of the brain such as cognitive, memory as well as sleep (Buzsáki & Draguhn, 2004). Neuronal synchronization, which is differentiated into local and long-range synchrony at different levels (Jefferys et al., 2012b; Jiruska et al., 2013), is associated with electrical activity, and chemical synaptic transmission, which is reflected by local field potentials (LFP) and make a significant contribution to the electroencephalographic (EEG) activity of the brain (Timofeev et al., 2012). However, alteration of such synchronization, which can result in abnormal activity within the brain, can be associated with a variety of neurological disorders (Uhlhaas & Singer, 2010). Epilepsy is one of those neurological disorders that can be significantly contributed by an altered neuronal synchronization (Popovych & Tass, 2014). Therefore, the coordination of neuronal activity becomes pathological in an epileptic brain and plays a crucial role in the generation of epileptiform activity which results in seizures in MTLE (McIntyre & Gilby, 2006).

1.3. Epileptiform Activity

In the epileptic brain, pathological neuronal synchronization, which can impair brain functions, has been investigated in limbic structures which are seizure-prone areas in the brain, and specifically, it has been reported that seizure occurrence could be a consequence of hyperexcitability due to the malfunction or imbalance in inhibitory GABAergic and excitatory glutamatergic synaptic transmission (Haliski & White, 2015; Huberfeld et al., 2011). Moreover, several studies have found that seizures could arise from the synchronization of inhibitory interneurons population (Avoli & de Curtis, 2011; de Curtis & Avoli, 2016; Gnatkovsky et al., 2008). In the epileptic brain, synchronization can sometimes become pathological through various mechanisms. One such mechanism involves excitation between hippocampal pyramidal cells, especially in the CA3 subfield of the hippocampus, which can produce a synchronized epileptiform depolarization (Köhling et al., 2000; Jefferys et al., 2012b). Under specific conditions, pyramidal cells can fire bursts of action potentials, recruiting a critical number of neurons to produce epileptiform activity (Traub & Wong, 1982). A second synaptic mechanism involves a network of inhibitory interneurons that can contribute to ictogenesis by entraining populations of inhibitory neurons to generate a hypersynchronous activity (Mann & Mody, 2008; de Curtis & Gnatkovsky, 2009). The synchronous activity of interneuron networks by releasing GABA neurotransmitters which bind to $GABA_A$ receptors, can accumulate intracellular Cl^- through the functions of cation-Cl^--co-transporter (CCC), KCC2 which subsequently leads to an increase in extrusion of Cl^- and K^+ into the extracellular environment (Rivera et al., 2005; Viitanen et al., 2010).

Excessive intraneuronal activity can thus lead to an increase in extracellular K^+ concentrations, depolarizing neurons and recruiting them into the seizure activity (Traynelis & Dingledine, 1988; Zuckermann & Glaser, 1968). On the other hand, synchronous interneuronal activity can regulate the activity of principal cells. Recovery from such inhibition causes neuronal excitation, which generates epileptiform discharges (Jefferys et al., 2012a). Also, EEG recording might be used to record epileptiform activity from epileptic patients and animal models; ictal and interictal discharges are examples of these abnormal events, which reflect the aberrant synchronization of neuronal networks (Avoli & de Curtis, 2011; Behr et al., 2014; Cohen et al., 2002; Engel, 2001).

Interictal discharges are short hypersynchronous events (de Curtis & Avanzini, 2001), that appear on the EEG activity of epileptic patients and in animal models of epilepsy. Researchers have been able to examine these interictal events in animal models of limbic seizures that are electrographically similar to those seen in MTLE patients to investigate their contributions to seizures (Jensen & Yaari, 1988).

In previous *in vitro* studies, interictal discharges in brain slices during bath application of the K^+ channel blocker, 4AP, which can enhance both inhibitory and excitatory transmission, have indicated two different types of interictal discharges (Avoli et al., 1996a). The first type of interictal, which can initiate at various sites of the brain, and propagate to other brain regions, are slow long-lasting discharges that occur at a low rate (Perreault & Avoli, 1992; Voskuyl & Albus, 1985). The second type originates in the CA3 region of the hippocampus and quickly propagates to the entorhinal cortex (EC) through the CA1 hippocampal region and subiculum and returns to the hippocampus via the dentate gyrus (DG) (Perreault and Avoli, 1992; Morris et al., 1996; Barbarosie & Avoli, 1997). This type is characterized by fast discharges and has a higher occurrence rate (Benini et al., 2003). According to pharmacological studies, slow interictal discharges are mainly associated with a GABA-mediated depolarization (Avoli et al., 1996b), whereas fast interictal discharges are associated with the activity of glutamatergic receptors (Perreault & Avoli, 1991).

On the other hand, ictal discharges are a long series of large-amplitude EEG spikes caused by hypersynchronous neuronal network activity (Avoli et al., 1996a; Brückner & Heinemann, 2000). Ictal discharges can be distinguished from baseline activity as well as various types of interictal discharges with a large amplitude which occur regularly between seizures (de Curtis et al., 2012; Schwartzkroin & Prince, 1980). A previous study has shown that inhibitory interneuron networks can be associated with the generation of ictal and interictal events by facilitating hypersynchronous activity. For example, the synchronous activity of the interneuronal network imposes the firing of the large population of principal cells resulting in the generation of the hypersynchronous firing of principal cells (Mann and Mody, 2008).

Electrophysiological investigations of ictal discharges induced by 4AP in *in vitro* preparations have revealed several similarities between ictal onset events and slow interictal discharges (Avoli

& de Curtis, 2011). Furthermore, both slow interictal discharges and those receding ictal discharges have been shown to be accompanied by transient increases in extracellular concentrations of K^+ which are $GABA_A$ receptor-dependent (Avoli et al., 1996a; Morris et al., 1996). These studies also have supported previous findings obtained from *in vitro* hippocampal slice preparations in the guinea-pig (Barolet & Morris, 1991; Uva et al., 2008).

Experimental reports show that the tendency of fast interictal discharges to inhibit the transient elevations in extracellular K^+ associated with slow interictal discharges, which have been shown to contribute to the ictal onset, appears to be the basis for their propensity to ictogenesis control (Avoli et al., 1996a). Fast interictal discharges, which are mostly glutamatergic but are also contributed by $GABA_A$ and $GABA_B$ receptor currents, may restrict excessive levels of synchronization by controlling GABA release and the associated increases of extracellular K^+ ions during slow interictal events, consequently affecting ictogenesis. One previous study has also supported this proposition in which electrical stimulation of para-hippocampal structures of the limbic system comparable to frequencies of fast interictal discharges can control the occurrence of ictal discharges in that region (Avoli et al., 2013).

In addition, intracellular recordings obtained from principal neurons have indicated that these ictal events are triggered by long-lasting GABAergic mediated potentials that reflect the synchronous activity of those interneurons (Avoli & de Curtis, 2011). These discoveries are supported by studies that indicate depression of ictal discharges when $GABA_A$ receptors are antagonized (Avoli et al., 1996b).

