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ABSTRACT

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Chronic and acute seizure activity affects NKCC1 and KCC2 expression and alters GABA-induced inhibition in the adult and developing hippocampus

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Abstract

Temporal lobe epilepsy is a relatively common nervous system disorder characterized by abnormal activity in the hippocampus, often caused by a decrease in GABA inhibition. GABA_A receptors are Cl⁻ ion channels whose effects are mediated by inward Cl⁻ rectifying (excitatory) NKCC1 and outward Cl⁻ rectifying (inhibitory) KCC2 cotransporters. The expression of these cotransporters is modified by both aberrant neural activity and “critical periods” in development. We hypothesized the hippocampal class V seizures would cause a permanent upregulation of NKCC1 and/or downregulation of KCC2. We also hypothesized rats with seizures during postnatal days 20-30 would cause long term dysfunction in hippocampal expression of these cotransporters. Western blotting results suggest that increased propensity for seizures is due to permanent changes in NKCC1 levels, which subsequently reduces GABA_A-mediated inhibition. Furthermore, developing rats that experience a seizure at P25 display decreased KCC2 expression levels both 24 hours and 90 days after the event, with little change in NKCC1 expression—with similar physiological effects as in adults.

Introduction

Temporal lobe epilepsy (TLE) is a relatively common nervous system disorder characterized by abnormal and excessive synchronous activity originating in neuroplastic regions of the brain, such as the hippocampus (Delpire 2000, Palma *et al.* 2006, Fisher *et al.* 2005, Sutula and Ockuly 2006). These aberrant electric discharges result in seizures and dysfunctional behavior, motor activity, autonomic function, or consciousness (Delpire 2000). Evidence suggests that this hyperexcitability and hypersynchronization stems from a disruption of the delicate balance between excitatory and inhibitory synaptic activity (Fisher *et al.* 2005, Jin *et al.* 2004). Many studies focus on the decrease or loss of receptor-mediated inhibition—particularly that mediated by γ -aminobutyric acid (GABA) (Delpire 2000, Jin, *et al.* 2004). This neurotransmitter is the primary inhibitory signal in the adult nervous system; it counteracts

excitatory neurotransmitters (e.g. glutamate) and prevents the spread of neuronal excitability (Delpire 2000). Indeed, much research demonstrates that even modest reductions in GABAergic inhibition of cortical neurons result in chronic models of epilepsy (Roper *et al.* 1997, Jin, *et al.* 2004, Dudek and Sutula 2007).

Kindling is the animal model for the most common form of TLE. In the rat model, repetitive application of electrical stimuli to the brain induces afterdischarges (ADs; i.e. persistence of response after cessation of stimulation) and seizure behavior. As the animal experiences additional ADs, the duration of these episodes increase and the strength of the stimulus required to cause network synchronization decreases. Paralleling this trend, the severity and state of seizure activity increases, developing from focal (class I and II) seizures to “secondary generalized tonic-clonic convulsions” (class V) and eventually to “recurring spontaneous seizures” (Sutula and Ockuly 2006). These neuronal changes are considered essentially permanent, and reflect the progressive reorganization of neuronal circuits (Sutula and Ockuly 2006). Thus, kindling provides an effective model to study the physiological changes characteristic of progressive TLE (Okabe *et al.* 2003, Sutula and Ockuly 2006). Indeed, studies show initial kindling-induced seizures increase GABA-dependent inhibition, but this inhibition is reduced with the emergence of class V seizures (Sayin *et al.* 2003). Therefore, like temporal lobe epilepsy, the increasing susceptibility to synchronization in kindling may be due to a loss of GABA-mediated inhibition (Okabe *et al.* 2003).

GABA_A receptors are Cl⁻ specific, ligand-gated ion channels that mediate the effects of GABA (Payne *et al.* 2003). In the mature brain, neurons maintain low intracellular Cl⁻ concentrations ($[Cl^-]_i$) compared to extracellular concentrations ($[Cl^-]_o$), so the equilibrium potential (E_{Cl}) of the membrane is lower than the resting cell potential (V_m) (Jin *et al.* 2004).

Thus, the activation of GABA_A receptors results in an influx of Cl⁻, which hyperpolarizes the membrane, causing an inhibitory postsynaptic potential (IPSP) (Payne *et al.* 2003, Dudek and Sutula 2007). However, under certain physiological (e.g. development) and pathophysiological (e.g. epilepsy) conditions, [Cl⁻]_i > [Cl⁻]_o, and E_{Cl} > V_m. Thus, GABA_A receptor activation elicits an excitatory postsynaptic potential (EPSP) (Payne *et al.* 2003, Jin *et al.* 2004). Consequently, though GABA is typically considered an inhibitory neurotransmitter, it may have hyperpolarizing or depolarizing effects depending on the relationship between [Cl⁻]_i and [Cl⁻]_o (Payne *et al.* 2003, Jin *et al.* 2004, Galanopoulou 2007).

Neuronal cation-chloride cotransporters (CCCs) play a pivotal role in the gradient between [Cl⁻]_i and [Cl⁻]_o and hence GABAergic functions (Payne *et al.* 2003, Jin *et al.* 2004, Galanopoulou 2007). *Figure 1* displays the two main CCC isoforms found in rat and human neuronal tissues: Na⁺-K⁺-Cl⁻-Cl⁻ isoform 1 (NKCC1) and K⁺-Cl⁻-Cl⁻ isoform 2 (KCC2) (Payne *et al.* 2002). These transporters use cation gradients generated by Na⁺-K⁺ ATPases to alter [Cl⁻]_i without the net movement of charge across the membrane (electroneutrally balanced by K⁺ and/or Na⁺) (Payne *et al.* 2002, Galanopoulou 2007). *Figure 1* also shows how such cotransporters dictate the effects of GABA. KCC2 transporters move one Cl⁻ and one K⁺ out of the cell to decrease [Cl⁻]_i; therefore, the opening of GABA_A channels are more likely to result in hyperpolarizing EPSPs. Conversely, NKCC1 transporters move two Cl⁻, one Na⁺, and one K⁺ into the cell, which increases [Cl⁻]_i and increases the likelihood of depolarizing IPSPs (Payne *et al.* 2002).

