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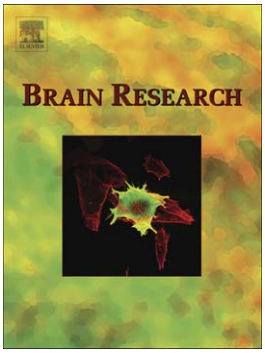

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TITLE

GABAergic compensation in connexin36 knock-out mice evident during low-magnesium seizure-like event activity

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Abstract

Gap junctions within the cerebral cortex may facilitate cortical seizure formation by their ability to synchronize electrical activity. To investigate this, one option is to compare wild-type (WT) animals with those lacking the gene for connexin36 (Cx36 KO); the protein that forms neuronal gap junctions between cortical inhibitory cells. However, genetically modified knock-out animals may exhibit compensatory effects; with the risk that observed differences between WT and Cx36 KO animals could be erroneously attributed to Cx36 gap junction effects. In this study we investigated the effect of $GABA_A$-receptor modulation (augmentation with 16 μM etomidate and blockade with 100 μM picrotoxin) on low-magnesium seizure-like events (SLEs) in mouse cortical slices. In WT slices, picrotoxin enhanced both the amplitude (49% increase, $p=0.0006$) and frequency (37% increase, $p=0.005$) of SLEs; etomidate also enhanced SLE amplitude (18% increase, $p=0.003$) but reduced event frequency (25% decrease, $p<0.0001$). In Cx36 KO slices, the frequency effects of etomidate and picrotoxin were preserved, but the amplitude responses were abolished. Pre-treatment with the gap junction blocker mefloquin in WT slices did not significantly alter the drug responses, indicating that the reduction in amplitude seen in the Cx36 KO mice was not primarily mediated by their lack of interneuronal gap junctions, but was rather due to pre-existing compensatory changes in these animals. Conclusions from studies comparing seizure characteristics between WT and Cx36 KO mice must be viewed with a degree of caution because of the possible confounding effect of compensatory neurophysiological changes in the genetically modified animals.

Section

Neurophysiology, neuropharmacology and other forms of intercellular communication

Keywords

Gap junction, GABA, connexin36, seizure, interneuron, cortex

Abbreviations

Cx36 KO: connexin36 knock-out

WT: wild-type

SLE: seizure-like event

1. Introduction

Gap junctions are transcellular channels that allow direct electrical and ionic coupling between adjacent cells. Neuronal gap junctions in the adult mammalian cerebral cortex are formed predominantly between GABAergic inhibitory interneurons (Deans et al. 2001; Liu and Jones 2003; Markram et al. 2004; Baude et al. 2007), but their function remains poorly understood. One of the avenues that has been explored for investigating their function has been to compare wild-type (WT) animals with genetic knock-outs lacking the gene for connexin36 (Cx36 KO) (Deans et al. 2001; Cummings et al. 2008); the protein that forms GABAergic interneuronal gap junctions (Deans et al. 2001). One of the concerns with utilizing genetically modified knock-out animals is the possibility of compensatory effects to functional systems not directly related to the gene in question. The risk is that observed differences between WT and Cx36 KO animals could be erroneously attributed to Cx36 gap junction effects.

Compensatory changes in GABAergic interneuron morphology (De Zeeuw et al. 2003) and function (Cummings et al. 2008) have been described within the central nervous system of Cx36 KO mice. Inhibitory post synaptic potentials recorded from striatal medium spinal neurons of Cx36 KO mice are prolonged and resistant to $GABA_A$-receptor blockade (Cummings et al. 2008). These effects are unlikely to be due to direct gap junction effects because they are preserved during exposure to gap junction blocking drugs (Cummings et al. 2008).

An idea that has gained credence in recent years is that gap junctions, by their ability to synchronize electrical activity between neurons, may contribute to seizure formation within the neocortex. Cx36 KO animals have not been utilized greatly in this area of research. Given the aforementioned limitations attached to the use of Cx36 KO animals, we were interested in determining whether compensatory effects, observed at the cellular level in previous studies,

may be expressed at a neuronal population level in the context of neocortical seizure activity. Because functional compensation in Cx36 KO mice has been localized to the $GABA_A$-receptor (Cummings et al. 2008), in this study we compared the effect of $GABA_A$-receptor modulation (etomidate augmentation and picrotoxin antagonism) on seizure-like-events (SLE) in neocortical slices from WT and Cx36 KO mice; utilizing the low-magnesium seizure model. The main aim of this study was to determine whether compensation in Cx36 KO mice is evident in cortical slice seizure-like population activity. Our results show that GABAergic modulation of SLE activity is altered in Cx36 KO mice; and that these effects are very likely due to compensatory mechanisms.

