Thalamocortical relationships and network synchronization in a new genetic model "in mirror" for absence epilepsy

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Abbreviations: 4AP: 4-aminopyridine; ACSF: artificial cerebrospinal fluid; BR/Orl: non-epileptic mice; BS/Orl: epileptic mice; CBX: carbenoxolone; CNQX: 6-cyano-7-nitro-quinoxaline-2,3-dione disodium salt; CPP: 3,3-(2-carboxypiperazine-4-yl)-propylphosphonate; CRTS: Cortical responses to thalamic stimulations; DiI: perchlorate of 1,1' -dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine; GAERS: Genetic Absence Epilepsy Rat from Strasbourg; GJ: Gap Junctions; ILA: Ictal-like activities; Kyn: kynurenic acid; nRT: nucleus reticularis thalami; SWD: spike-wave discharges; TRCS: Thalamic responses to cortical stimulations; VB: ventrobasal thalamus.

Abstract:

Electroencephalographic generalized spike and wave discharges (SWD), the hallmark of human absence seizures, are generated in thalamocortical networks. However, the potential alterations in these networks in terms of the efficacy of the reciprocal synaptic activities between the cortex and the thalamus are not known in this pathology. Here, the efficacy of these reciprocal connections is assessed *in vitro* in thalamocortical slices obtained from BS/Orl mice, which is a new genetic model of absence epilepsy. These mice show spontaneous SWD, and their features can be compared to that of BR/Orl mice, which are free of SWD. In addition, since gap junctions may modulate the efficacy of these connections, their implications in pharmacologically-induced epileptiform discharges were studied in the same slices.

The thalamus and neocortex were independently stimulated and the electrically-evoked responses in both structures were recorded from the same slice. The synaptic efficacy of thalamocortical and corticothalamic connections were assessed by measuring the dynamic range of synaptic field potential changes in response to increasing stimulation strengths. The connection efficacy was weaker in epileptic mice however, this decrease in efficacy was more pronounced in thalamocortical afferents, thus introducing an imbalance in the reciprocal connections between the cortex and thalamus. However, short-term facilitation of the thalamocortical responses were increased in epileptic mice compared to non-epileptic animals. These features may favor occurrence of rhythmical activities in thalamocortical networks. In addition, carbenoxolone (a gap junction blocker) decreased the cumulative duration of 4-aminopyridine-induced ictal-like activities, with a slower time course in epileptic mice. However, the 4-aminopyridine-induced GABA-dependent negative potentials, which appeared to trigger the ictal-like activities, remained.

Our results show that the balance of the reciprocal connections between the thalamus and cortex is altered in favor of the corticothalamic connections in epileptic mice, and suggest that gap junctions mediate a stronger cortical synchronization in this strain.

1. **Introduction**

Typical absence seizures are characterized by brief periods of impaired consciousness concomitant with generalized synchronous bilateral spike-wave discharges (SWD) on electroencephalographic recordings (EEG). SWD are generated in thalamocortical networks without participation of the hippocampus (Danober *et al*., 1998; Meeren *et al*., 2005).

Many studies were aimed at determining which structure (cortex or thalamus) was the pacemaker for the generation of generalized SWD in absence epilepsy (Meeren *et al*., 2005). Experiments in slices have ascribed the generation of absence seizures to alterations in (1) the thalamic neuronal intrinsic properties and (2) the synaptic interactions between the thalamic nuclei (Huguenard and Prince, 1994; Crunelli and Leresche, 2002; Budde *et al.,* 2005). However, studies with genetic rodent models of absence seizures, and in particular the genetic absence epilepsy in rats from Strasbourg (GAERS) (Danober *et al*., 1998) and Wistar Albino Glaxo/Rijswjk (WAG/Rij) rats (Coenen and van Luijtelaar, 2003) have provided increasing evidence that the cortex exerts a leading role in the initial phase of SWD, and leads to synchronization of neuronal firing in thalamocortical networks (Avoli *et al.,* 1983; Steriade and Contreras, 1998; Pinault, 2003; Meeren *et al.,* 2005; Polack *et al.,* 2007, 2009). Following the initial phase of the epileptic seizure, it has been found that both structures are necessary for the maintenance of the epileptic activities (Danober *et al*., 1998). In this context, the relative efficacy of the reciprocal connections between cortex and thalamus may be an important parameter to promote generation and maintenance of SWD. This factor can be studied in slices in which these connections are still physiologically active, both in epileptic and non-epileptic animals. Such slices are very difficult to obtain in rats but can be prepared in mice, mainly due to larger rat

brain sizes, which reduces the maintenance of reciprocal connections between the cortex and thalamus in a given slice thickness.