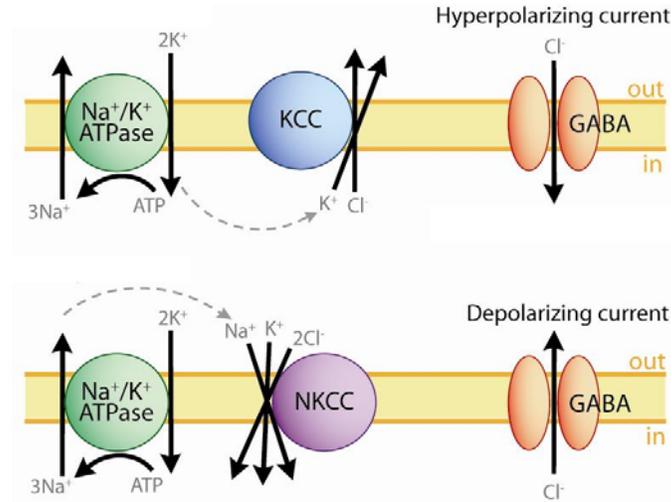


Figure 1: Action of NKCC and KCC ion cotransporters found in rat neurons. Arrows indicate the direction of transport for each of the ions. KCC ion transporters use the K⁺ gradient produced by a Na⁺/K⁺ ATPase to move Cl⁻ out of the cell. The net efflux of Cl⁻ results in hyperpolarizing currents across GABA_A receptors, which serve as neurotransmitter-gated Cl⁻ channels. NKCC also uses secondary active transport (Na⁺ gradient generated by the ATPase) to drive the net influx of Cl⁻ into neurons. The actions of these cotransporters result in depolarizing currents through ion channels. Figure adapted from Payne, *et al.* 2002.

In concordance with the above observations, GABAergic functions change from excitatory to inhibitory during early neural development as a function of changing [Cl⁻]_i. In immature neurons, [Cl⁻]_i is high, and GABA_A receptor responses are depolarizing. The purpose of this hyperexcitability during postnatal maturation is to activate voltage-sensitive calcium channels; the release of calcium triggers numerous Ca²⁺-mediated effects that facilitate important processes in the developing brain, such as neuronal proliferation and synaptogenesis (Galanopoulou 2007). However, during early postnatal maturation, [Cl⁻]_i (and subsequently, E_{Cl}) decreases. Hence, in the adult brain, GABA is inhibitory (Payne *et al.* 2002, Yamada 2004, Dzhala *et al.* 2005).

Unsurprisingly, developmental patterns of expression of NKCC1 and KCC2 shown in *Figure 3* parallel these changes in GABAergic function (Payne *et al.* 2002, Jin *et al.* 2004, Dzhala *et al.* 2005, Galanopoulou 2007). The period of this transition varies between individual regions of the brain (Delpire *et al.* 2000, Wang *et al.* 2002). Wang *et al.* observed high levels of

NKCC1 mRNA in hippocampal neuroepithelium of P1 rats (2002). Furthermore, studies performed by Dzhala *et al.* show that NKCC1 protein expression in rat cortex is relatively high directly after birth, peaking between postnatal day (P) three to P14. This expression level is reduced by P21 through adulthood (P90). Conversely, KCC2 exhibits low expression levels in hippocampal neurons between P3-P14, but dramatically increases on P15 and continues through adulthood (Payne *et al.* 2002, Wang *et al.* 2002, Dzhala *et al.* 2005). Thus, in very early postnatal life, inward-rectifying NKCC1 is the principle CCC, presumably leading to high $[Cl^-]_i$ in hippocampal neurons and high E_{Cl^-} . Ultimately, GABA is excitatory. However, after the second postnatal week, outward-rectifying KCC2 cotransporters increase, $[Cl^-]_i$ and E_{Cl^-} decrease, making GABA inhibitory (Ueno *et al.* 2001, Yamada *et al.* 2004, Dzhala *et al.* 2005)

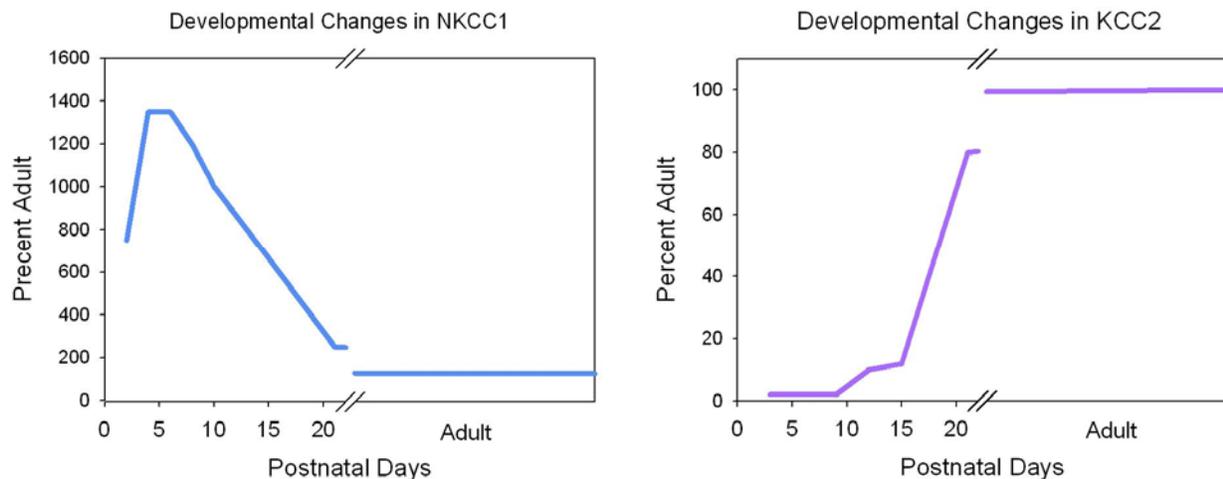


Figure 2: Developmental regulation of NKCC1 and KCC2 in rat cortical neurons. NKCC1 (left) and KCC2 (right) are expressed as a percentage of final, adult levels. Studies performed by Dzhala *et al.* and Wang *et al.* indicate NKCC1 was higher between postnatal day (P)3-P14 compared to P12 through P90 (adult). KCC2 displayed an opposite pattern: Expression levels of this protein were low between P3-P14 compared to P21 through P90 (figure adapted from Dzhala *et al.* 2005).