2. Results

Comparison of WT and Cx36 KO seizure-like-event characteristics

Seizure-like events were reliably generated with removal of magnesium from the aCSF (see Figure 1). The seizure-like events typically consisted of a large depolarisation, followed immediately by a 4-7 Hz oscillation of varying length, but no longer than 3 s. The amplitude of the initial depolarisation was usually larger than the oscillation that followed. Baseline SLE characteristics were compared between all WT (n=37), Cx36 KO (n=36) and mefloquin-treated WT (n=42) slices. On average, event amplitude was lower in Cx36 KO and mefloquin-treated WT slices compared to WT, but neither of these differences were statistically significant (p=0.06 for both).

GABAergic blockade – picrotoxin effects (Figure 2)

Picrotoxin (100 μM) applied to cortical slices from WT mice induced highly reproducible changes in SLE characteristics, with a mean(SD) 49(33)% increase in event amplitude (p=0.0006) and 37(34)% increase in event frequency (p=0.005). These results are in keeping with the expected pro-seizure effect of $GABA_A$-receptor blockade. In Cx36 KO slices, a similar picrotoxin-mediated increase in event frequency was observed (19(19)% increase, p=0.002); however the amplitude effect was eliminated (p=0.63). There was no significant difference in the magnitude of the frequency change between WT and Cx36 KO slices (p=0.1).

To test whether the lack of amplitude effect in the Cx36 KO slices was due to Cx36 gap junction blockade, WT slices were pre-treated with mefloquin (5-25 μM) prior to picrotoxin delivery. In this case, an increase in both SLE amplitude (27(39)%, p=0.001) and frequency (19(17)%, p<0.0001) was observed, the magnitudes of which were not statistically different from those recorded from WT slices in the absence of mefloquin. These results indicate that acute pharmacological Cx36 blockade does not alter the effect of $GABA_A$-receptor blockade on SLE activity; and that the reduction in amplitude responsiveness to GABA blockade in Cx36 KO animals is likely to be the result of a compensatory effect.

GABAergic augmentation – etomidate effects (Figure 3)

Etomidate (16 μM) applied to cortical slices from WT mice induced a mean(SD) 18(28)% increase in event amplitude (p=0.003) and 25(24)% decrease in event frequency (p<0.0001). In Cx36 KO slices, a similar etomidate-mediated reduction in event frequency was observed (16(22)% decrease, p=0.0006); however, the amplitude effect was eliminated (p=0.8). There was no significant difference in the magnitude of the frequency change between WT and Cx36 KO slices (p=0.2).

To test whether the lack of amplitude effect in the Cx36 KO slices was due to Cx36 gap junction blockade, WT slices were pre-treated with mefloquin (5-25 μM) prior to etomidate delivery. In this case, an increase in SLE amplitude (20(32)%, p=0.003) was observed. When compared across all slices, the magnitude of the increase was not significantly different to that seen in WT slices in the absence of mefloquin (p=0.8). However, in a sub-group of slices etomidate was

perfused with and without mefloquin in the same slice. In those cases where an increase in event amplitude was observed with etomidate on its own (7/12 slices), the corresponding amplitude increase during combined etomidate/mefloquin delivery was consistently lower (mean(SD) 44(49)μV difference, p=0.06; one-sample t-test). This effect occurred irrespective of the order of drug delivery. A significant reduction in event frequency (21(22)% decrease) was also observed during combined etomidate and mefloquin delivery ($p<0.0001$), the magnitude of which was not significantly different from that recorded from WT slices in the absence of mefloquin.

While a modest effect attributable to Cx36 gap junction blockade is evident in these results, the discrepancy in findings between WT slices pre-treated with mefloquin and Cx36 KO slices further implicates the presence of compensatory mechanisms in the KO animals.

Effect of picrotoxin and etomidate on SLE waveform morphology

As shown, picrotoxin and etomidate ($GABA_A$ antagonist and agonist, respectively) both effected an increase in SLE amplitude when delivered to WT slices. This paradoxical finding was explored further by comparing the effect of each agent on the profile of the first population "spike" in each event. The width of the waveform was reduced by etomidate from 17(5) ms to 14(3) ms ($p<0.01$), but was unaltered by picrotoxin (18(3) ms before and after drug delivery, p=0.8). The reduction in spike width during etomidate delivery was also reflected in an increased gradient of the spike upslope (795(387) μV/ms compared to 503(270) μV/ms, p=0.0001) and downslope (-518(-322) μV/ms compared to -271(-180) μV/ms, p=0.002). In contrast, no significant change in spike slope was evident following picrotoxin delivery. These results are

shown graphically in Figure 4; and point to different mechanisms of amplitude enhancement by each agent.

