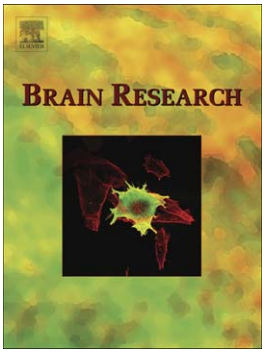

Connexin36 knockout mice display increased sensitivity to pentylenetetrazol-induced seizure-like behaviors

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TITLE:

Connexin36 knockout mice display increased sensitivity to pentylenetetrazol-induced seizure-like behaviors

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RUNNING TITLE: Cx36 gap junctions modulate seizure threshold

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TABLES: 1

Abstract (215 wds.)

Objective: Large-scale synchronous firing of neurons during seizures is modulated by electrotonic coupling between neurons via gap junctions. To explore roles for connexin36 (Cx36) gap junctions in seizures, we examined the seizure threshold of connexin36 knockout (Cx36KO) mice using a pentylenetetrazol (PTZ) model.

Methods: Mice (2-3 months old) with Cx36 wildtype (WT) or Cx36KO genotype were treated with vehicle or 10-40 mg/kg of the convulsant PTZ by intraperitoneal injection. Seizure and seizure-like behaviors were scored by examination of video collected for 20 minutes. Quantitative real-time PCR (QPCR) was performed to measure potential compensatory neuronal connexin (Cx30.2, Cx37, Cx43 and Cx45), pannexin (PANX1 and PANX2) and gamma-aminobutyric acid type A ($GABA_A$) receptor α1 subunit gene expression.

Results: Cx36KO animals exhibited considerably more severe seizures; 40 mg/kg of PTZ caused severe generalized (≥grade III) seizures in 78% of KO, but just 5% of WT mice. A lower dose of PTZ (20mg/kg) induced grade II seizure-like behaviors in 40% KO vs. 0% of WT animals. There was no significant difference in either connexin, pannexin or $GABA_A$ α1 gene expression between WT and KO animals.

Conclusion: Increased sensitivity of Cx36KO animals to PTZ-induced seizure suggests that Cx36 gap junctional communication functions as a physiological anti-convulsant mechanism, and identifies the Cx36 gap junction as a potential therapeutic target in epilepsy.

SECTION: 8 - Disease-related neuroscience

KEYWORDS: epilepsy, Cx36, gap junction, PTZ, interneuron

1. Introduction

Seizures are commonly thought to result from an imbalance between excitation and inhibition, resulting in aberrant large-scale synchronized electrical activity in large populations of neurons. Recently there has been increased interest in the role of gap junctional intercellular communication in modulating this synchronized activity (Gajda, et al. 2006, Traub, et al. 2004).

Gap junctions are constructed from approximately 20 members of the connexin protein family by aggregation of six connexin proteins at the cell membrane, forming a connexon (or hemichannel), which associates with a connexon on a neighboring cell to form a mature gap junction. Many different connexin proteins are expressed in the various brain cell-types. Immunolabelling studies indicate that connexin36 (Cx36) is the predominant gap junction protein expressed in neurons (Condorelli, et al. 2000, Degen, et al. 2004, Rash, et al. 2000, Sohl, et al. 1998). Other connexins with minor expression in mouse neurons include connexin30.2 (Cx30.2) (Kreuzberg, et al. 2008), and connexin45 (Cx45) (reviewed in Sohl, et al. 2005).

Direct electrotonic communication via gap junctions may modulate seizure generation and maintenance. (Carlen, et al. 2000, Draguhn, et al. 1998, Perez-Velazquez, et al. 1994). In vivo seizure models, where cell-to-cell communication via gap junctions is manipulated before the administration of proconvulsant drugs, have suggested roles for gap junctional intercellular communication in ictogenesis acting via axonal excitatory-excitatory gap junctions (Traub, et al. 2002). Gap junction blockade with both octanol and carbenoxolone has been shown to be protective against penicillin-induced ictogenesis in an in vivo rat seizure model (Bostanci and Bagirici 2006). Using a pentylenetetrazol (PTZ) seizure model, Nassiri-Asl and co-workers found that Cx36 gap junction blockade with quinine increased seizure latency and decreased seizure severity. These protective effects of quinine were counteracted by trimethylamine (TMA), which alkalizes cells to open gap junction channels (Nassiri-Asl, et al. 2008, Nassiri-Asl, et al. 2009). These in vivo findings suggest a central role for gap junctions in setting the ictogenic threshold. Similar results have been found in hippocampal slice experiments. Gap junction channel blockade using carbenoxolone resulted in a decrease in seizure-like events (Kohling, et al. 2001, Medina-Ceja, et al. 2008). Low