We therefore investigated the efficacy of the reciprocal thalamocortical connections in a novel genetic model of absence epilepsy, including (1) BS/Orl (epileptic) mice and (2) control BR/Orl mice (non-epileptic) (Rinaldi *et al.*, 2002). These two strains are derived from an eight-way cross and have been selected according to their respective resistance and sensitivity to convulsions, which were induced by a single dose of a methyl β-carboline-3-carboxylate. Methyl β-carboline-3-carboxylate is a β-carboline, which is an inverse agonist at the benzodiazepine site of the $GABA_A$ receptors (Chapouthier *et al.*, 1998). It turns out that beyond their interest for studying the beta-carboline-induced seizure activity, these two lines represent a model for absence epilepsy (susceptible *vs* resistant). This fact is not surprising since different studies have suggested a close relationship between absence epilepsy and beta-carboline effects (Marescaux *et al.*, 1987; Vergnes *et al.*, 2001). In brief, bilateral spontaneous (without injection of any drug) SWD occur in all adult BS/Orl mice at the cortex and thalamus but never at the hippocampus, and this feature lasts throughout life. The frequency of these SWD is approximately 1 per min when the animal is in a calm waking state, while their duration varies between 1 and 8 seconds and the intra burst frequency is 8 ± 1 Hz. The SWD are concomitant with an arrest of the locomotor activities although they are sometimes accompanied with a slight movement of the whiskers and a fall of the head. The SWD are suppressed with anti-absence seizure drugs (ethosuximide and sodium valproate) while they are exacerbated by carbamazepine, which is known to aggravate absence seizures. In contrast, the BR/Orl strain does not display any spontaneous SWD even under the effect of drugs known to induce SWD (Rinaldi *et al.*, 2002).

We prepared functional thalamocortical slices in which the reciprocal thalamocortical relationship (between the somatosensory cortex and ventrobasal thalamus (VB)) was preserved. To test the "efficacy" of these connections in epileptic and non-epileptic mice we established input-output curves following electrical stimulations and measured the amplitude of the responses in each structure in each mouse model. Only slices where both cortical and thalamic responses were obtained have been included in this study.

In rat models of absence epilepsy the synchronized oscillations, which give rise to SWD's, have been shown to occur in deep cortical layers (Pinault, 2003; Polack *et al*, 2007, 2009) where neurones present an increased synaptic excitability in WAG/Rij rats (D'Antuono *et al.*, 2006). In this context, we tested the ability of the thalamocortical network, in particular at these deep cortical layers (Vb and VI), to generate pharmacologically-induced rhythmical activities to detect possible alterations related to absence epilepsy. 4-aminopyridine (4AP), a potassium channel blocker, is known to induce synchronized activities in thalamocortical networks (Tancredi *et al*., 2000; D'Arcangelo *et al*., 2002). We therefore characteristised the epileptiform activities induced *via* bath-application of 4AP in thalamocortical slices, which were prepared from epileptic and non-epileptic mice. Since gap junctions (GJ) have been shown to modulate epileptiform synchronous activities (Nakase and Naus, 2004), here we investigated their role in thalamocortical synchronization by observing the effects of a GJ blocker on these activities, since interneuronal connexins are strongly expressed in the thalamocortical system of mice (Liu and Jones, 2003).

2 Results

2.1 Visualization of reciprocal fibers between cortex and thalamus

The preservation, at least partial, of the fibers connecting the thalamus and cortex was first assessed in thalamocortical slices using fluorescent tracers. We processed only slices where cortical or thalamic responses to thalamic or cortical electrical stimulations respectively have been obtained. After fixation, a crystal of carbocyanine (DiI) was independently inserted into (1) the cortex and (2) thalamus. The cortical site, where carbocyanine was inserted, displayed an intense red fluorescence with a non-specific halo, and the marker migrated from the deposit site along the axons. In Fig. 1 fibers were visualized 10 days after the deposition of the DiI crystal. Here we observed that fibers initially running along the lower cortical layer, inside the white matter, skirt around the hippocampus and reach the VB through the nucleus Reticularis Thalami (nRT). Fibers connecting the thalamus and cortex were observed in all slices investigated with this protocol (n=6; 4 thalamic deposits and 2 cortical deposits).