3. Discussion

While the possibility of compensatory effects in genetically modified knock-out animals is well recognized, few studies have attempted to quantify such effects in the Cx36 KO strain. The main finding of this study was that GABAergic effects on SLE amplitude were eliminated in mice lacking Cx36 gap junctions; but were unaffected in wild-type slices in which Cx36 gap junctions were blocked pharmacologically. The implication is that compensation effects in the knock-out animals significantly altered GABAergic modulation of SLE activity. This is the first time that compensation in Cx36 KO mice has been demonstrated at the level of population seizure activity. These findings highlight an important potential source of confounding in studies utilizing genetic knock-out animals to investigate the role of Cx36 gap junctions in modulating seizure processes.

This study found some evidence for Cx30 gap junction modulation of SLE amplitude. While mefloquin pretreatment did not eliminate the etomidate-induced increase in SLE amplitude, it did appear to limit the magnitude of the increase slightly (although it did not reach traditional levels of statistical significance). We suggest that interruption of the inhibitory network syncitium by blockade of Cx36 gap junctions disrupts the precision of action potential timing in excitatory pyramidal cell networks; the result is impaired action potential synchronization and a reduction in the amplitude of excitatory population events.

Compensatory mechanisms in the Cx36 KO mice

The nature of the compensation effect in Cx36 KO mice is not known. The results of our study, while clearly showing expression of compensation effects at the level of population seizure-like

activity, does not directly answer the question of where the compensation is occurring. We have found no evidence for up-regulation of other gap junction proteins in the Cx36 KO cerebral cortex (data not shown) and it would appear that compensatory effects may reside in morphological and electrophysiological neuronal properties independent of gap junction up-regulation (De Zeeuw et al. 2003). For example, Cummings, Yamazaki, Cepeda, Paul, and Levine (Cummings et al. 2008) have shown that inhibitory post synaptic potentials (IPSPs) recorded from Cx36 KO striatal spiny neurons are resistant to $GABA_A$-receptor blockade with bicuculline. This is consistent with our finding that cortical $GABA_A$-receptor blockade with picrotoxin had a reduced effect in slices from Cx36 KO mice. Cummings and colleagues speculate on the possibility of changes in $GABA_A$-receptor subunit composition and/or reduced clearance of GABA from the synaptic cleft; hypotheses that are yet to be tested. Neurosteroids, being one of the main autocrine endogenous modulators of GABA activity, might also be altered in Cx36 KO animals (Belelli and Lambert 2005). $GABA_A$-receptor subunit alteration seems unlikely on the basis of the differing specificities of the two drugs tested in the present study. Etomidate is a highly specific $GABA_A$-receptor agonist that targets β2 and β3-containing receptors (Reynolds et al. 2003; O'Meara et al. 2004). Picrotoxin on the other hand is a more general-acting $GABA_A$ antagonist and its action does not appear to be subunit-specific (Bell-Horner et al. 2000). If the compensation resided in a change in $GABA_A$-receptor subunit composition, one would expect picrotoxin to have similar effects in WT and Cx36 KO slices; which was not the case in this study. Whatever the mechanism, the implication is that GABAergic inhibition is somehow more robust in the Cx36 KO.

The effects of GABA blockade and augmentation on SLE frequency and amplitude

Our results clearly indicate that the process of triggering a population epileptiform burst – as estimated by the *frequency* of the SLEs – is different from the subsequent processes that determine local spread of depolarisation which, in turn, influence the *amplitude* of the SLE. Hippocampal data shows that population epileptiform bursts are initiated when a threshold network firing frequency is reached (de la Prida et al. 2006). Activation of single cells were able to precipitate the initiation of the next event and reduce the inter-burst interval, as could an increase in neuronal excitability (de la Prida et al. 2006). The reduction in inter-burst interval was not due to a change in the firing frequency threshold, but a reduction in the time to threshold. Extrapolating to the results of our neocortical study, the changes in SLE frequency in WT slices following picrotoxin and etomidate probably resulted from altered neuronal excitability; causing threshold firing to be achieved sooner with picrotoxin GABA blockade, or later with etomidate induced GABA augmentation. That parallel changes in event frequency were evident in the Cx36 KO animals suggests comparable $GABA_A$-receptor function in both genotypes.