Fast interictal discharges have been shown to control the propensity of para-hippocampal structures such as EC to generate ictal discharges (Swartzwelder et al., 1987). This phenomenon was reported in brain slices during bath application of 4AP or Mg^{2+}-free ACSF, where ictal discharges were eventually abolished from the EC, whereas fast interictal spikes continued to occur for several hours (Barbarosie & Avoli, 1997). Consequently, as ictal discharges can be recorded in the EC of brain slices obtained from rodents, I devoted my research efforts to this brain region.

1.4. Entorhinal Cortex (EC)

The EC is a structure in the medial temporal lobe which is reciprocally connected with the hippocampus proper (Amaral et al., 1987). The EC comprises six layers and is divided into superficial layers and deep layers (Insausti et al., 1995). Moreover, morphologically it is divided into medial and lateral entorhinal cortices (Uva et al., 2004). EC plays an important role in memory functions such as memory formation and consolidation in the normal brain (Kitamura & Inokuchi, 2014), as well as in pathological conditions such as MTLE (Vismer et al., 2015). The main inputs of the EC come from the piriform cortex and hippocampus; however, the main outputs of this region is the hippocampus which results in the contribution of EC to the seizure propagation (Jones & Lambert, 1990; Sewards & Sewards, 2003; Vismer et al., 2015).

A decrease in EC volume of MTLE patients and animal models of this disorder caused by cell loss during epilepsy has also been shown by numerous research in the field of volumetric analysis (Bonilha et al., 2003; Bartolomei et al., 2005; Scholl et al., 2013). Such neuronal loss consequences in wiring modification which increases the level of neuronal excitability and epileptiform activity (Vismer et al., 2015).

According to several studies, other limbic structures, specifically the EC, contribute to the generation of MTLE seizures (Elkommos et al., 2015). This part of the temporal lobe has an essential role in processing higher-order sensory information and sending them to the hippocampus (Fyhn et al., 2004). Evidence shows numerous reciprocal connections between the EC and other regions of the brain (Barbarosie and Avoli, 1997). These results indicate that the EC might be a potential area for initiating and distributing epileptic activities related to MTLE. In general, a vast amount of *in vitro* research reported that EC could generate ictal events (Avoli et al., 1996b).

Several investigations reported that the subiculum, which connects the EC and hippocampus, may regulate the propagation of epileptiform activity. Axons from the EC propagate through the perforant pathway to the granule cells of the DG for such propagation. DG granule cells send their mossy fiber-containing axons to CA3 pyramidal cells, which in turn send them through the Shaffer collaterals to the CA1 area. By sending a projection via the subiculum to the EC, CA1 finally completes this circuit. (Barbarosie et al., 2000; Barbarrosie and Avoli, 1997).

Other research indicates that when more atrophy is observed in EC, more interaction between EC and hippocampus in seizure generation is expected (Bernasconi et al., 2001). These results might be interpreted considering research in EC pathology in the generation of seizure activities. In addition, in terms of histological results and correlations between EC and pathological measures, neural cell loss is observed in superficial layers of the EC, and animal works with models of MTLE reported that the neural loss is especially observed in layer 3 of EC (Scholl et al., 2013; Du et al., 1995) that is in line with human studies (Du et al., 1993).

1.5. Animal Model of Mesial Temporal Lobe Epilepsy

During the last decades, animal models of epileptic disorders improved our understanding of the underlying mechanisms of epilepsy and epileptic seizures. However, despite many efforts in this line of research and the discovery of new drugs, these models might not provide specific targets for drug-resistant seizure activities (Semah et al., 1998; Wiebe, 2000). In particular, for MTLE, the main procedure includes respective surgery, and more research is needed in this area. Both *in vivo* and *in vitro* seizure models allow the replication of epileptiform activity in simplified experimental preparations and can provide insights into the pathophysiology. In this thesis, I investigated the *in vitro* preparations utilized in epilepsy research.

The *in vivo* chronic animal models of epilepsy have been used to study the mechanisms of epileptogenesis. *Status epilepticus* (SE) which is the seizure activity of more than 10 minutes duration can be induced by several procedures, including the locally or systematically injection of kainic acid as well as pilocarpine, followed by a latent period before the onset of the first refractory spontaneous seizure (Lévesque and Avoli, 2013; (Vicedomini and Nadler, 1987).

On the other hand, *in vitro* preparations have become a powerful method for investigating the underlying mechanisms of ictogenesis (Raimondo et al., 2017). These preparations can be mostly obtained from mammalian brain slices. *In vitro* epileptiform activity can be evoked by performing ionic or pharmacological manipulations on preparations from normal animals, by using tissue from animals which have experienced an epileptogenic *in vivo* insult, or by using genetic models of epilepsy with an *in vitro* phenotype (Hablitz, 1984; Wong and Traub, 1983).

In vitro epileptiform activity has been elicited from brain preparations derived from multiple mammalian species such as mice, rats, and mice as well as epileptic humans following neurosurgical resections (Raimondo et al., 2017; Peyrache & Destexhe, 2019). Rats have been the most commonly used species, with multiple different strains being employed. However, the relative ease of modifying the mouse genome and the recent widespread use of transgenic mice strains have increased the popularity of mouse models for experimental use. Nonetheless, the choice of species will depend on the specific scientific question asked. Using this *in vitro* preparation, investigators have elucidated some of the intrinsic neuronal and synaptic properties that appear to be involved in the generation of burst activity and hyperexcitability typical of the epileptic brain.

Several experimental models used certain drugs to induce depolarization shifts linked to the interictal activity observed in EEG of epileptic human patients. These models are appropriate for understanding the generation of MTLE which indicate that many factors contribute to such activities, including inhibition reduction in the brain, potentiation of post-synaptic excitatory potentials, activation of NMDA receptors, and excitation synchronization. Based on these models, there is extensive evidence that convulsant chemicals used in these models reduce synaptic inhibition that is obtained with GABA (Gören et al., 2001).

Based on previous studies of *in vitro* slice preparations, using various drug treatments to control the extracellular membrane has helped to identify the underlying mechanisms of the initiation, propagation, and termination of epileptiform activity. In *in vitro* slice preparations, K^+ channel blockers such as 4AP and $GABA_A$ receptor antagonists such as bicuculline and gabazine are the most commonly used drugs for inducing epileptiform activity (Avoli & Jefferys, 2016).

Therefore, in order to investigate the pathophysiological mechanisms underlying the generation of epileptiform activity during the situation that synaptic inhibition is maintained, 4AP could be a suitable model to induce such discharges to take effect in normal or increased synaptic inhibition (Perreault and Avoli, 1992). In this thesis, 4AP bath application in an *in vitro* juvenile intact brain slice preparation was used to induce both ictal and interictal discharges.

1.6. Gamma-Aminobutyric Acid (GABA)

A few decades ago, the link between seizures and GABA was discovered with reports of seizures in infants given formula lacking pyridoxine which is a necessary coenzyme for the GABA synthesis from the glutamic acid (Coursin, 1954; Molony & Parmelee, 1954). Afterwards, several studies have demonstrated that some chemicals that might induce interictal and ictal discharges in *in vivo* (Ayala et al., 1973; Dichter & Spencer, 1969) and *in vitro* (Schwartzkroin & Prince, 1977) preparation are $GABA_A$ receptor antagonists. Additionally, it was established that interictal events (Dingledine & Gjerstad, 1980; Schwartzkroin & Prince, 1980) and ictal discharges (Ben-Ari et al., 1979; Kostopoulos et al., 1983) were significantly associated with a reduction in GABA-related inhibition.