dose mefloquine (<20 μM) is thought to specifically block homotypic Cx36 (and Cx50) gap junctions, and also decreases seizure-like events in cortical brain slices (Cruikshank, et al. 2004). In contrast to these findings, our group has previously reported that, in neocortical slices, gap junction blockade with low-dose mefloquine and carbenoxolone actually increases seizure frequency and amplitude (Voss, et al. 2009). However all the pharmacological gap junction blockers have a number of off-target effects that make interpretation of these results problematic.

Genetic knockout animals are accepted biological models for the elucidation of complex physiological systems. Accordingly, examination of seizures in Cx36KO animals may yield a clearer view of roles for Cx36 gap junctions in seizure propensity. The purpose of this study was to further define the role of Cx36 gap junctions in PTZ-induced seizures by examining differences in seizure responses between WT and Cx36KO animals.

2. Results

There were marked differences in seizure propensity between WT and Cx36KO animals in response to PTZ injection (Fig. 1). At the highest PTZ dose used (40 mg/kg) WT animals rarely exhibited the higher seizure grades, with just 1 of 19 (5%) animals displaying (Grade IV) generalized clonic-tonic seizures; in contrast, 34 of 34 (100%) Cx36KO animals displayed some form of seizure-like behavior and 28 of 34 (82.4%) animals had full generalized clonic-tonic seizures ($p<0.001$, Fisher's exact test). A similar trend was seen in data collected from animals treated with low dose (20 mg/kg) PTZ. After 20mg/kg injection, all (5/5) WT animals displayed grade I seizure-like behavior only; whereas this same dose produced grade I seizure-like behavior in three Cx36KO animals and grade II seizure-like behavior in the other two Cx36KO animals (NS). A lower dose of 10 mg/kg failed to elicit any seizure-like response in animals of either genotype. No difference in propensity to seizure according to gender was seen in animals of either genotype (p=0.36)

Figure 1 here.

Table 1 here.

To examine the possibility for compensatory expression of neural connexins, pannexin or GABA receptor related genes in Cx36KO animals, QPCR was performed to measure neuronal connexin (Cx30.2, Cx37, Cx43 and Cx45), pannexins (PANX1 and PANX2) and the gamma-amino-butyric acid A receptor ($GABA_A$) alpha-1 subunit transcripts (Table 1). For all transcripts examined, no strongly statistically ($p \leq 0.05$) or biologically significant (≥1.5-fold expression level change) differences were detected between WT and Cx36KO animals. Levels of both PANX1 and PANX2 were somewhat reduced in KO animals (0.78 fold and 0.70 fold respectively) compared to WT animals but neither expression difference was statistically significant (p=0.22 and p=0.52 respectively).

3. Discussion

Our primary finding was that animals lacking connexin36 gap junctions had a markedly lowered seizure threshold for generalized tonic-clonic seizures compared to WT animals. This finding contrasts with the view that gap junction closure protects against seizures. Clearly the functional interactions between gap junctions, chemical synapses, and intrinsic neuronal and glial currents are complex and subtle; and await the development of a comprehensive quantitative theory for their proper description. However, at a qualitative level our results are in accord with known neurobiology. It is well-established that Cx36 is the predominant connexin in the inhibitory interneuron network (Connors and Long 2004; Fukuda 2007, Fukuda, et al. 2006). It is therefore easy to envisage that absence of Cx36 gap junction communication amongst these (soma-targeted basket-cell type) inhibitory neurons could result in slower, and less effective, inhibitory control of excitatory runaway activity (Trevelyan, et al. 2006); and hence the predisposition to generalized seizures that we found in the Cx36KO mice. Figure 2a is a cartoon depicting possible connections between populations of neurons and spread of seizure activity. (For the purposes of illustration, we ignore the intrinsic mechanisms of control of neuronal excitability.) This schema is supported by anatomical data recently described by Caputi and co-workers (Caputi, et al. 2009) and relies on the networks of inhibitory interneurons as described by Beierlein et al. (2000). The reason for the inclusion of this diagram in our paper is to highlight the, slightly counterintuitive, fact that the chemical synapses and electrical synapses within the inhibitory-inhibitory polysynaptic pathway have functionally *opposite effects*. Spread of activity within the inhibitory syncitium via *gap junctions* will increase inhibitory soma-targeted cell (I2) activity and *limit* seizure spread. Conversely *chemical* (GABAergic) synaptic activity amongst the interneurons is inhibitory. By "inhibiting-the-inhibitory" neurons (I2), chemical synaptic activity can augment seizure spread. Since Cx36KO mice lack Cx36 gap junctions, these animals are entirely dependent on the chemical inhibitory synaptic pathways to limit seizure spread.