Neuronal development during these early stages can be disrupted by aberrant activity or excitation. For example, studies show that seizure activity in young animals may lead to long-term behavioral and cognitive impairment (Cilio *et al.* 2003, Sayin *et al.* 2004). However, the pathophysiological effects of early seizures seem to vary as a function of age and developmental

stage, pointing to specific “critical periods” in neuronal maturation (Cilio *et al.* 2003). These stages seem to result in maturation of inhibition. In particular, the dentate gyrus of the hippocampus undergoes considerable development after birth (Sayin *et al. under review*). A study by Sayin *et al.* (Figure 3) found rats that experienced seizures during P20-30 displayed long-term reduction of inhibition and increased seizure susceptibility in adulthood (2006). This study concluded that “seizures during P20-30 irreversibly modified functional properties of inhibitory circuitry and capacity for plasticity in the [dentate gyrus]” (2006).

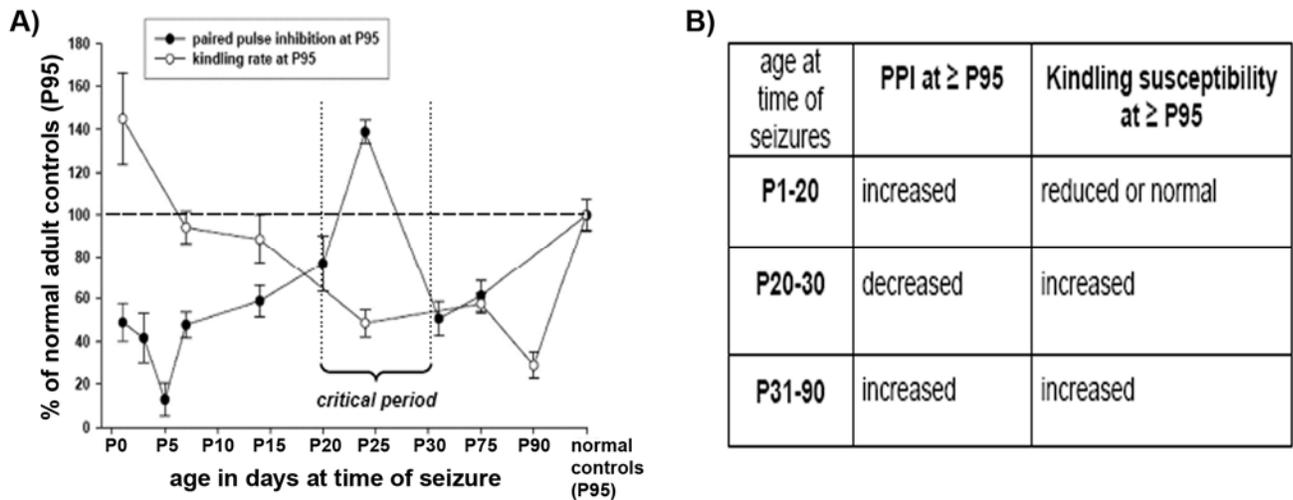


Figure 3: Relationships between long-term effects of seizures during development on paired pulse inhibition and seizure susceptibility in adulthood. **A)** Long-term effects of seizures on PPI in the dentate gyrus and susceptibility to kindling in P95 adult rats that experienced seizures evoked by hyperthermia, PTZ, and KA at ages P1-P90. The long-term alterations in PPI and susceptibility to kindling induced by postnatal seizures evoked by different methods varied systematically as function of age. Seizures during P20-30 reduced PPI in adulthood and were associated with acquisition of increased sensitivity to kindling persisting into adulthood. Seizures at P31-90 increased PPI but also increased sensitivity to kindling. **B)** Summary of the long-term patterns of alterations in PPI and kindling susceptibility as a consequence of seizures during postnatal periods of P1-20, P20-30, and P31-90 (Sayin *et al. under review*)

Given these observations, how do chronic class V seizures change the short and long term expression of NKCC1 and KCC2 in adult rats? Furthermore, do seizures during certain “critical periods” of neuronal development cause dysfunctional expression of NKCC1 or KCC2 and long-term seizure susceptibility?

Because of the intimate relationship between CCCs and the excitatory/inhibitory actions of GABA_A receptors, it is hypothesized that the loss of GABA-mediated inhibition characteristic of epileptogenic activity is due to a modification of normal NKCC1 and/or KCC2 expression levels. Notably, the hippocampi of KCC2 knockout mice are exceptionally hyperexcitable; these animals die immediately after birth, presumably by GABA-induced excitation (Payne *et al.* 2003, Muñoz *et al.* 2006). Interestingly, NKCC1-null mice or mice with 20-30% of normal KCC2 expression levels display no physiological aberrations (Payne *et al.* 2003). Furthermore, drugs that target NKCC1 or KCC2 also point to their role in GABAergic functions. Hochman *et al.* showed that epileptiform activity in hippocampal slices induced by a GABA_A agonist was counteracted by furosemide, a NKCC1 inhibitor (1995). Further, selective inhibition of NKCC1 by bumetanide in neonatal rats increased GABA-induced inhibition (Dzhala *et al.* 2005). Notably, bumetanide served as a superior anticonvulsant in neonatal rats than phenobarbital, a common drug used to treat epilepsy in adult humans (Dzhala *et al.* 2005).

We hypothesized that the hippocampi of adult rats that experienced class V seizures would display a permanent upregulation of NKCC1 and/or downregulation of KCC2 compared to unkindled rats. We also hypothesized rats that experience seizures during the critical period (postnatal days 20-30) would show long term dysfunction in hippocampal NKCC1 and/or KCC2 expression compared to rats that did not experience seizures. To test this hypothesis, we used Western blotting and densitometry to quantify the expression level of NKCC1 and KCC2 in adult rats (> P90) that had experienced from three to spontaneous (unelicited) class V seizures and those that had experienced ADs only compared to rats that had experienced no seizures. These groups were sacrificed either 24 hours or three months after their last seizure event. To analyze the developmental effects of seizures on NKCC1 and KCC2, we used similar techniques

to quantify expression levels in animals that had experienced a seizure event on P1, P25, and P35 (sacrificed either 24 hours or 90 days after seizure activity).