In the adult mammalian brain, releasing GABA from the axonal end of interneurons inhibits neuronal firing by activating two different types of GABA receptors including, $GABA_A$ and $GABA_B$ (Farrant & Kaila, 2007; Cherubini, 2012). GABA activates the $GABA_A$ receptor channels which leads to Cl^- inward flow and consequently inhibits neuronal firing by hyperpolarizing the membrane of the cell (Galanopoulou, 2008). Notably, employing various antagonists of $GABA_A$ receptor-mediated currents contributes to the onset of ictogenesis in the limbic areas such as EC, and they are correlated with marked increases in extracellular levels of K^+ (Isaeva et al., 2010; Galanopoulou, 2008).

During postnatal development, instead, GABA, by binding and activating the $GABA_A$ receptor, depolarizes and excites targeted cells by an outward flux of Cl^- (Ben-Ari et al., 1989). Such a higher intracellular level of Cl^- is affected by the different expressions and activities of the CCCs, such as NKCC1, and KCC2, which mediate the Cl^- uptake and extrusion, respectively (Kaila et al., 2014). However, downregulation of NKCC1 expression during development results in Cl^- extrusion, and consequently causes the switch of GABA action from depolarizing to hyperpolarizing (Rivera et al., 1999).

While the excitatory action of GABA plays an essential role in neuronal development and synaptogenesis in neonates, it also accounts for the increased seizure susceptibility and poor efficiency of GABAergic anticonvulsants in developing brains (Kahle & Staley, 2008). Bumetanide, an NKCC1 and KCC2 inhibitor related to loop diuretics, has already been used in

animal models to decrease epileptiform activity in both *in vitro* slice preparations and *in vivo* in animal models of neonatal seizures (Dzhala et al., 2012; Savardi et al., 2021). The role of NKCC1 in controlling the excitatory action of GABAergic neurotransmission in neonates makes it a potential target for developing a new anticonvulsant strategy to treat neonatal seizures by using bumetanide (Kahle & Staley, 2008; Löscher et al., 2013). It, therefore, helps to understand the mechanisms involved in seizure initiation/generation to develop more effective, mechanism-targeted therapeutic interventions and to identify efficient predictive parameters (Kahle & Staley, 2008).

1.7. Cation-Chloride Co-transporters (CCCs)

As described in the previous section, the intracellular concentration of Cl^- in the nervous system determines the polarity of the GABA-mediated neurotransmission (Schulte et al., 2018). As a consequence of the neuronal Cl^- gradients reversal, GABAergic neurotransmission shifts from excitatory to inhibitory throughout development (Ben-Ari, 2014). Therefore, the maintenance of a transmembrane ionic gradient for Cl^- is critical for preserving fast synaptic transmission through ligand-gated ion channels. In terms of $GABA_A$ receptor signaling, the intracellular Cl^- level is regulated by the CCCs (Chamma et al., 2012).

The CCCs encoded by the solute carrier12 (SLC12) superfamily genes are secondarily active transporters proteins (Medina et al., 2014). These co-transporters actively modulate the transport of Cl^- anions across the cell membrane along with Na^+ and K^+ cations. Therefore, they play important roles in physiological processes such as regulation of ion absorption and maintaining intracellular Cl^- homeostasis and neuronal excitability (Chew et al., 2019; Kaila et al., 2014). There are three large groups of CCCs family proteins: K^+-Cl^- co-transporters (KCC1-4); Na^+-K^+-Cl^--co-transporters (NKCC 1 and 2); and Na^+-Cl^- co-transporter (NCC) (Medina et al., 2014; Payne et al., 2003). The two primary and main CCC families in the central nervous system (CNS) are NKCC1 and KCC2, which by accumulating and extruding Cl^-, respectively, play a major role in maintaining intracellular Cl^-. KCC2, a Cl^- exporter, uses K^+ gradients for maintaining intracellular Cl^- at a lower level. In comparison, NKCC1 mediates Cl^- influx using Na^+ gradients across the cell membrane to maintain homeostasis (Delpire, 2000; Gamba, 2005; Kahle et al., 2008).

KCC2 expression is limited to the central nervous system and can be found in the dendrites and cell body of neurons, and also in various cells such as interneurons and cortical pyramidal cells, spinal cord (Barthó et al., 2004; Benarroch, 2013; Blaesse et al., 2009; Kahle & Staley, 2008; Kanaka et al., 2001). The expression of KCC2a, one of the KCC2 variants, does not often change throughout life. In contrast, in rodents, KCC2b expression increases primarily during the first week after birth (Uvarov et al., 2007).

On the other hand, NKCC1, which was first identified in ehrlich cells, expression is widely distributed throughout the body, and it is not limited to the brain. In the nervous system, it can be widely found in various parts of neurons, such as in the axonal and somatodendritic components as well as in the brain endothelial and glial cells (Benarroch, 2013; Kahle & Staley, 2008). Recent studies have reported that NKCC1 is downregulated throughout development and also, the expression of KCC2 increases in an adult brain (Aronica et al., 2007; Dzhala et al., 2005; Plotkin et al., 1997). It is believed that such developmental decreases in NKCC1 expression along with increases in KCC2 levels are indeed what contribute to the E_{GABA} transition from depolarizing to hyperpolarizing condition during the development of CNS (Benarroch, 2013; Cherubini et al., 1991; Rivera et al., 1999). Additionally, according to a previous study, NKCC1 could also facilitate seizures in the developing brain (Dzhala et al., 2005) by accumulating intracellular Cl^- in the hippocampal pyramidal neurons, thereby reducing the inhibition mediated by $GABA_A$ receptors. Nonetheless, NKCC1 and KCC2 blocker, bumetanide, can prevent seizure-induced neuronal Cl^- accumulation (Dzhala et al., 2010).

1.8. Roles of NKCC1 and KCC1 in MTLE

Based on several studies, NKCC1 and KCC2 malfunctioning has been associated with different neurological disorders, including brain ischemia and epilepsy (Löscher et al., 2013; Miles et al., 2012) and is considered to be a critical regulator of GABAergic receptors in the brain (Huberfield et al., 2007). In this regard, disturbances in NKCC1 and KCC2 with unknown causes might contribute to ineffective inhibition.

According to earlier studies, upregulation of NKCC1, and a deficiency in KCC2 expression increase the intracellular concentration of Cl^- in a developing brain. Such an increase in the intracellular level of Cl^- results in an outflow of Cl^- ions and subsequently leads to neural depolarization. On the other hand, downregulating in NKCC1 increasing KCC2 expression results in a net Cl^- influx (Löscher et al., 2013; Markadieu & Delpire, 2014).

Further evidence suggests that the downregulation of NKCC1 changes the equilibrium potential of Cl^- from depolarization to hyperpolarization in neonates (Hübner et al., 2001). In addition, at postnatal day 5 (P5), under-developed neural cells in neonates accumulate Cl^- ions leading to depolarization (Jansen et al., 2010). These findings are in line with animal models that showed depolarizing GABAergic signaling at P4-14 in rats (Galanopoulou, 2008). These results were also obtained regarding the areas that contribute to MTLE such as the hippocampus (Dzhala et al., 2005). It is also reported that GABA has depolarization effects in neonate rats, and in the postnatal period, increased KCC2 changes this effect to hyperpolarization in developed neural cells (Rivera et al., 1999).