In support of our results, there is other experimental evidence that Cx36 gap junction ablation increases seizure propensity. Pais and co-workers (Pais, et al. 2003), working in vitro using hippocampal slices, found increased inter-ictal discharges in Cx36KO mice as compared to WT mice. In a recent study by Yang and colleagues, uncoupling of inhibitory interneurons was shown to cause secondary excitatory effects in

pyramidal cells (Yang, et al. 2007). Furthermore, our group has recently reported in the isolated cortical slice model that gap junction blockade with low-dose mefloquine and carbenoxolone increases seizure-like event frequency and amplitude (Voss, et al. 2009). There is also genetic evidence supporting the idea that Cx36 malfunction is pro-convulsant, with alterations in the sequence and expression level of Cx36 implicated in the seizure phenotype. A single nucleotide polymorphism (SNP) in the Cx36 gene is associated with juvenile myoclonic epilepsy (JME) (Hempelmann, et al. 2006). The authors found that the T allele of a SNP at position 588 of the transcript (C588T) was more frequent in JME patients than in controls (35% in patients vs. 29.7% in the control group, p= 0.016). The mRNA transcript for Cx36 has been found to be reduced by 44% in response to kainate treatment, both acute and in kindling (Sohl, et al. 2000); although levels were unchanged in an in vitro bicuculline model of epilepsy (Samoilova, et al. 2003).

Figure 2 here.

Whilst PTZ has multiple synaptic and membrane effects – including modulation of inward cation currents (Fowler and Partridge 1984), sodium-potassium ATPase activation (Bignami, et al. 1966) and reduced membrane resistance (Gross and Woodbury 1972) – it would seem likely that its predominant action is to reduce synaptic GABAergic inhibition (Pellmar and Wilson 1977). The following explanation refers to the diagram and nomenclature in figure 2. The actions of PTZ are to reduce GABA-mediated chloride conductance non-specifically at both the I2-E2, I3-E2, ('ie'), and the I1-I2 ('ii') chemical synapses. Because the I1-I2 electrical gap-junction connections are preserved in the WT mice, the (pyramidal cell suppressing) I2-E2 activity is maintained to some degree – reducing runaway excitation and seizure spread. In contrast, the Cx36KO animals have no mechanism to maintain the (pyramidal cell suppressing) I2-E2 activity; which may explain the surprisingly large difference in seizure propensity observed between WT and Cx36KO animals. Thus we could reasonably speculate that interneuron gap junctions might act as a "physiological buffer" against the development of seizures when there are conditions of stress or malfunction in the GABAergic synaptic system.

There are many other possible explanations for our observations. Although we did not find any change in expression mRNA for the alpha subunit of the $GABA_A$ receptor, there could a myriad of possible post-translational compensatory effects. Other studies have indicated that the pro-seizure effects of PTZ are dependent upon thalamo-cortical interactions (Mirski and Ferrendelli 1986, Mirski, et al. 2003). The GABAergic reticular nucleus of the thalamus, in particular, is densely connected by Cx36 gap junctions (Condorelli, et al. 2000) and it is possible that a similar synergistic interaction between PTZ and Cx36 ablation may be evident at this sub-cortical nucleus. Another possible explanation for the reduced PTZ seizure-threshold in Cx36KO mice is that compensatory expression of other gap junction genes may occur and that this may increase predisposition to seizure. However, we could find no evidence for significant changes in the transcription of any of the gap junction-forming proteins we investigated.

In summary, we have found that Cx36KO mice are markedly more sensitive to PTZ-induced generalized seizure than mice with functional Cx36 gap junctions. This calls into question the generally held view that increased neuronal gap junction communication promotes seizures.