2.2 Cortical responses to thalamic stimulations (CRTS)

2.2.1 Cortical responses to thalamic stimulations: single shock stimulations

Cortical responses to thalamic stimulation displayed (Fig. 2B) an early negative component, defined as N1, which remained when kynurenate (Kyn) (blocker of glutamatergic synaptic responses) was applied to the bath. N1 was therefore considered as an axonal response. N1 was followed by a synaptic negative component, defined as N2, which disappeared upon Kyn application. N1 could be mono- or bi-phasic with an average onset latency of 0.9 ± 0.1 ms in BR/Orl slices (n=8) and 0.7 ± 0.1 ms in BS/Orl slices (n=7). N2 had a latency-to-peak of 4.7 ± 0.3 ms for BR/Orl (n=8) and 7.6 ± 2 ms for BS/Orl (n=7). N2 was largest in the primary somatosensory cortex with an average amplitude of -210 ± 24 µV (n=8) in BR/Orl and -65.9 ± 9.2

µV (n=7) in BS/Orl (p<0.05). The average maximal duration of N2 was 30.5±4.8 ms (n=8) in BR/Orl and 49.9±7.9 ms (n=7) in BS/Orl (p<0.05) (Table 1).

2.2.2 Cortical responses to thalamic stimulations: comparison of excitability indices

We determined the threshold intensity and the connection efficacy for the fiber and synaptic responses. There was no significant difference in connection efficacy for the fiber responses between both strains. The threshold intensity of the synaptic components was not significantly different (0.1±0.01 mA for BR/Orl; n=8 and 0.12±0.03 mA for BS/Orl; n=7). However, the connection efficacy was significantly (p<0.01) lower in BS/Orl slices (232.5±38.6 % of increase/mA; n=7) than in BR/Orl slices (432.5±53.4 % of increase/mA; n=8) (Fig. 3A), showing that the thalamocortical afferences are relatively less effective in the epileptic mice.

2.2.3 Cortical responses to thalamic stimulations: paired-pulse stimulations

To detect possible differences in depression or facilitation of responses between the two strains we used a paired pulse protocol to determine the paired pulse ratio (PPR). The PPR was defined as the ratio: amplitude of the second synaptic response divided by the amplitude of the first synaptic response. Both stimulations of the paired stimuli were identical (duration=80 µs, I=I_{50}). In addition, the PPR was calculated for different interstimulus intervals (5 ms-100 ms).

In both strains we always observed a facilitation of the second response for intervals ranging from 5 to 100 ms (Fig. 3A). The PPR at short intervals (5 and 10 ms) was significantly (p<0.05) larger in BS/Orl (n=7) compared to BR/Orl (n=6). At intervals above 100 ms, no facilitation occurred (data not shown). We never observed any depression of the response to the second stimulation, even at higher stimulation intensities. To determine whether this lack of depression was due to the absence of intracortical inhibition in these thalamocortical slices, we

applied double shock stimulations to the white matter underlying the somatosensory cortex and recorded in the upper cortical layers. At an intensity of 1mA, we measured the 1st and 2nd synaptic responses at stimuli intervals of 5, 10 and 20 ms (Fig. 2C). Here we reported that the amplitude of the 2nd response was greatly reduced in comparison to the 1st response, thus indicating that intracortical inhibition was still functional in these slices.

2.2.4 Cortical responses to thalamic stimulations: repetitive stimulations at the frequency of the SWD

To determine if there was a propensity of the thalamocortical circuits to generate activities at the SWD frequency, we stimulated the thalamus at 7-8 Hz (I=I$_{50}$). In both strains, rhythmic activities could not be induced during or after each train of stimulations, and the evolution of the response amplitude during each train was not different (BR/Orl, n=6 and BS/Orl, n=7).

2.3 Thalamic responses to cortical stimulations (TRCS)

2.3.1 Thalamic responses to cortical stimulations: single shock stimulations

Thalamic responses to cortical stimulation were recorded in a restricted zone of the VB, corresponding to the ventroposterolateral thalamus according to the atlas of Paxinos and Watson (1988). These responses displayed (Fig. 2B) an early fiber component (N1) and a late synaptic part (P2 and N3) that disappeared with Kyn. N1 could be mono or biphasic (depending on the slice) with an onset latency of 0.8±0.1 ms for BR/Orl (n=7) and 1.5±0.2 ms for BS/Orl (n=7), with an average duration of 3±0.2 ms for BR/Orl (n=7) and 2.5±0.3 ms for BS/Orl (n=7), and an average amplitude of -157±27 µV in BR/Orl (n=7) and -111±13 µV in BS/Orl (n=7). The differences in latency and amplitude were significant (p<0.05). The second component was a monophasic positive phase (P2) with latency-to-peak of 5.8±0.4 ms for BR/Orl (n=7) and 5.9±0.2

ms for BS/Orl (n=7). The third component was a negative phase (N3) that could be mono or biphasic (depending on the slice). N3 had a latency-to-peak of 21.8± 2.6 ms for BR/Orl (n=7) and 19.7±1 ms for BS/Orl (n=7). This phase had an average amplitude of -46±7 µV for BR/Orl (n=7) and -38±5 µV for BS/Orl (n=7) and lasted 134± 21.5 ms (n=7) and 112.7±11.7 ms (n=7) for BR/Orl and BS/Orl respectively (Table 2).