Genetic factors that decrease NKCC1 and KCC2 in mice also enhance the intracellular concentration of Cl^-, and hyperexcitability is observed in these animal models (Dzhala et al., 2008). Human studies also suggest that gene expression factors related to NKCC1 influence electrophysiological measures in patients with epilepsy (Kahle et al., 2014; Schulte et al., 2018). Several reports demonstrated that NKCC1 enhancement and reduction in KCC2 are related to mRNA and protein levels in seizure models of adult animals (Palma et al., 2006; Kahle et al., 2014). Therefore, Cl^- plasticity impairment contributes to the development of epileptiform activity in later stages (Sivakumaran & Maguire, 2016).

In this regard, decreased GABAergic inhibition is indicative of seizure activity mechanisms (Talos et al., 2012). It might be stated that NKCC1 blockage suppresses interictal discharges based on the GABAergic system in MTLE. In general, one might conclude that the excitatory aspects of the GABAergic system are responsible for MTLE epileptogenicity. Indeed, excitation induced by GABA has been shown to be caused by alterations in NKCC1 and contribute to the precipitation of epileptic activity in MTLE (Blumcke et al., 2013; Palma et al., 2006). Moreover, Pathak et al. (2007) similarly demonstrated a decrease in KCC2 expression and an increase in E_{GABA} in the rat

hippocampus. As a result, decreased KCC2 expression disrupts intracellular Cl^- and subsequently reduces inhibition. Therefore, more research and investigation are required to investigate the role of $GABA_A$ receptor-mediated signaling in epileptiform synchronization as well as its contribution to ictogenesis.

1.9. Research Rationale

Over the past few decades, new research has revealed some of the primary mechanisms and factors of epileptiform activity. The molecular and pharmacological mechanisms of epilepsy have been extensively studied *in vitro* using animal slices and human epileptic tissues. Furthermore, recent studies have demonstrated that GABAergic mechanisms can considerably contribute to the epileptiform activity (Khazipov, 2016).

A precise balance between NKCC1 and KCC2 activity is necessary for GABA neurotransmitter actions such as inhibitory GABAergic signalling in the adult brain, and for excitatory GABAergic signalling in the developing brain (Delpire, 2000). However, alterations in the balance of NKCC1 and KCC2 activity determine the switch from depolarizing to hyperpolarizing effect of GABA during the first postnatal week (Huberfield et al., 2007). And in juvenile rat brains (10-25 day-old), such switch may not be complete thus making neuronal networks hyperexcitable due to residual GABA-mediated depolarizations. Nowadays, a loop diuretic class of drug, bumetanide, is the only clinically available drug with selectivity for NKCCs, and KCCs and therefore has played a potential role as a method for treatment of seizures and prevention of epilepsy (Kahle et al., 2008; Löscher et al., 2013).

According to prior research, downregulating the activity of either NKCC1 or both NKCC1 and KCC2 with low and high concentrations of bumetanide, respectively, alters interictal and ictal synchronization in juvenile EC in a way that is different from what was reported in adult brain slices (Hamidi & Avoli, 2015). Moreover, according to electrophysiological recordings of *in vitro* from epileptic human brain tissues, bumetanide modifies epileptiform discharges generated by subicular neurons (Cherubini et al., 2022). In my research, both interictal and ictal events were produced by in *in vitro* EC intact brain slice preparations of juvenile rats.

Therefore, I hypothesized that NKCC1 and KCC2 activities in the juvenile rat (22-23 day-old) might contribute to the generation of ictal and interictal discharges in the EC during the application of 4AP and antagonizing the activity of these co-transporters could thus modify ictal and interictal discharges.

Chapter 2: Experimental Procedures and Methods

2.1. Animals

All experimental procedures presented in this thesis were approved by the Animal Care Committee of McGill University before use and conducted in accordance with the protocols and standard procedures of the Canadian Council on Animal Care. All efforts were made to reduce the number of animals and their suffering. I used 32 slices from 7 juvenile male Sprague-Dawley rats obtained from Charles River laboratories. The rats were housed in cages under a standard rodent vivarium environment with a 12-h light/12-h dark lights cycle. Food and water were available to all animals without restriction.

2.2. Brain Slice Preparation and Maintenance

Juvenile, male Sprague-Dawley rats (22-23 day-old; Charles River Laboratories, Saint-Constant, QC, Canada) were sacrificed under isoflurane anesthesia. The brain was then carefully extracted and placed in a cold (1–3°C) oxygenated ACSF solution containing (in mM): 124 NaCl, 2 KCl, 2 $CaCl_2$, 2 $MgSO_4$, 1.25 KH_2PO_4, 26 $NaHCO_3$, 10 D-glucose. A vibrating microtome is used to cut horizontal brain slices (450 μm thick) including the EC. Afterwards, the slices were transformed into a recovery interface tissue chamber for constantly perfused with oxygenated ACSF (95% O_2, 5% CO_2) with a pH of 7.4 at 31–32°C temperature. 4AP (50 μM), and bumetanide (10 μM or 50 μM) were continuously bath applied with a 1-2 ml/min flow rate. Sigma-Aldrich Canada was supplied the chemicals (Oakville, Ontario, Canada).

2.3. Electrophysiological Recordings

After 1 hour of continuous ACSF perfusion, ACSF-filled glass pipettes (1B150F-4; World Precision Instruments, Sarasota, Florida, USA; tip diameter 10 μm, resistance 5-10 MΩ) were connected to amplifiers and used to record field potentials. In these experiments, Interictal

discharges were characterized by slow spikes that occurred every 21.5 ± 1.50 s and had a duration of 2.5 ± 0.15 s in the EC. Moreover, ictal discharges recorded from EC occurred every 236.2 ± 25.9 s and lasted 126.5 ± 5 s. The electrodes for recording were placed in the deep layer of the lateral EC. By using PCLAMP 9.2 software (Molecular Devices) field potential signals were sampled and recorded at 5000 Hz (Molecular Devices Digidata 1322A, Palo Alto, CA, USA). Afterwards, data were analyzed with CLAMPFIT 9.2 (Molecular Devices).

2.4. Statistical Analysis

In order to analyze the duration and occurrence rate of both interictal and ictal discharges, CLAMPFIT 9.2 was used (Molecular Devices). The period between the first deviation of the discharge from baseline and its return to baseline was used to characterize the duration of both interictal and ictal discharges. To find variations across experimental conditions in the duration and occurrence rate of both ictal and interictal discharges, raw data were converted to Z-scores, and I applied one-way ANOVAs followed by Tukey post-hoc tests. Statistical analyses were performed in MATLAB R2021a (MathWorks, Natick, MA) with a level of significance of $p < 0.05$. The number of slices used for analysis is shown by n, and results were presented as mean ± standard error of the mean (mean ± SEM).