4. Experimental procedure

4.1 Animals for seizure study

Male and female two to three month old C57/Bl6:129sv mice were used in this study. Cx36KO mice were gifted by Prof. David Paul (Deans, et al. 2001). The PCR strategy detailed by the authors was used to confirm the genotype of WT and Cx36KO F1 animals. Animals were housed in 12h light: dark conditions with full access to water and food. All housing conditions, feeding regimes and the experiments described here were approved by the Waikato University's animal ethics committee (protocol #740) and every effort made to minimize pain and discomfort to the animals during the experiments. To confirm that the animals used in our experiments did not exhibit spontaneous seizures we observed recorded video footage collected from a control group of five WT and five KO untreated animals for a period of 180 min – no seizure activity was observed during this period.

4.2 Drug preparation

Pentylenetetrazol (Sigma-Aldrich cat#: P6500) was prepared by dissolving in sterile (lipopolysaccharide-free) phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4, pH 7.4) to a final concentration of 12 mg/ml.

4.3 Pentylenetetrazol (PTZ) seizure model

Animals were weighed and then injected intraperitoneally with the pentylenetetrazol (PTZ) preparation (see section 2.2 above) at PTZ doses of 10 mg/kg (to five wildtype and five Cx36KO animals), 20 mg/kg (to five wildtype and five Cx36KO animals) and 40 mg/kg (to 19 WT and 34 Cx36KO animals). The 40 mg/kg dose used was 20% below the reported LD50 (I.P.) for PTZ; this ensured a low mortality rate (1 of 62 animals used) during the experiment. Smaller doses (10-20 mg/kg) have been reported to induce absence-type seizures (Marescaux, et al. 1984) but are normally considered sub-convulsive for generalized clonic-tonic seizures (e.g. see Itoh and Watanabe 2009, Sansig, et al. 2001).

After injection, behavior of treated animals, including occurrence of all seizure-like behaviors and generalized clonic-tonic seizures, was scored for 20 min from collected video using the scale defined by Hu et al. (1998) as follows: grade 0—no response;

grade I—staring, front- or hind-limb pawing, grade II—staring (≥10sec), rearing, nodding, front- and hind-limb pawing; grade III: staring, rearing, nodding, pawing, jumping, wobbling; falling; grade IV— status epilepticus and/or death. Scores of III or IV are equivalent to generalized clonic-tonic seizures. PBS vehicle-only control injections (100µl) were performed on three WT and three KO animals. Chemicals were purchased from Sigma Ltd, NZ.

4.4 Tissue sampling and cDNA preparation

Tissue was collected from six sites in the neocortex of each of 12 adult (2-3 m.o.) mice (six WT and six Cx36KO animals) by dissection into cold sterile nuclease-free PBS immediately. These tissues were from animals that had not been injected with PTZ or any other drug or vehicle. The tissues were minced using a #10 scalpel blade, placed in a 1.5 mL tube and tissue lysis carried out by the addition of 1 mL of Tri-reagent (Sigma, NZ) and tituration ~10X using a fine-bored 1 mL pipette tip until the tissue was completely lysed. The samples were then processed according to the manufacturer's instructions and resultant total nucleic acids resuspended in 20 µL of DEPC-water. A DNase treatment was carried out using Turbo DNase (Ambion, NZ; cat: AM2238) and then cDNA was produced from 2 µg of this DNA-free RNA in a 20 µL reaction using SuperscriptIII (Invitrogen cat: 18080-051). All protocols were performed according to the manufacturer's instructions.

4.5 Realtime quantitative rtPCR (QPCR)

Each 20µl QPCR reaction consisted of 0.2µM of each of the appropriate intron-flanking forward (F) and reverse (R) primers (Cx30.2_F GCTGTCGCCAGACCTGCTA, Cx30.2_R CTGGTGCATGGAGTAGATGAC, Cx37_F GCGCAGCTTCGGCCACG, Cx37_R AGATGAAGAGCACCGTTAACCAG, Cx43_F AGACAGGTCTGAGAGCCTGAAC, Cx43_R GCGAAAGGCAGACTGTTCATC, Cx45_F CAGGAGTTCTGGTGAACAG, Cx45_R GGCTGCTCTGTGTTGCACAC, PANX1_F CTCTGCTGCTCATCTCGCTG, PANX1_R GAGTATGGCAAACAGCAGTAG, PANX2_F CTGGTCTTCACCAAGAACTTCG, PANX2_R GCGTAGGGCAGGAACTTGTG, GABRA_F TGCGTATCACAGAGGATGGCACTC, GABRA_R CCCATCTTCTGCTACAACCACTGAAC, RNApolII_F