Materials and Methods

Adult Kindled Animals

Adult male Sprague Dawley rats (Harlan, Madison, WI) were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) and stereotaxically implanted with an insulated, stainless-steel bipolar electrode for stimulation and recording. The electrode was implanted in either the olfactory bulb (9 anterior, 1.2 lateral, 1.8 ventral with respect to bregma) or the perforant path (8.1 mm posterior, 4.4 mm lateral, 3.5 ventral with respect to bregma), and was fixed to the skull with acrylic.

After a 1 week recovery period prior to the surgical implantation, the unrestrained awake implanted rats received twice daily kindling stimulation (5 days per week) with a 1 sec train of 62 Hz biphasic constant-current 1msec square wave pulses. The stimulation was delivered at the lowest intensity that evoked an AD. Evoked behavioral seizures were classified according to standard criteria and ranged from class I seizures (behavioral arrest) to class V seizures (bilateral tonic-clonic motor activity with loss of postural tone) (Sutula and Steward, 1986), which are comparable with partial complex seizures with secondary generalization. Kindled rats were killed at a minimum of 24 hours after the last evoked seizure for Western blot analysis. All procedures were reviewed and approved by the Research Animal Resources Committee (University of Wisconsin).

Postnatal Animals

Seizures were induced in male Sprague Dawley (Harlan, Madison, WI) rats by administration of kainic acid (described below) on specific postnatal (P) days: P1, P25, and P35. Kainic acid was dissolved in saline and administered subcutaneously to rats in 1 mg/kg dosages.

Control animals were injected with saline solution of equivalent volume. Rats were observed for seizure activity for approximately 1-2 hours and returned to their litters or cages for normal animal care. Rats that experienced seizures and the corresponding controls (saline-injected) were sacrificed either 24 hours later or when they reached adulthood (> P90). All procedures were in accordance with the Research and Animal Care Committee of the University of Wisconsin.

Isolation of Hippocampi

At the appointed time after the evoked seizure or injection of saline, rats were anesthetized with isoflourane, euthanized by decapitation, and the brain was removed and placed in 4°C saline solution. The hippocampi of both hemispheres were then removed by blunt dissection and immediately frozen in liquid nitrogen. Samples were stored at -80°C.

Tissue Extraction

Hippocampi of seizure-induced and control rats were placed in isolation buffer consisting of 0.22M mannitol, 0.075M sucrose, 1mM EGTA, and protease inhibitors at 4°C, then homogenized and centrifuged for 3 minutes in 5000rpm. The supernatant was then collected and centrifuged at 10,500rpm for 8 minutes. The resulting pellet was then resuspended in 20µL SDS lysis buffer and 0.2µL protease inhibitor per 0.01g of initial hippocampal tissue. Each sample was then sonicated for 15 seconds and centrifuged at 12,000rpm for 30 minutes. The resulting supernatant was collected and stored at -80°C.

Western Blots

The supernatant protein extracted by the method above was analyzed by the BCA method using a DC Protein Assay (Bio-Rad Laboratories) and BioMate 3 UV-Vis Spectrophotometer (Thermo Scientific). Samples were then prepared with 50µg total protein, 5µL 5x SDS loading buffer containing 10% β-mercaptoethanol, and diluted to 25µL total volume. The protein

samples were heated for > 3 minutes at 95°C. The resulting lysates were separated on a Tris-HEPES-SDS 4-20% gradient gel (Pierce) in 1x SDS-Hepes running buffer at 500V and 35mA for two hours.

The resulting gel separation was then transferred onto PVDF transfer membrane (0.45µm, Pierce) soaked briefly in methanol prior to use. Transfers were performed overnight at 23V at 4°C. Blots were incubated in 5% nonfat milk in TBST (150mM NaCl, 20mM of 1M pH 7.5 Tris buffer, and 0.1% Tween 20) for one hour (25°C) or overnight (4°C) with shaking, for 1-2 hours in primary antibody to NKCC1 (T4 mouse monoclonal antihuman colonic T84 epithelial Na⁺ - K⁺ - Cl⁻ co-transporter, unconjugated) or KCC2 (rabbit polyclonal anti-K⁺ - Cl⁻ co-transporter, unconjugated), rinsed 3x in 150mM TBST, incubated with HRP conjugated secondary IgG for 1 hour, rinsed 3x in 150mM TBST, and visualized by enhanced chemiluminescence (ECL Western Blotting Substrate, Pierce) in the linear range.

Analysis

Quantification of the resulting images was performed using ImageJ image processing and analysis software. Band intensities were normalized within animals by comparison with blot images that probed for actin. The band intensities of NKCC1 or KCC2 for experimental animals were expressed relative to their respective controls. These values were averaged within each experimental group; significant differences were determined using ANOVA analysis ($p \leq 0.05$).

Results

Figure 4 displays the relationship between number of class V seizures and the band intensity of NKCC1 protein (relative to animals that had not experienced seizures). Animals used for this data were sacrificed within 24 hours of their last class V (CIV) seizure. The representative Western blot (4A) shows that NKCC1 protein expression is upregulated in animals that experienced at least three class V seizures compared to control animals (no seizures). A

compilation of data from multiple Westerns (*Figure 4B*) shows that NKCC1 expression increases with induction of CIV seizures, and that this increase becomes statistically significant ($p \leq 0.05$) after 19-24 CIVs. Furthermore, data trends also suggest that there is a direct relationship between CIV seizures and the expression of NKCC1. Sample sizes for each group are as follows: control (n = 6), 3CIV (n = 4), 6-11CIV (n = 5), 19-24CIV (n = 5), 34-50CIV (n = 4), 69-75CIV (n = 2), spontaneous (n = 3).