The amplitude effects reported in this study are more difficult to interpret. The amplitude of individual population events depends upon recruitment of neuronal units into the burst event (van Drongelen et al. 2003; Stefanescu and V.K. 2008) and/or enhanced synchronization of existing units (Durand and Warman 1994). The increase in event amplitude during picrotoxin exposure is likely to be due to recruitment of new units secondary to an increase in neuronal excitability. The etomidate-mediated increase in event amplitude, on the other hand, is more likely to result from enhanced synchronization, as general anaesthetics reduce neuronal excitability but are known to increase local neuronal synchrony (Erchova et al. 2002). While we were not able to directly measure neural synchrony in this study, etomidate caused a narrowing

and sharpening of the SLE population activity; evidence of enhanced synchronicity of neuronal depolarisation. The increase in event amplitude seen with etomidate may also be a manifestation of the capacity of this drug to promote seizure activity (Modica et al. 1990b; Voss et al. 2008). The question is why these effects should be abolished in the Cx36 KO tissue? One possibility may reside in spike frequency adaptation. Spike frequency adaptation is the termination of action-potential firing during prolonged depolarization and has been shown to be reduced in Cx36 KO fast spiking interneurons (Deans et al. 2001). The resultant enhancement of feedback inhibition in the Cx36 KO following the initiation of a population burst could limit the recruitment of pyramidal cells into the event, curtailing event amplitude. This would explain why event amplitude, but not frequency, was affected in the Cx36 KO animals.

Drug dosage

The diffusion profile of mefloquin into brain tissue is not known, although its high lipophilicity suggests it would probably diffuse relatively rapidly. The mefloquin dose and delivery regime used in this study (pre-treatment period of at least 20 minutes at 5μM) was chosen on the basis of a previous study in which 5-10 μM mefloquin delivery for 20 minutes was shown to induce reproducible changes in low-magnesium-induced cortical SLE activity (Voss et al. 2009). These effects were attributed to Cx36 gap junction blockade because they were abolished in Cx36 KO slices. This low dose would have limited effects on other gap junctions, particularly the astrocytic gap junction Cx43 and non-gap-junctional off-target effects would also be minimal (Cruikshank et al. 2004). However, to cover the possibility of slower than expected diffusion of mefloquin into the slice tissue, in a subset of etomidate and picrotoxin experiments, mefloquin pre-treatment was extended to 1 hour at 25μM (n=4). In these cases, the changes in SLE

amplitude and frequency in response to etomidate/picrotoxin delivery were indistinguishable from those observed at the lower mefloquin dose; confirming that the lack of effect of mefloquin was not due to an insufficient amount of drug reaching the tissue recording site.

Etomidate is a general anaesthetic drug that, at anaesthetic concentrations, has highly specific $GABA_A$-receptor agonist effects (O'Meara et al. 2004). In this study, etomidate was delivered at an aCSF concentration of 16 μM. Etomidate tissue concentration is calculated to reach only 15-20% of its maximum after 10 minutes (Benkwitz et al. 2007), giving an estimated tissue concentration at the recording site in our experiments of 2.4 to 3.2 μM. This approximates a clinical burst suppression dose (De Paepe et al. 1999). At this dose, the possibility of off-target GABA effects is unlikely. For example, concentrations greater than 10 μM are required to induce presynaptic and postsynaptic actions, such as block of L-type calcium (Takahashi and Terrar 1994) and sodium channels (Lingamaneni and Hemmings 2003) and inhibition of brain nitric oxide synthase activity (Galley and Webster 1996). That etomidate at the dose used acts via the $GABA_A$-receptor is further corroborated by the finding that pre-treatment of slices with picrotoxin completely eliminates the etomidate effect on SLE amplitude and frequency (adult c57bl6 mice, data not shown).

Implications

Our results are consistent with the suggestion that the Cx36 KO mice have an increased GABAergic tone as a compensation for their lack of interneuron gap junctions. Therefore, results from studies comparing seizure characteristics between wild-type and connexin36

deficient mice must be viewed with a degree of caution because of the possible confounding effect of compensatory neurophysiological changes in the genetically modified animals. Ideally, knock-out based research results should be supported by alternative strategies to manipulate the subcellular structure under investigation. Our study has focused on GABAergic modulation of seizure activity; however the possibility of more wide-reaching compensatory changes should also be considered.

4. Experimental Procedure

All methods were approved by the Animal Ethics Committee at the University of Waikato.

Cortical slice preparation

Neocortical slices were prepared from mixed sex C57/Bl6/129SV adult WT and Cx36 KO mice (the latter gifted by professor David Paul, Harvard University). Following anaesthesia with carbon dioxide, the mice were decapitated and the brain rapidly removed and chilled in ice-cold artificial cerebrospinal fluid (aCSF), modified for cerebral protection according to Nowak and Bullier (Nowak and Bullier 1996). The aCSF contained 92.7 mM NaCl, 24 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 3 mM KCl, 19 mM $MgCl_2$, 0 mM $CaCl_2$, and 25 mM D-glucose, saturated with carbogen (95% O_2; 5% CO_2). The brain was held in ice-cold aCSF and sectioned in the coronal plane into 400 μm thick slices using a vibratome (Campden Instruments, UK). The slices were given a minimum of one hour for recovery at room temperature (approximately 22ºC) in carbogenated low-magnesium aCSF composed of: 124 mM NaCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 5 mM KCl, 0 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM D-glucose. The slices were subsequently transferred to an immersion-style recording chamber and perfused continuously with carbogenated low-magnesium aCSF at room temperature. The low-magnesium aCSF was delivered via two sealed 50 ml syringes, each driven by an infusion pump at a flow rate of 2.5 ml/min (giving a total flow rate of 5 ml/min). All solutions were replaced after no more than 1 week of storage at 1-4°C.