TGCGCACCATCAAGAGAGTG, RNApolII_R GCCAGGACACTCGGTCATG) - primers pairs were designed to flank at least one intron and were all checked for target gene specificity by BLAST (www.ncbi.nlm.nih.gov/BLAST/) searching. 0.75U of Thermo-Start *Taq* polymerase (Thermo), and 4 µM SYTO82 (Molecular Probes), 1X PCR reaction buffer (Thermo NZ ltd), 5 µl (representing approximately 60 ng) of cDNA. Realtime quantitative PCR (rtQPCR) was performed using a Corbett Rotor-Gene 6000 (Corbett Life Science, NZ Ltd) for 45 cycles of 94°C 20 sec, 60°C 20 sec, 72°C sec. After normalization of the Ct values with reaction efficiency using LinReg software (Ramakers, et al. 2003), the data files were examined using the 2^[-ΔΔCt] method (Livak and Schmittgen 2001) for comparative gene expression using RNApolII as a house-keeping gene.

4.6 Statistical analysis

Frequency of seizure-like events was compared using Fisher's exact test with significance assumed if p value ≤ 0.05. For QPCR analysis, Student's t-test was performed to test for the significance of differences between WT and Cx36KO animals. When comparing connexin, pannexin and $GABA_A$ α1 subunit mRNA transcript levels between the WT and Cx36KO groups, fold difference for biologically significant change was assumed to be ≥1.5-fold.

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Ethical approval

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Conflict of interest statement

None of the authors have any conflicts of interest to disclose.

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Figure 1. Seizure grade in wildtype (WT) and Cx36 knockout (Cx36KO) mice treated by intraperitoneal injection of PTZ. Legends indicate seizure grade in roman numerals (1A: 20 mg/kg PTZ treatments, n= 5 for both WT and Cx36KO and 1B: 40 mg/kg treatments n=19 and 34 for WT and Cx36KO, respectively.

Figure 2. 2a.) Diagram of possible connections involved in seizure spread. Two excitatory (triangles, E1 and E2) and three inhibitory (circles, I1, I2 and I3) neurons are shown. Chemical synaptic pathways are shown in solid lines (or dotted in 2b and 2d, if their activity is much reduced); and gap-junction mediated pathways are shown in dashed lines. In brackets we label the synapses from pre-synaptic to post-synaptic cells. (e.g. an '(ie)' synapse is a GABAergic synapse that originates from an inhibitory neuron onto an excitatory neuron). Pathways originating from excitatory cells (glutamatergic and gap junction mediated) are in red and from inhibitory cells (GABAergic and Cx36 gap junction mediated) in blue. The inhibitory neuron labels are based on the nomenclature of Caputi et al. (2009). Parvalbumin-positive cells are in light blue (multipolar-bursting (MB) and fast spiking (FS)), and calretinin-positive cells in dark blue (multipolar-calretinin-positive (MCR)). The MB cells target the layer 2/3 pyramidal cells. There are further between-layer complexities that have not been included for the sake of clarity – such as input from layer 4 pyramidal cells onto I2, and inclusion of bipolar-calretinin-positive cells which also target I2.

Figures 2b, c, and d have focussed on the inhibitory interneuronal pathways relevant to the proposed explanation for our results. 2b) the chemical synaptic pathway, 2c) the electrical synaptic pathway; and 2d) the Cx36KO synaptic pathway.

Table 1. Connexin, pannexin and $GABA_A$ receptor α1 subunit gene expression in WT and Cx36KO mouse neocortex.