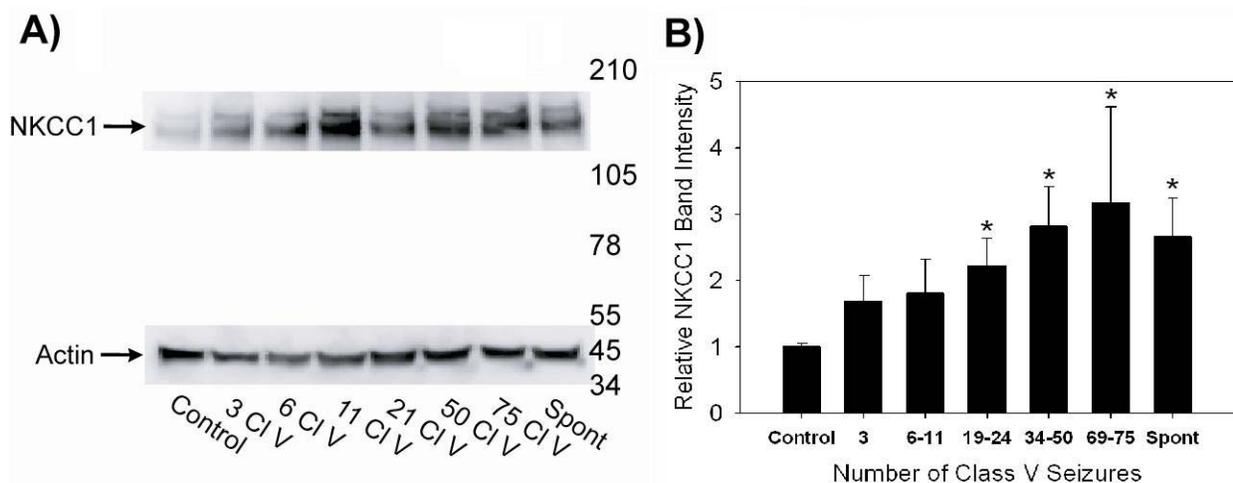


Figure 4: Relative NKCC1 band intensity for kindled rats compared to control (unkindled) samples, sacrificed within 24 hours of the last class V (CIV) seizure. **A)** Illustrative Western blot. Trends indicate that NKCC1 expression is relatively low in unkindled rats, but increases with induction of CIV seizures. **B)** Compilation of Western blot data of relative NKCC1 band intensity. Results show that hippocampal NKCC1 expression is higher in rats that experience CIV seizures compared to rats that had not experienced seizures. Trends may also suggest that the increase of NKCC1 expression in hippocampus extracts is directly related to the number of CIV seizures an animal experiences. *Indicates a statistical difference from the control ($p \leq 0.05$).

Figure 5 shows data for relative band intensities of KCC2 protein as a function of CIV seizures (sacrificed within 24h of the last CIV seizure). The representative Western blot (5A) shows that KCC2 protein expression does not appreciably change compared to control animals with no seizures. Compiled data (*Figure 4B*) substantiates this observation; however, a statistically significant difference from the control ($p = 0.039$) occurs for animals that experienced spontaneous CIV seizures. No KCC2 data was obtained for the 6-11CIV or 69-

75CIV groups; therefore these groups were omitted from the figure. Sample sizes are as follows: control (n = 3), 3CIV (n = 2), 19-24CIV (n = 4), 34-50CIV (n = 2), spontaneous (n = 2).

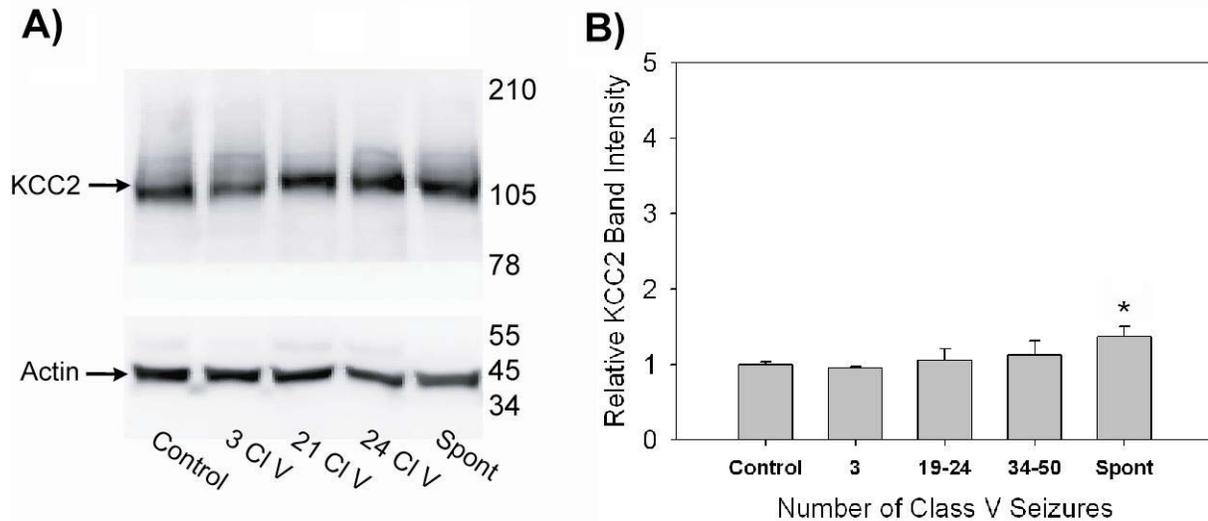


Figure 5: Relative band intensities of KCC2 for kindled rats compared to control (unkindled) samples. Animals used for this data were sacrificed within 24h of their last class V (CIV) seizure. Western blot analysis showed that KCC2 expression did not appreciably change for rats that experienced up to 50 class V seizures. No KCC2 data was obtained for the 6-11CIV or 69-75CIV groups; therefore these groups were omitted from the figure. *Indicates a statistical difference from the control ($p \leq 0.05$).

Figure 6 juxtaposes the data in *Figures 4* and *5* to contrast the relative NKCC1 and KCC2 band intensity as a function of CIV seizure.