Electrophysiological recording

A single 50 μm teflon-coated tungsten wire was positioned in the cortex for recording spontaneous local field potential activity. A silver/silver-chloride disc electrode served as a common reference and bath ground. The recording set-up was enclosed within a grounded Faraday cage. The neocortical signal was amplified (1000x, A-M Systems, USA) and bandpass filtered (1 and 3000Hz) before analog-digital conversion (Power 1401, CED, UK) and recording on computer (Spike2, CED, UK) for later analysis.

Drug preparation

Etomidate (16 μM) (Hypnomidate, Janssen-Cilag, Belgium), picrotoxin (100 μM) (Sigma, USA) and mefloquin (5-25 μM) (Sigma, USA) solutions were prepared by adding the appropriate amount of the drug directly to pre-prepared, carbogenated low-magnesium aCSF. In the case of mefloquin and picrotoxin, dimethyl sulphoxide (DMSO) was added to a final concentration of no more than 0.1% to aid solubility. This concentration of DMSO does not affect SLE activity in cortical slices (data not shown). All drug solutions were delivered via the double-syringe perfusion system described above.

Experimental protocols

Once transferred to the recording chamber, a stable pattern of SLE activity was established and recorded for at least 10 min in all slices before proceeding with the experimental protocols as follows:

1. In **WT** (n=11, from 3 animals) and **Cx36KO** tissue (n=12, from 2 animals), 100 µM picrotoxin was delivered for 20 min, followed by wash-out with low-magnesium aCSF for 20 min.

   In a further group of **WT** animals (n=17, from 4 animals), slices were pre-treated with 5 µM (n=14) and 25 µM (n=3) mefloquin for at least 20 min, followed by 100 µM picrotoxin as described above. Mefloquin perfusion was continued during picrotoxin delivery.

2. In **WT** (n=26, from 6 animals) and **Cx36KO** (n=24, from 5 animals) tissue, 16 µM etomidate was delivered for 10 min, followed by wash-out with low-magnesium aCSF for 20 min.

   In a further group of **WT** animals (n=25, from 7 animals), slices were pre-treated with 5 µM (n=21) or 25 µM (n=4) mefloquin for at least 20 min, followed by 16 µM etomidate as described above. Mefloquin perfusion was continued during etomidate delivery.

For those experiments utilising mefloquin pre-treatment, the drug was perfused into the recording bath prior to the slices being transferred from the holding chamber. Thus, the effect of mefloquin on low-magnesium SLE activity could not be assessed in this study (but has been documented previously (Voss et al. 2009)).

Statistical analysis

In order to statistically compare the drug effects, the average values of the SLE amplitude and frequency were calculated for the 5 min prior to drug delivery, and then compared to the

corresponding average value during the 15 min window, beginning 10 min following the start of drug infusion. Drug effect was quantified as percent change and analysed using the one-sample t-test. In addition, for slices from WT mice, the effect of etomidate and picrotoxin on shape of the initial "population spike" in each SLE was determined by measuring spike width and the gradient of the spike up- and down-slopes. The Kolmogarov-Smirnov test was used to confirm data normal distribution. Unless otherwise stated, the data is expressed as mean percent change and mean(SD). $P<0.05$ was considered statistically significant.

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Figure Legends

Figure 1. Seizure-like event (SLE) activity induced by removal of magnesium from the aCSF. Compressed view (top) showing multiple SLEs and expanded view (bottom) showing a single SLE.

Figure 2. Graph showing the effect of picrotoxin (100 μM) on seizure-like event (SLE) amplitude (top) and frequency (bottom) in wild-type (WT), connexin36 knock-out (Cx36 KO) and mefloquin-pretreated wild-type (WT + Mef) slices.

Figure 3. Graph showing the effect of etomidate (16 μM) on seizure-like event (SLE) amplitude (top) and frequency (bottom) in wild-type (WT), connexin36 knock-out (Cx36 KO) and mefloquin-pretreated wild-type (WT + Mef) slices.

Figure 4. Effect of etomidate (top) and picrotoxin (bottom) on the shape of the initial population "spike" in each seizure-like event. Note the narrower profile following etomidate delivery. There was no significant difference in the baseline amplitudes ($p=0.13$). The heavy lines represent the mean and lighter shading the distribution of individual events.