Gene name	Gene symbol	Fold difference (Cx36KO vs. WT)	*p* value
Connexin30.2	Cx30.2	1.03	0.56
Connexin37	Cx37	1.15	0.32
Connexin43	Cx43	1.11	0.56
Connexin45	Cx45	1.10	0.50
Pannexin1	PANX1	0.78	0.22
Pannexin2	PANX2	0.70	0.52
$GABA_A$ receptor α1	GABRA1	1.05	0.49

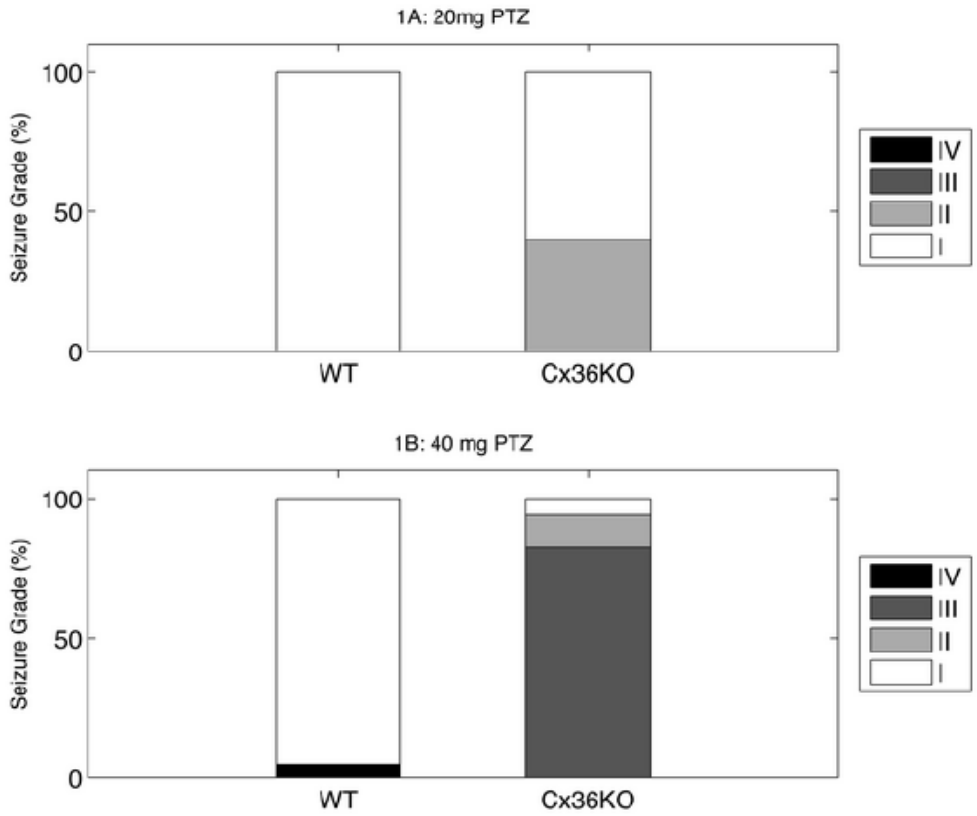
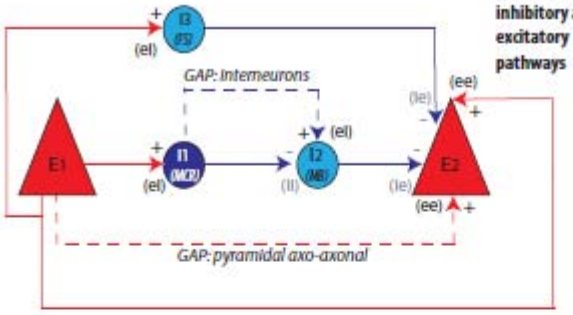
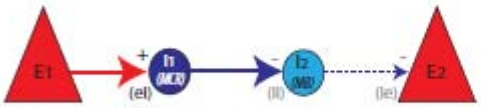


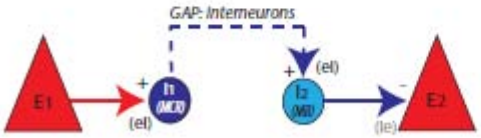
Figure 1



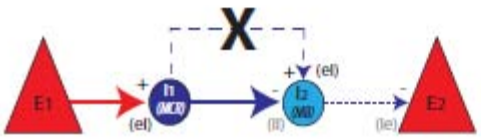
2a. Diagram of inhibitory and excitatory pathways



2b. The chemical synaptic pathway:
Activation of I1–>
Inhibition of I2–>
Allows activation of E2.
(Increased Seizures)



2c. The electrical synaptic pathway:
Activation of I1–>
Activation of I2–>
Prevents activation of E2.
(Decreased Seizures)



2d. The Cx36KO synaptic pathways:
Activation of I1–>
Inhibition of I2–>
Allows activation of E2.
(Increased Seizures)

Figure 2