Chapter 3: Results

3.1. 4-Aminopyridine-Induced Epileptiform Activity in Entorhinal Cortex

In this study, I recorded field potential activity from the EC region of intact brain slices (n=32 slices) obtained from 7 juvenile (22-23 day-old) rats during 4AP (50 μM) application (Fig. 1A). As illustrated in Fig. 1B,C, both interictal and ictal epileptiform events were induced in EC of juvenile rats by applying 4AP.

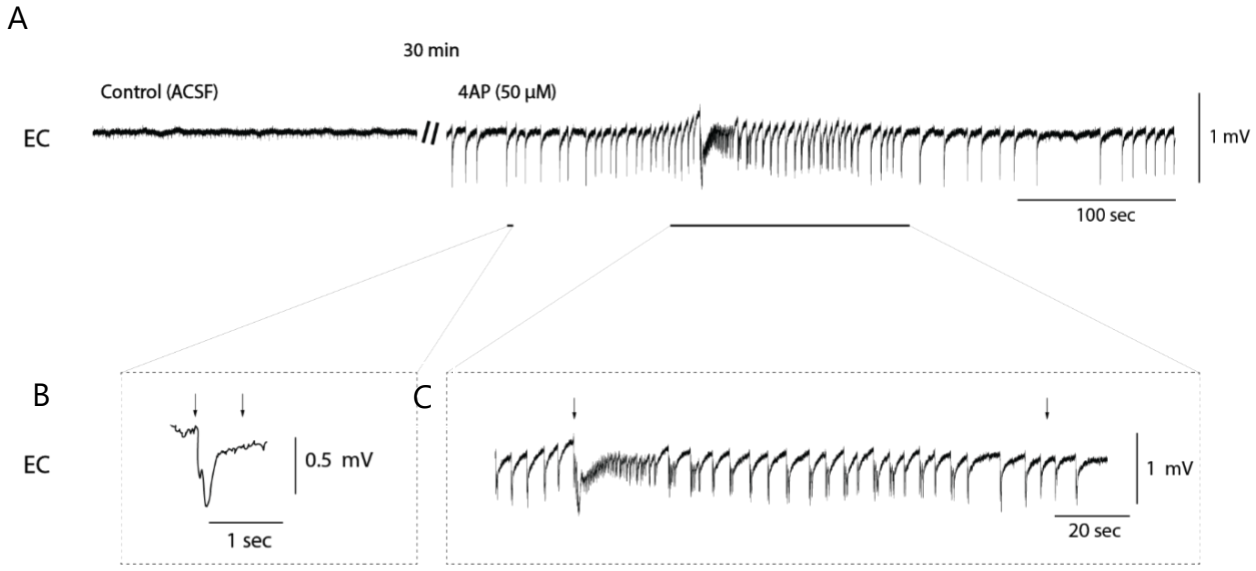

Figure 1. A: Field potential recordings obtained from EC during application of normal ACSF and 4AP in an intact brain slice that generated interictal and ictal discharges in the EC. **B:** Expanded sample of interictal discharges recorded in EC shown in **A**. **C**: Expanded sample of ictal discharges recorded in EC illustrated in **A**.

3.2. Effects of 10 µM bumetanide on Epileptiform Activity Induced By 4-AP

I investigated the effects of bumetanide which has a low affinity for KCC2 and a high affinity for NKCC1, on the 4AP-induced epileptiform discharges recorded from EC. It has been reported that at low doses (10 µM), bumetanide blocks NKCC1 whereas, at higher concentrations (50 µM), it inhibits both NKCC1 and KCC2 (Löscher et al., 2013). As shown in Fig. 2, blocking NKCC1 with 30 min application of bumetanide (10 µM) on the 4AP-induced epileptiform discharges recorded from the juvenile EC, significantly reduced the occurrence rate of interictal events without changing their duration. In addition, this procedure increases the duration of ictal events while it decreases the occurrence rate of ictal events in EC (n = 17 slices; ** $p < 0.01$).

In Fig 3. I also compared the duration and the average number of ictal and interictal events during 4AP (50 µM) and the application of bumetanide (10 µM). Application of bumetanide (10 µM; n=17) resulted in a significant reduction in the average number of interictal events without changing their duration (Fig. 3C,D; ** $p < 0.01$). In addition, this procedure increased simultaneously the duration of ictal events while it decreased the occurrence rate of ictal events in EC (Fig. 3A,B; * $p < 0.05$).

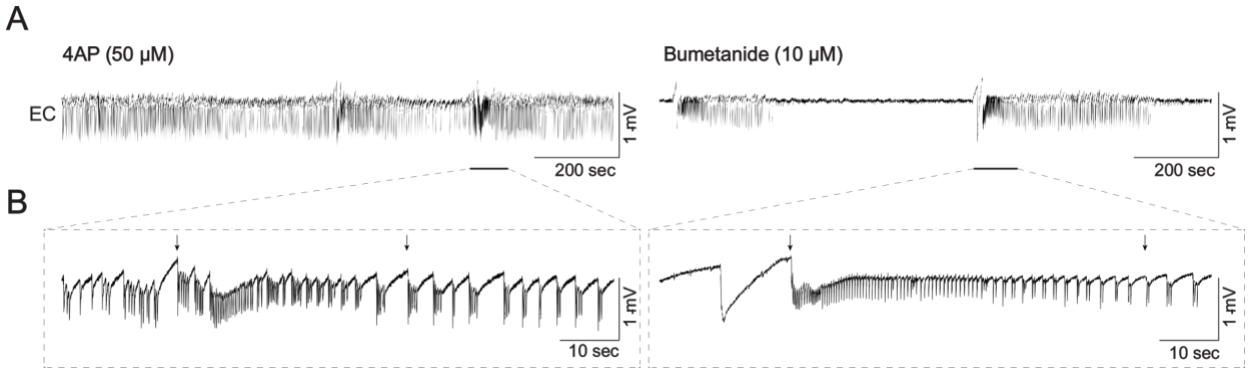

Figure 2. A: Field potential recordings obtained from the EC area during the application of 4AP (50 µM) and bumetanide (10 µM). Note that blocking NKCC1 with low doses of bumetanide significantly reduces interictal discharges in EC whereas ictal discharges continue to occur. **B:**

Enlarged portions of the recordings are shown in insets with traces that are further expanded at the start of an ictal discharge during 4AP after application of bumetanide.