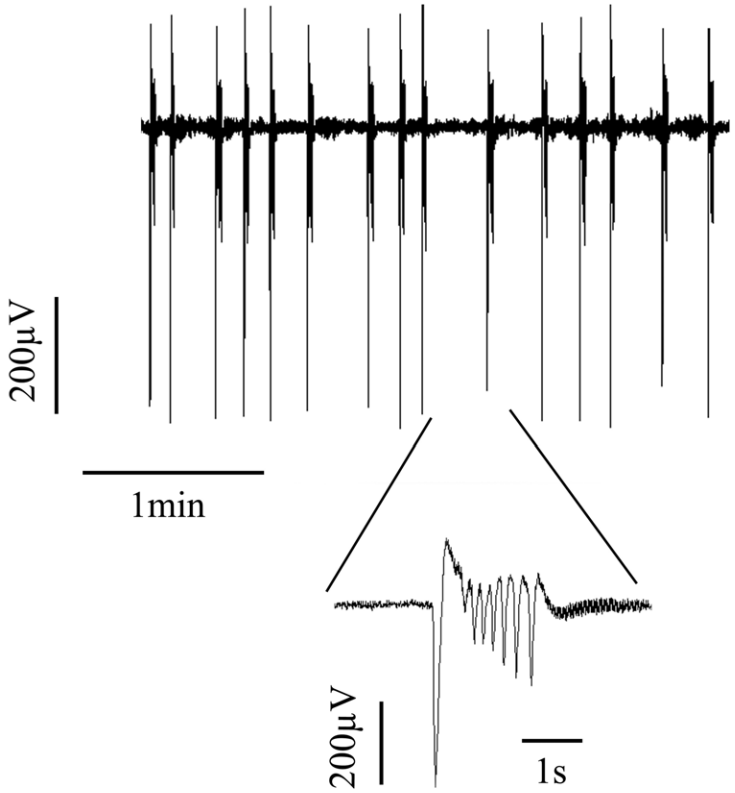

Figure 1

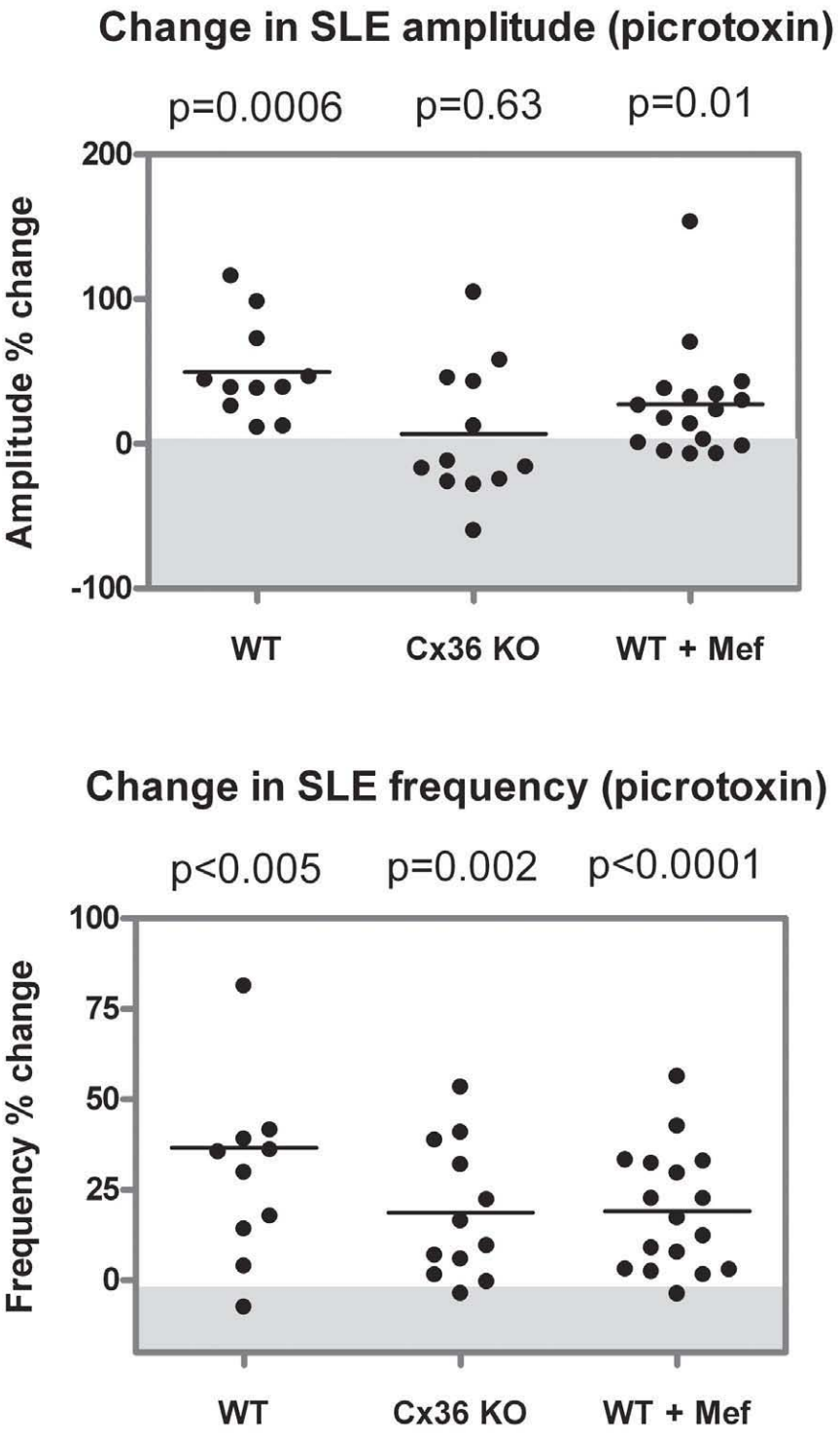

Figure 2

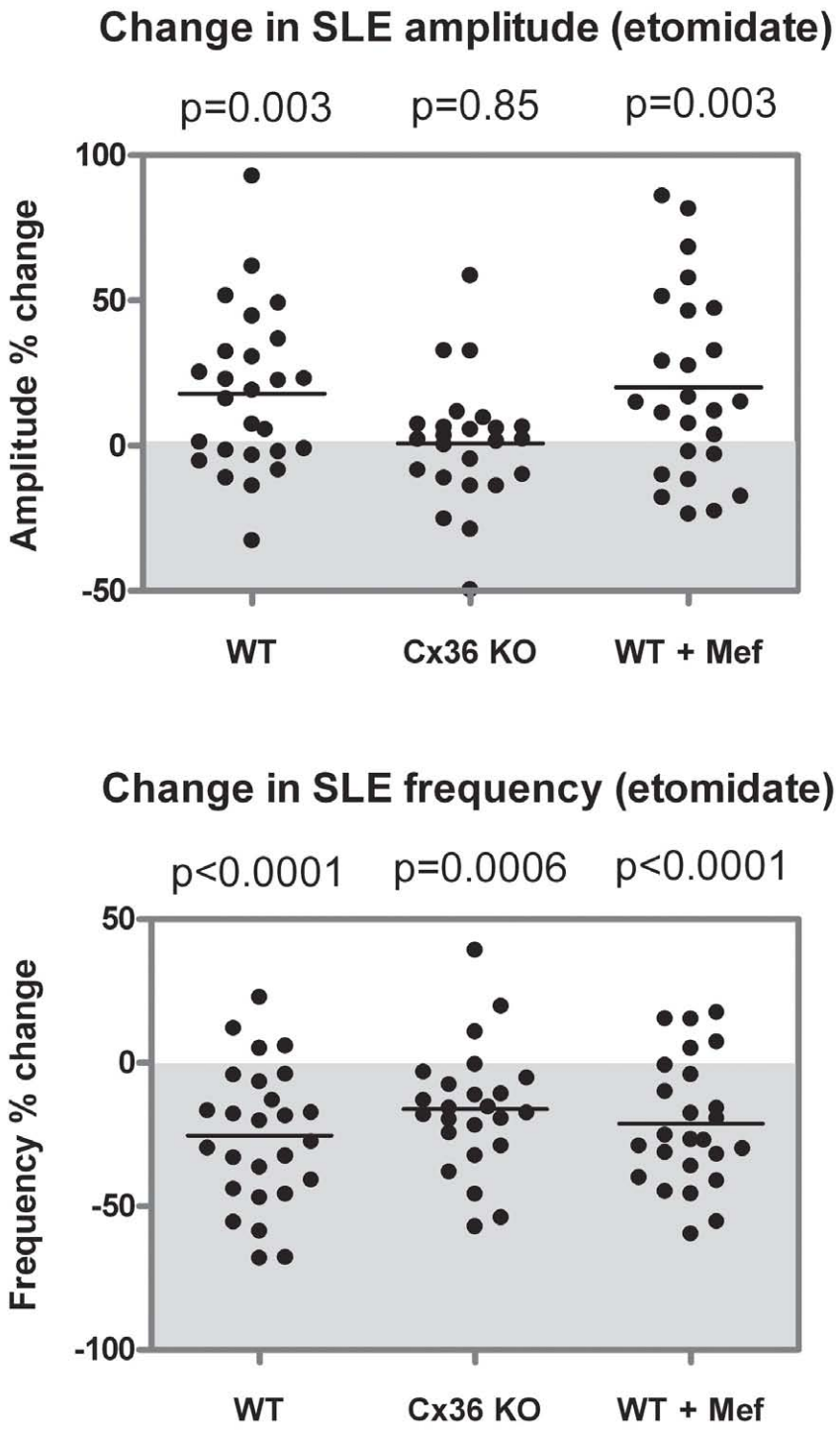

Figure 3