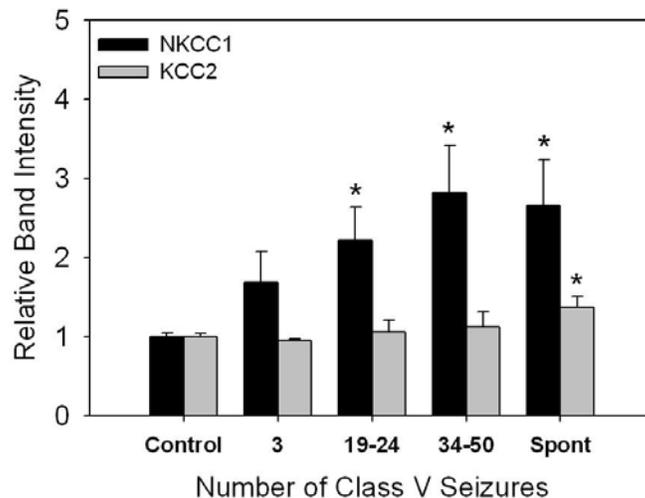


Figure 6: Relative NKCC1 band intensity for kindled rats compared to control (unkindled) samples, sacrificed within 24 hours of the last seizure. KCC2 expression remains relatively constant, while NKCC1 increases with induction of CIV seizures. No KCC2 data was obtained for the 6-11CIV or 69-75CIV groups; therefore these groups were omitted from the figure. * Indicates a statistical difference from the control ($p \leq 0.05$).

Figure 7 shows the relative NKCC1 and KCC2 band intensities for rats that experienced ADs (class I, II, III, and IV) compared to 3 CIVs. The representative Western blot (Figure 7A) indicates that NKCC1 and KCC2 expression do not appreciably change with AD activity. However, compiled data (7B) may indicate that NKCC1 band intensities are slightly (but not statistically) lower in AD animals. KCC2 band intensities did not display an appreciable difference in either AD or 3 CIV animals compared to the control. Sample sizes for NKCC1 are as follows: control (n = 6), AD (n = 4), 3CIV (n = 4). For KCC2: control (n = 3), AD (n = 4), 3CIV (n = 2).

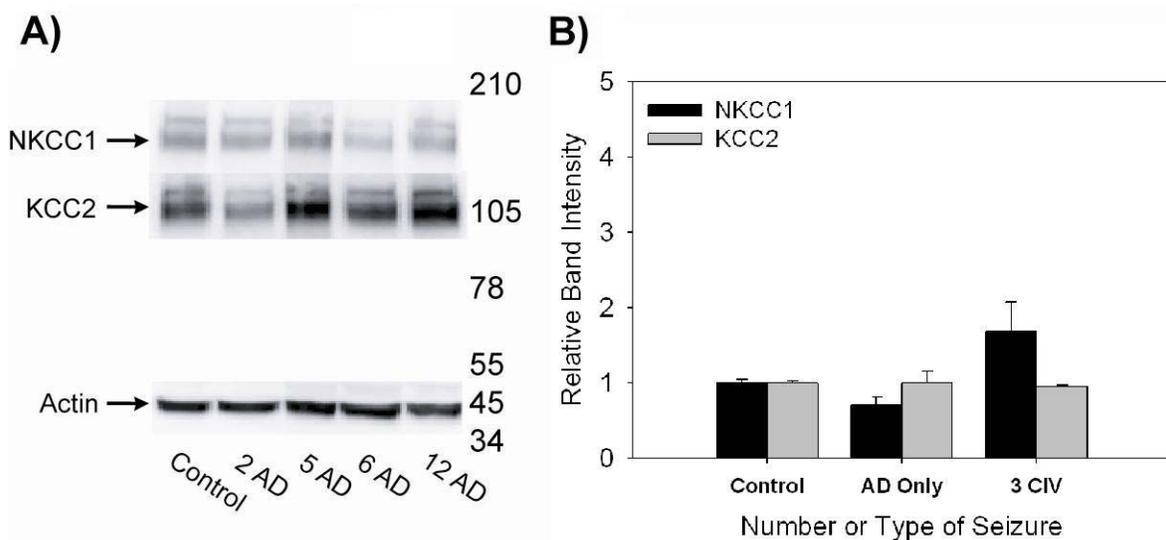


Figure 7: Relative NKCC1 and KCC2 band intensities for kindled rats compared to control (unkindled) samples, sacrificed within 24 hours of the last seizure. Trends indicate that NKCC1 expression is upregulated when rats experience a total of 3 CIV seizures, but may be downregulated with less severe seizure activity (after discharge, AD). KCC2 expression remains relatively stable regardless of seizure intensity.

We also investigated the longevity of these changes in cotransporter expression.

Figure 8 shows the relative NKCC1 and KCC2 band intensities for rats that experienced ~20-30 CIVs, sacrificed 24 hours or three months after their last seizure activity, respectively. Trends indicate that NKCC1 band intensities remained elevated in rats that had not experienced seizures for three months. Furthermore, the NKCC1 density appears higher than the 24 hour tissue. Conversely, analysis of KCC2 band intensities show that expression levels do not change

substantially either 24 hours or three months after the last induced CIV seizure. Sample sizes for NKCC1 are as follows: control (n = 6), 19-34 CIV (n = 6), 3 Month 23-27 CIV (n = 3). For KCC2: control (n = 3), 19-34 CIV (n = 5), 3 Month 23-27 CIV (n = 3).