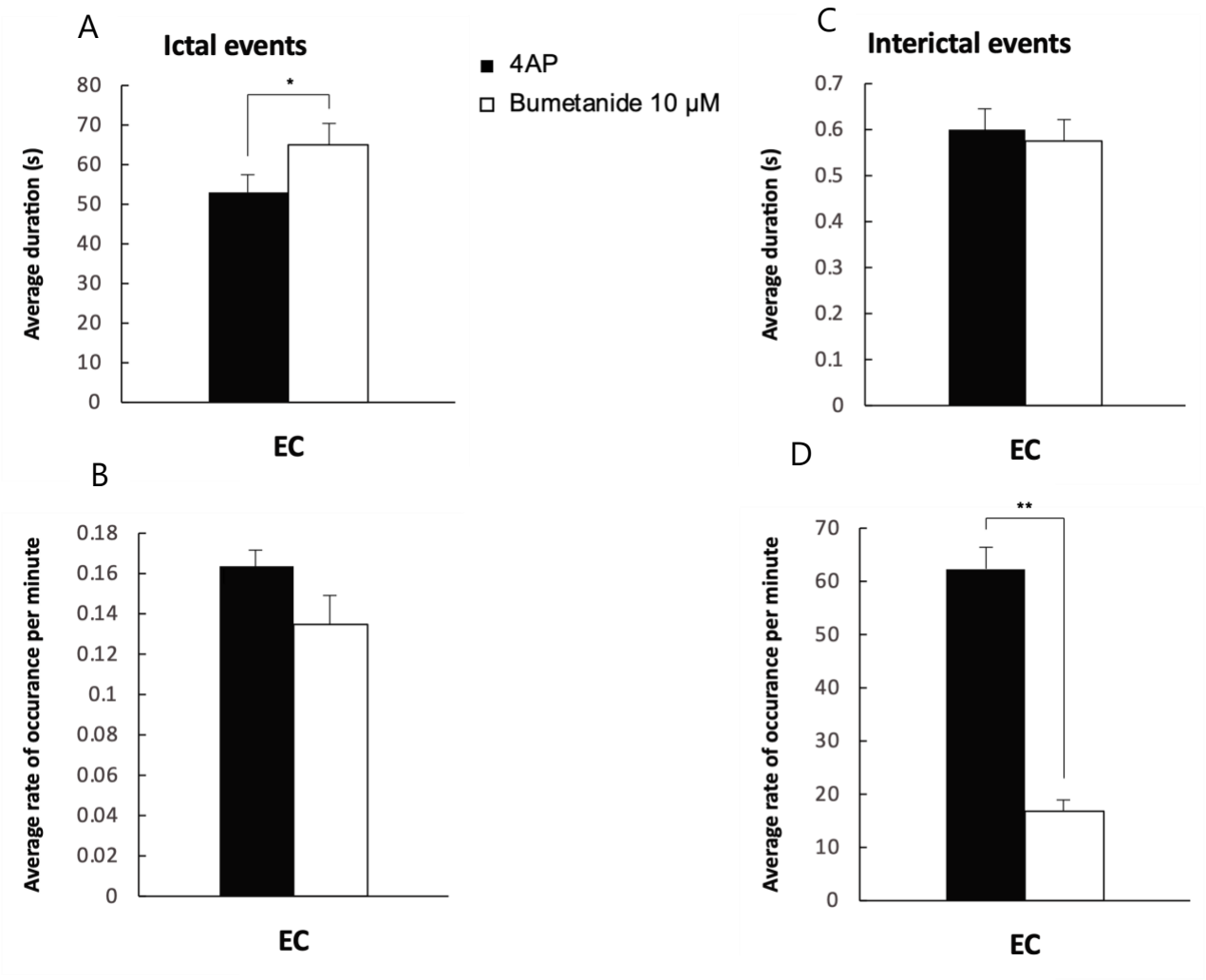

Figure 3. Bar graphs showing the average occurrence rate and duration of ictal and interictal discharges recorded from the EC under control conditions (4AP) and during application of 10 µM bumetanide. Note that blocking NKCC1 increases the duration (**A**) of ictal discharges in EC and does not have a significant effect on the occurrence rate (**B**) of ictal discharges. Whereas blocking NKCC1 with bumetanide (10 µM) does not influence the duration (**C**) of interictal discharge, the occurrence rate (**D**) of interictal events significantly decreases. * $p < 0.05$, ** $p < 0.01$.

3.3. Effects of 50 µM bumetanide on Epileptiform Activity Induced By 4-AP

Finally, I also blocked the activity of both NKCC1 and KCC2 with 50 µM bumetanide (Fig. 4). Blocking both NKCC1 and KCC2 with high concentrations of bumetanide (50 µM; n = 15 slices), induced a significant reduction in the ictal events' duration and rate of occurrence (Fig. 5A,B; ** $p < 0.01$; * $p < 0.05$). Also, while 50 µM bumetanide induced a slight reduction in the duration of the interictal discharges in EC, the rate of occurrence of interictal events slightly increased in the same region (Fig. 5C,D). These data are in agreement with what was reported in adult rats (Hamidi and Avoli 2015).

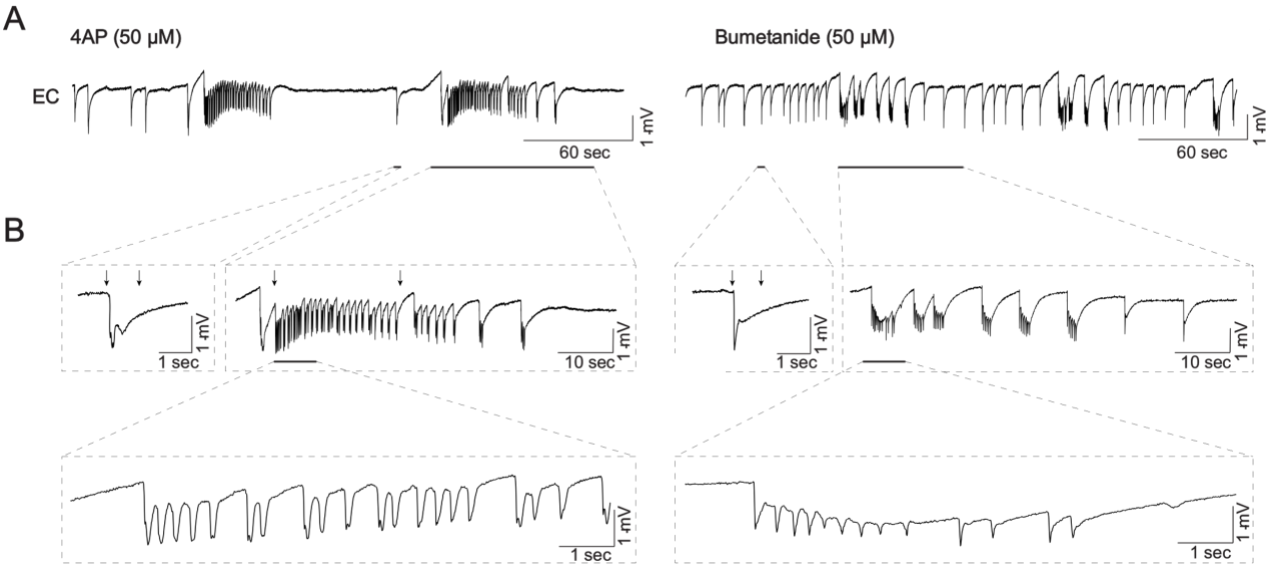

Figure 4. A: Demonstrated Effects induced by 50 µM bumetanide on the epileptiform discharges recorded from the EC during bath application of 4AP. **B:** Insets show examples of traces at an expanded time scale.