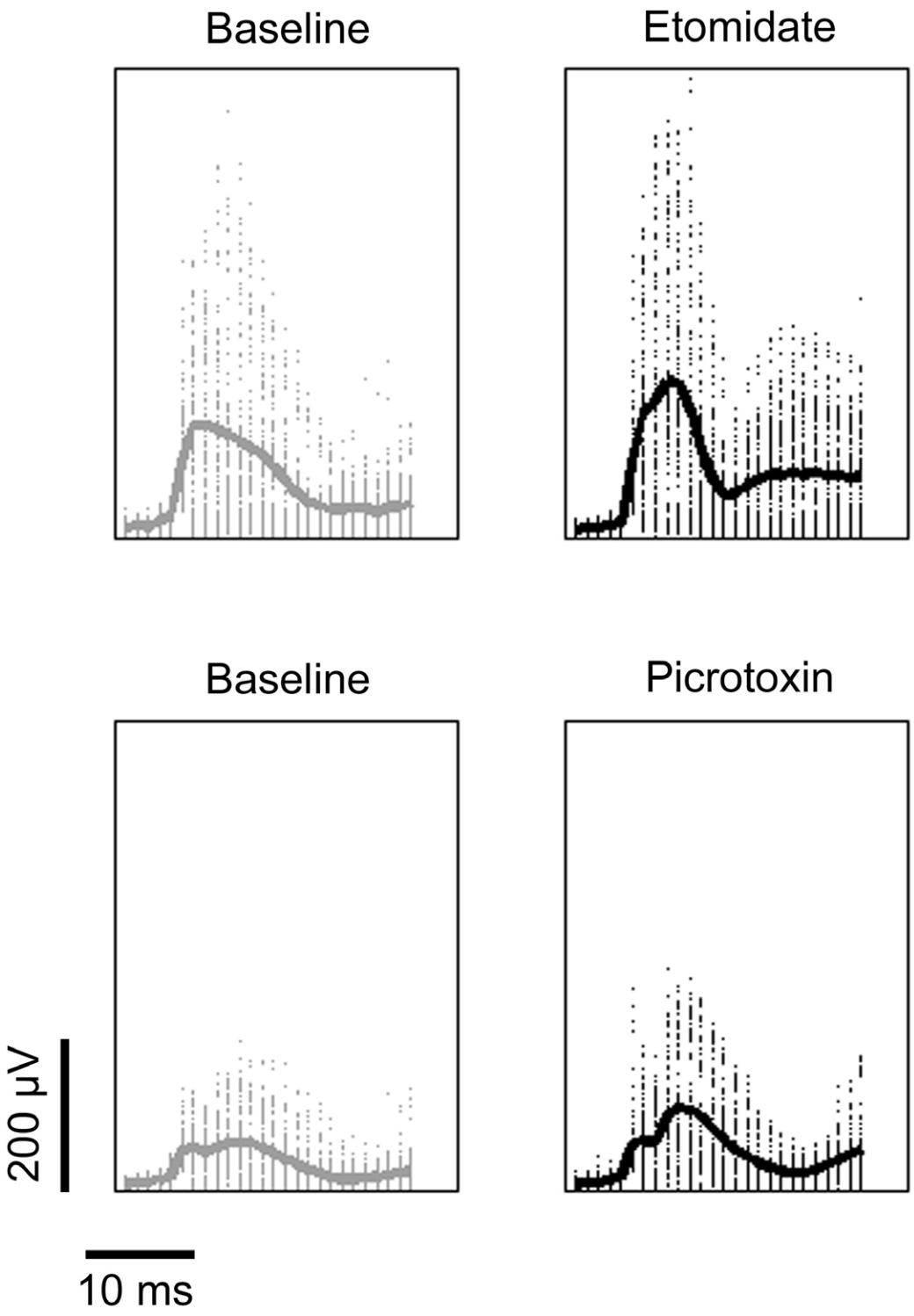

Figure 4

Research highlights

- GABA receptor augmentation and blockade increases SLE amplitude in cortical slices
- SLE amplitude augmentation is abolished in connexin36 knock-out mice
- GABAergic compensation to SLE activity is evident in connexin36 knock-out mice