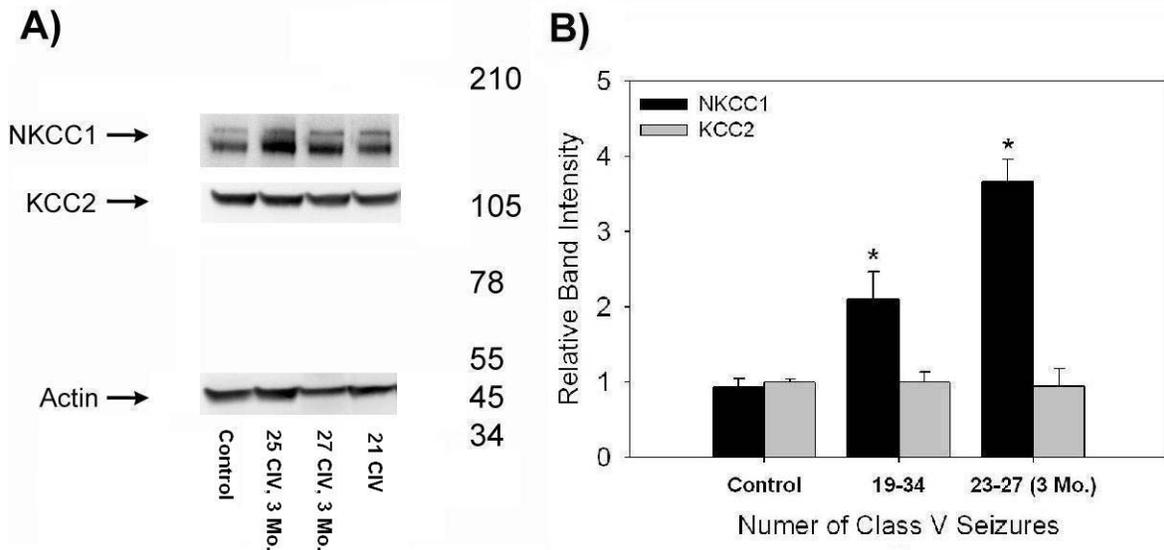


Figure 8: Relative NKCC1 and KCC2 band intensities for kindled rats compared to control (unkindled) samples, sacrificed 24 hours or 3 months after induction of the last CIV seizure, respectively. Data shows that NKCC1 expression remains elevated in rats that have experienced ~20-30 CIVs for up to three months after the last induced seizure. *Indicates a statistical difference from the control ($p \leq 0.05$).

Finally, we collected preliminary data on the effects of seizures during various stages of development, focusing on the aforementioned “critical period” between P20-30. Notably, *Figure 9* shows rats that experience seizures on P25 display decreased levels of KCC2 protein both 24 hours (*Figure 9A*) and 90 days (*Figure 9B*) after the event, with little change in NKCC1 levels. Conversely, seizures on P1 and P35 do not show an appreciable trend in either cotransporter. However, no significant difference arose to substantiate this qualitative observation. See *Appendix* for n values of each group.

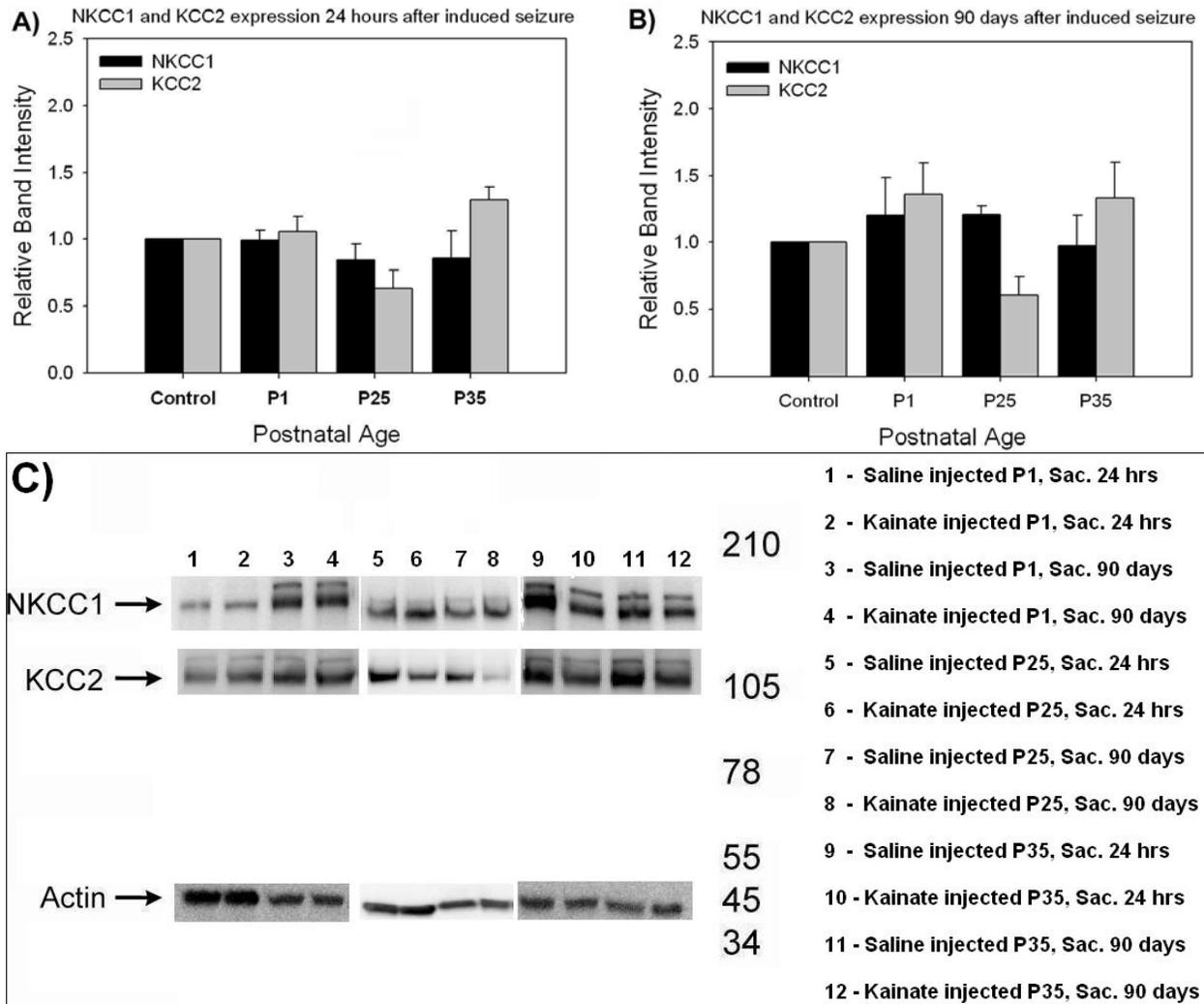


Figure 9: Relative NKCC1 and KCC2 band intensities for rats injected with kainic acid on a specific postnatal (P) day, relative to control animals injected with saline at the same stage of development. **A)** Animals sacrificed 24 hours after an induced seizure. Trends show rats that experience a seizure on P25 display lower KCC2 levels 24 hours after the event. **B)** Animals sacrificed 90 days after an induced seizure. Results show rats that have a seizure on P25 display decreased KCC2 density 90 days after the event. **C)** Representative Western blots for the experimental groups.