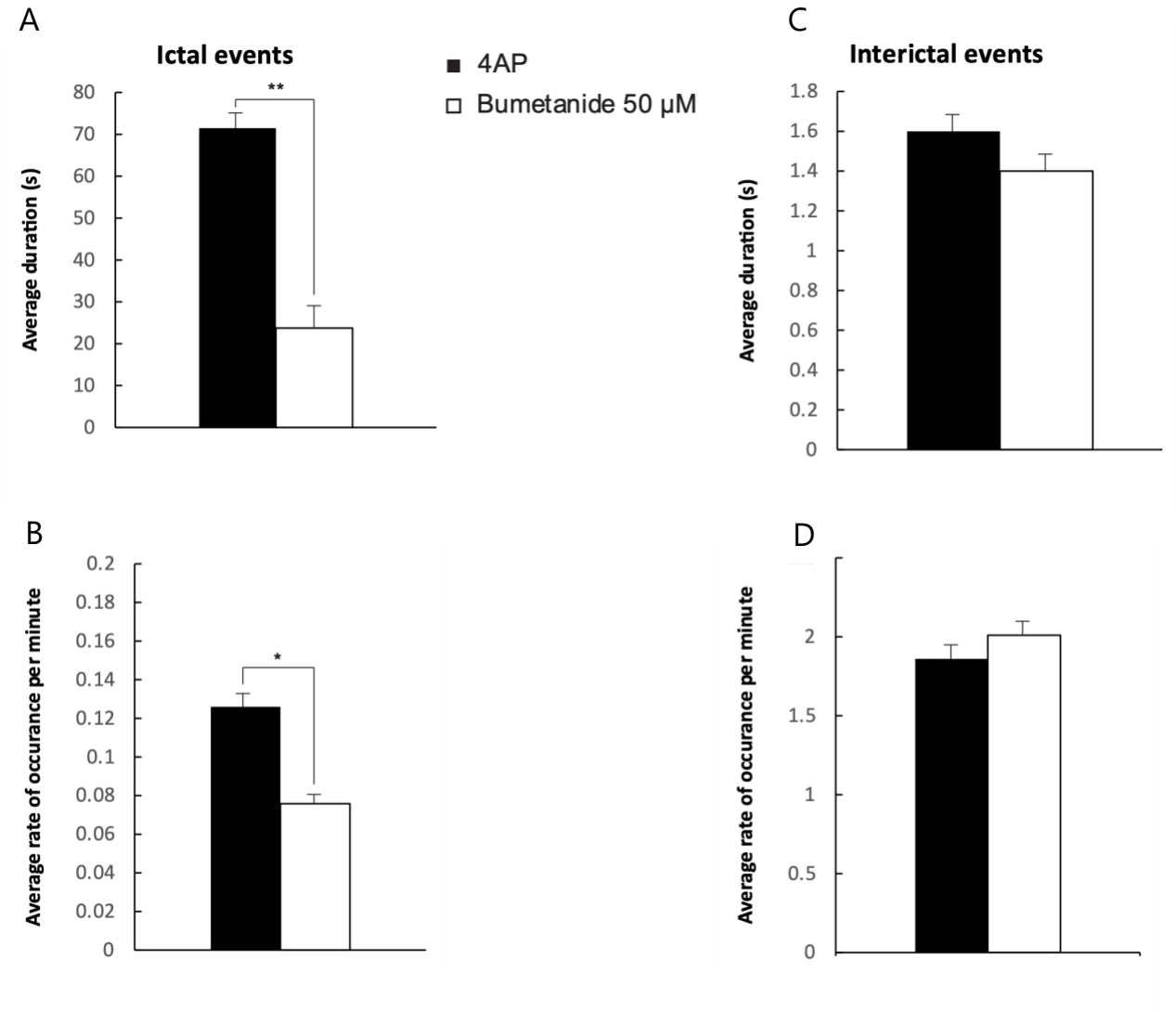

Figure 5. Bar graphs showing the average occurrence rate and duration of ictal and interictal discharges recorded from the EC under 4AP treatment and during 50 µM bumetanide application. Note that blocking NKCC1 and KCC2 significantly decreases the duration (**A**) and occurrence rate (**B**) of ictal discharges in EC. Meanwhile blocking NKCC1 and KCC2 with bumetanide (50 µM) slightly modulates the duration (**C**) and occurrence rate (**D**) of interictal events. * $p < 0.05$, ** $p < 0.01$.

Chapter 4: Discussion

Research over the last decades proposed several underlying mechanisms of epileptogenesis. Specifically, *in vitro* investigation suggested that the GABAergic system has an important role in seizure activity and neural synchronization that is observed in MTLE (Avoli & de Curtis, 2011; Schwartzkroin & Prince, 1980). Electrophysiological recordings of *in vitro* brain slices revealed that bumetanide acts as a specific blocker of NKCC1 and might reduce interictal discharges (Dzhala et al., 2005; Hamidi & Avoli, 2015). Thus, the antagonistic action of the bumetanide on NKCC1 might have beneficial effects on the treatment of several brain disorders such as epilepsy. The structural and functional properties of NKCC1 make bumetanide a potential medication for the treatment of MTLE.

In this study, bath application of 4AP induced two types of epileptiform activities, interictal and ictal activities in *in vitro* juvenile rat brain tissue containing the EC. At the next stage of the experiment, bumetanide with different dosages was used as an NKCC1 and KCC2 blocker in the EC brain slices that manifested epileptiform activity. It was hypothesized that blockage of NKCC1 and KCC2 through the function of bumetanide would decrease the number of ictal and interictal discharges. This research project might give us more insight into the mechanisms of epileptiform synchronization and might provide basic knowledge regarding the treatment of epilepsy. Therefore, field potential activity from the EC of brain slices (n=32) obtained from 7 juvenile (22-23 days old) rats were recorded during the 4AP (50 μM) application. Application of 4AP induced interictal and ictal events in EC of juvenile rats. Bumetanide (10 μM; 50 μM) was applied to determine its effects on epileptiform discharges recorded from EC.

4.1. NKCC1 and KCC2 in Neonatal Epilepsy

Neonatal epileptiform activities might have damaging consequences for the developmental brain. The results of seizure activity during development consists of epilepsy generation, and cognitive and motor problems (Ben-Ari & Holmes, 2006). Available antiepileptic drugs imperfectly modulate these seizure activities. In the developed brain, medications such as barbiturates act as

GABA agonists that might decrease epileptiform activity (Galanopoulou, 2008). These medications increase the frequency or the duration of the opening of GABA Cl^- (GABAA) channels (Schulte et al., 2018). In mature neural cells, GABA acts as a hyperpolarization factor and has inhibitory effects due to the influx of Cl^-. However, GABA is considered an excitatory factor in the developmental brain (Ben-Ari, 2002). This excitatory effect is mediated by increased intracellular Cl^- ions that are produced through the enhanced activity of NKCC1. Under-developed neural cells have an excess of Cl^- ions, so GABA enhances Cl^- efflux that in turn, depolarizes the neuron (Ben-Ari, 2002).

The depolarization influences of the GABAergic system might have a specific role in lowering the epileptiform activity threshold (Khazipov et al., 2015). This process, thus, makes anticonvulsant drugs ineffective among neonates. The diuretic loop medication, bumetanide downregulates the activity of NKCC1, and rodent reports have shown that this process decreases seizures in juvenile models (Nardou et al., 2009). From a theoretical perspective, downregulation of Cl^- obtained through inhibitory action of NKCC1 would lead to blockage of excitation induced by the GABAergic system (Löscher, et al., 2013). In this regard, the $GABA_A$ channels that operate with Cl^- ions might short circuit excitatory actions. These actions might mimic the inhibitory effect of GABA that is observed in the developed brain of adult individuals (Kahle & Staley, 2008).

4.2. Modulation of Epileptiform Activity by Blocking NKCC1 and KCC2 Activity

The present research results show that blocking NKCC1 and KCC2 activity via bumetanide leads to modulation of ictal and interictal discharges. Specifically, the 30-minute perfusion of bumetanide (10 μM) significantly reduced the mean number of interictal events in EC brain slices ($n = 17$). Interestingly, the duration and occurrence rate of ictal events and the duration of interictal events were not significantly modulated.

The current study results might be interpreted by the fact that depolarization effects mediated by $GABA_A$ receptors might be modulated by bumetanide. The antiepileptic modulation of bumetanide is presumably associated with the decreased intracellular concentration of Cl^- in neural

cells. Since our findings showed bumetanide can control the seizure activity in EC from juvenile rats, this modulatory effect might be attributed to the inhibition of NKCC1 and KCC2. Previous results indicated that co-administration of phenobarbital, which is considered to be an effective antiepileptic medication, with bumetanide led to improved seizure activity in immature rat slices (Dzhala et al., 2008). This combination method in medication administration shows bumetanide-induced modulation in the Cl^- concentration in immature neural cells. In line with our findings, Kahle et al. (2009) have reported that the clinical application of bumetanide might represent antiepileptic modulation in humans (Kahle et al, 2009). However, it is reported that due to the structural properties of bumetanide, this molecule has an almost 500-fold higher affinity for these cation- Cl^- co-transporters.

Nonetheless, the modulations of blocking NKCC1 with bumetanide and its influences on seizure disorders remain a controversial issue in the literature. Some authors proposed that bumetanide leads to the reduction of epileptiform activity that is induced by increases in K^+ concentration (Dzhala et al., 2008). Others point to the fact that this medication might increase seizures through low Mg^{2+}-ACSF (Kilb et al, 2007). Hamidi & Avoli (2015) showed that NKCC1 and KCC2 antagonist controls seizures that are induced by 4AP and increase excitatory processes in neural cells at later periods of development. This finding contributes to the literature that reported that the excitatory modulation of GABA produced mostly under the influence of NKCC1 and KCC2 should be considered an important factor in ictogenesis. More specifically, our results showed that bumetanide might have an effective influence on the epileptiform activity of MTLE in juvenile rats.

In addition, it is shown that bumetanide administration led to attenuation of epileptiform activity in a clinical setting with human cases. Eftekhari et al. (2013) reported that two out of three patients with MTLE demonstrated amelioration of seizure activity after the treatment. Specifically, they investigated MTLE patients that were resistant to common medications. A considerable reduction in the number of epilepticus events was observed after bumetanide administration. Interestingly, during the administration of bumetanide, two of the patients were completely seizure-free and they also represented an improvement in follow-up phases. This result demonstrated that bumetanide might be a suitable alternative for the treatment of drug-resistant patients with MTLE. It was

previously reported that at lower doses, bumetanide is a safe diuretic and might not represent severe side effects in children (Donovan et al., 2015).

As shown in previous sections, a large amount of *in vitro* and *in vivo* animal model evidence points to the role of pathophysiology related to Cl^- ions in the immature brain in the generation of epileptiform synchronization. In this regard, bumetanide has gained much interest in this field. Congruent with the results of this thesis, *in vitro* research demonstrated that this medication-induced hyperpolarization affects neural cells (Dzhala et al., 2008; Dzhala et al., 2005). Furthermore, *in vivo* epileptiform activities were shown to be slowed down after bumetanide administration in mice (Sivakumaran & Maguire, 2016).

Chapter 5: Conclusions

In conclusion, I propose that blocking NKCC1 and KCC2 activity with bumetanide controls the 4AP-induced epileptiform discharges, which are known to mirror in part $GABA_A$ receptor. Activation of Cl^- permeable $GABA_A$ receptors stimulates neurons during development due to increased intracellular Cl^- levels and a depolarized Cl^- equilibrium potential. As net Cl^- outward neuronal transport grows, GABA becomes inhibitory.

The NKCC1 makes it easier for Cl^- to accumulate in neurons. Bumetanide, an NKCC1 and KCC2 inhibitor, lowers epileptiform activity in the EC part of brain slices *in vitro* and reduces electrographic seizures in newborn rats *in vivo* (Kahle & Staley, 2008). The degree of NKCC1 expression in the human and rat cortex compared to the expression of the Cl^- extruding transporter, KCC2, revealed that Cl^- transport in the prenatal human brain is as neonate as in the rat. Bumetanide may be effective in the treatment of newborn seizures since NKCC1 enhances seizures in the developing brain.

The results of the current research indicated that low dosage (10 μM) and high doses (50 μM) of bumetanide by antagonizing only NKCC1 and both NKCC1 and KCC2, respectively, led to anticonvulsant effects in terms of the number of ictal and interictal discharges. This finding might pave the way for future research to determine the dose-response of bumetanide. On the other hand, future works might consider the combined effects of bumetanide and other anticonvulsants in the treatment of seizure activity in immature neural cells.

Overall, the findings of the current work indicate that bumetanide might have reversed the Cl^- concentration gradient in neural cells such that membrane hyperpolarization and inhibition controlled the epileptiform activities. Blocking NKCC1 with low doses of bumetanide (10 μM; n=17 slices) significantly reduced the occurrence rate of interictal events without changing their duration. In addition, this procedure increases the duration of ictal events while it decreases the occurrence rate of ictal events in EC. These data suggest that in 22-day-old rat EC, NKCC1 co-transporter is still active in juvenile rats. Blocking both NKCC1 and KCC2 with high concentrations of bumetanide (50 μM; n = 15 slices), induced a significant reduction in the ictal events' duration and rate of occurrence. Also, while 50 μM bumetanide induced a slight reduction

in the duration of the interictal discharges in EC, the rate of occurrence of interictal events slightly increased in the same region. These findings are in agreement with what was obtained in adult rats (Hamidi & Avoli 2015).

In this thesis, it has been observed that bumetanide has an effective role in controlling seizures. Also, previous research in healthy and clinical populations also points to its therapeutic effects (Sivakumaran & Maguire, 2016). As a result, using such a molecule might be a rational method of treating MTLE in the immature brain. In addition, due to the fact that the expression of NKCC1 and KCC2 during the developmental phases is similar in humans and rodents, the findings of the current work might be extended to human participants in future research.

Therefore, neonates with recurrent seizure activity could be treated with bumetanide along with other doses of standard anticonvulsants. Nevertheless, the combination of adjunctive anticonvulsant with bumetanide shall be considered carefully and future work might be carried out to indicate the related side effects of NKCC1 and KCC2 inhibition in children. Regarding the fact that the GABAergic system is a critical factor in CNS development and synaptogenesis, NKCC1 and KCC2 inhibition might be considered with more caution. In addition, any treatment effect of using bumetanide in neonates shall be weighed regarding the detrimental influences of epileptiform activities in the developing brain.

All in all, the goals of my research objective will enhance our comprehension and understanding of the fundamental factors and mechanisms of epilepsy and will provide critical background and foundational information required for the advancement of effective therapeutic strategies for epilepsy. My objective was to determine whether the NKCC1 and KCC2 could influence the epileptiform activity induced by 4AP in juvenile EC.

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