Discussion

We hypothesized that the hippocampi of adult rats that experienced class V seizures would display a permanent upregulation of NKCC1 and/or downregulation of KCC2 compared to unkindled rats. The results in *Figures 4-8* support this hypothesis and suggest that the increased propensity for seizures observed in kindling is—at least, in part—due to changes in

chloride-cation cotransporter expression levels that reduce $[Cl^-]_i$, which subsequently reduces GABA_A-mediated inhibition.

Results in *Figure 4-6* show that the density of NKCC1 is higher in the hippocampi of animals that experienced at least three class V seizures compared to control animals with no seizures. Additionally, we see no apparent difference in KCC2 expression between animals that experience CIV seizures versus the unkindled animals. The increased expression of NKCC1 without compensatory increases in KCC2 resulted in higher $[Cl^-]_i$ and decreased E_{Cl} . These cellular changes thereby reduced the hyperpolarizing capacity of GABA_A receptors in hippocampal neurons. These effects decreased the number or strength of IPSPs, reduced inhibition in hippocampal neurons, and increased excitability and seizure activity. Importantly, the increased NKCC1 with unchanged KCC2 expression in *Figure 6* indicates dysfunction in hippocampal cotransporter expression in rats that have experienced class V seizures. Thus, these results may point to a cotransporter and $[Cl^-]$ -based mechanism for epileptogenesis.

Though a statistically significant increase in NKCC1 density (relative to unkindled rats) only appeared in rats that experienced more than ~20 CIV seizures, we anticipate larger sample sizes would expand the observed statistical difference to groups of rats that experienced fewer than 20 CIV seizures. Indeed, we qualitatively observe an increase in NKCC1 expression in both *Figure 4A* and *4B*.

We also observed a significant increase in KCC2 expression for spontaneous animals (*Figure 5B*). However, the small sample size ($n = 2$) of this group leads us to question the validity of these results. We project that further analysis with more animals will eliminate this difference. However, should later data substantiate the increased expression of KCC2 in these animals, we might attribute these alterations to the highly dysfunctional nature of spontaneous

hippocampal neurons. Namely, rats that experience spontaneous seizures do not require stimulation for induction of CIVs. Therefore, the increase in KCC2 expression we observe in *Figure 5B* may be due to a loss of neuronal protein regulation or—conversely—an acute compensatory mechanism to reduce hyperexcitability.

Results in *Figure 7* show rats that experienced AD (i.e. Cl I, II, III, or IV seizure behavior) did not display an appreciable change in KCC2 expression, with a slight decrease in NKCC1 expression. This trend coincides with those observed in *Figure 6*. Additionally, when we contrast AD and 3 CIV rats (*Figure 7B*), we observe a qualitative increase in NKCC1 expression after induction of CIV seizures. This apparent difference between AD and CIV animals may indicate that increased NKCC1 expression is an important component of the progression to CIV seizure activity. Significantly, these results coincide with previous investigations, which show initial kindling-induced seizures increase GABA-dependent inhibition, but this inhibition is reduced with the emergence of class V seizures (Sayin *et al.* 2003).

Figure 8 shows that NKCC1 expression remains high three months after the last kindling-induced CIV seizure. Thus, the elevated expression levels of NKCC1 in kindled animals are a lasting phenomenon. Furthermore, *Figure 8B* correlates with characteristics of both kindling and epilepsy: The neuronal changes induced by repeated seizures are essentially permanent (Sutula and Ockuly 2006). Interestingly, the three month tissue appears to have relatively higher NKCC1 expression levels compared to the 24 hour tissue. This result suggests that changes in cotransporter expression continue, even after cessation of stimulations. However, further analysis involving more animals with various numbers of CIV seizures is necessary to confirm this observation.

We also hypothesized rats that experience seizures during the critical period (postnatal days 20-30) would show long term dysfunction in hippocampal NKCC1 and/or KCC2 expression compared to rats of the same age that did not experience seizures. The results in *Figure 9* support this hypothesis: Rats that experience a kainate-induced seizure at P25 display decreased KCC2 expression levels both 24 hours and 90 days after the event, with little change in NKCC1 expression. These alterations would have the same overall effect as those described for *Figures 4-6*: increased $[Cl^-]_i$. However, in this case, decreased KCC2 expression reduces the outward movement of Cl^- and ultimately increases excitability of hippocampal neurons.

Most notably, the change in KCC2 expression is observed in rats injected at P25 but not P1 or P35. This data supports the theory of a critical period of neuronal development between P20-30. Furthermore, the decreased KCC2 expression in P25-injected animals is observed in adulthood ($> P90$). Thus, this result correlates with the study conducted by Sayin *et al.*, which found that seizures during P20-30 caused long-term reduction of inhibition in neural tissue (*under review*). Furthermore, the trends observed in *Figure 9* may show that decreased KCC2 expression is one of mechanisms that mediate the permanent alteration of inhibitory circuitry caused by developmental-stage seizures.

P1 and P35 animals displayed a high degree of variability for both NKCC1 and KCC2 expression levels. For example, some animals showed increased expression of a particular cotransporter, while other animals of identical age and treatment showed decreased expression. This discrepancy correlates with previous observations: The pathophysiological effects of early seizures are highly variable and depend on stage of development (Cilio *et al.* 2003, Sayin *et al.* 2004).

We observed long-term, increased NKCC1 expression in rats that experienced class V seizures. This change was not observed in animals that experienced ADs in kindling. Evidence from numerous studies suggests that the characteristic hyperexcitability found in kindling models and epilepsy stems from a disruption of the delicate balance between excitatory and inhibitory synaptic activity (Fisher *et al.* 2005, Jin *et al.* 2004). Thus, increased NKCC1 expression may be an important factor that mediates the progressive excitability characteristic of epileptogenesis. Interestingly, seizures during the critical period result in decreased KCC2 expression, leading to increased seizure propensity in adult life. Thus, the results of this study indicate that CCCs may play a fundamental role in GABAergic function and neuronal activity. Currently, medical treatments for epilepsy target the GABA system by augmenting GABA_A activity or inhibiting the removal of GABA from the synaptic cleft (Delpire 2000). However, this approach does not address the balance between internal and external [Cl⁻] that mediates the excitatory or inhibitory actions of the GABA_A receptor itself. The intimate relationship between neuronal excitability and cotransporter expression may be an important avenue for new anticonvulsant drug development.

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