2.3.2 Thalamic responses to cortical stimulations: comparison of excitability indices

There was no significant difference in the connection efficacy for the fiber responses between both strains. The threshold intensities of the stimulations, to obtain the synaptic components, were not significantly different (0.15±0.03 mA for BR/Orl; n=7 and 0.13±0.02 mA for BS/Orl; n=7). However, the connection efficacy for the synaptic response was significantly ($p<0.02$) lower in BS/Orl (191.2±17.8 % of increase/mA; n=7) relative to BR/Orl (251.3±14.8 % of increase/mA; n=7) (Fig. 3B). This shows that the efficacy of the corticothalamic afference is decreased in epileptic mice compared to non-epileptic mice.

2.3.3 Thalamic responses to cortical stimulations: paired-pulse stimulations

Following the second stimulation, which we applied at stimulation intervals of 5 to 100 ms, we did not observe any change in the amplitude of N3 (BR/Orl (n=7) and BS/Orl (n=7) mice (Fig. 3B)).

2.3.4 Thalamic responses to cortical stimulations: repetitive stimulations at the frequency of the SWD

To determine if there was a particular propensity of the thalamocortical circuits, to generate activities at the frequency of the SWD, we stimulated the cortex at 7-8 Hz ($I=I_{50}$). In

both strains, rhythmic activities could not be induced during or after each train of stimulations, and the evolution of the response amplitude during each train was not different (BR/Orl, n=7 and BS/Orl, n=7).

2.4 Comparison of the connection efficacy of corticothalamic and thalamocortical pathways

In non-epileptic mice (BR/Orl) we measured a significantly larger efficacy in the thalamocortical pathway compared to that of the corticothalamic pathway (cortical responses = 432.5±53.4 % of increase/mA, n=8; thalamic responses = 251.3±14.8 % of increase/mA, n=7; $p<0.01$). In contrast, in epileptic mice (BS/Orl) the efficacy of the two pathways, was not statistically different (cortical responses = 232.5±38.6% of increase/mA, n=7; thalamic responses = 191.2±17.8% of increase/mA, n=7; $p>0.05$), indicating a relative decrease in the efficacy of the thalamocortical afferences in epileptic mice.

2.5 Effect induced by 4-aminopyridine on thalamocortical slices

Spontaneous or self-sustained activities were never obtained after repetitive stimulations in these slices. We attempted to identify differences between epileptic and control mice in the pattern of the synchronized activities induced in the cortex and thalamus through 4AP bath application (50 µM). Five to 10 min after the application of 4AP we observed (1) GABA-dependent negative field potentials and (2) multiphasic interictal events. In addition, synchronous spontaneous ictal-like activities (ILAs), characterized by an initial tonic phase followed by afterdischarges (Fig. 4A and 4B), were generated within 10 to 40 mins, and their frequency became stable within 60 mins. These ILAs were recorded in the somatosensory, insular cortex and VB. The cortical ILAs were larger and more robust in the infragranular layers.

The ILAs recorded simultaneously in the VB and the somatosensory cortex were highly synchronized (Fig. 4B) with undetectable time lags, while those generated in the insular cortex occurred independently (not shown). Moreover, the ILAs observed in the VB were always much smaller than the cortical ILAs. In previous experiments in rat thalamocortical slices (Gigout *et al.*, 2006a) it was shown that the 4AP-induced ILAs recorded in the thalamus are in fact generated in the neocortex and recorded by volume conduction in the thalamus. We therefore considered the thalamic ILAs recorded in mice (where the distance between cortex and thalamus is smaller than in the rats) to mirror activities generated in the neocortex.

Each ILA was always preceded by a large GABA-dependent negative field potential. This observation has also been shown in entorhinal cortex (Lopantsev and Avoli, 1998; Fig. 4A). Such GABA-dependent negative potentials and interictal events could be observed in the cortex but never in the thalamus of all the slices.

Bath application of the NMDA receptor antagonist CPP (5µM) abolished the ILAs in 10-15 mins (n=3) and they could reappear after prolonged washout (60 to 120 min). CPP applications did not modify the interictal activities or the GABA-dependent negative field potentials (Fig. 4Cb). The latter were abolished by the GABA$_A$ receptor antagonist, bicuculline (25 uM) (n=2) (Fig. 4Cc).

2.6 Comparison of the 4-aminopyridine-induced spontaneous activities in BR/Orl and BS/Orl

For the two groups of mice we calculated the ratio for the number of thalamocortical slices generating neocortical ILAs during 4AP application. There was no significant difference between BS/Orl and BR/Orl slices for (1) ILAs generation, (2) latency of ILAs onset, (3) mean duration, (4) average frequency and (5) the cumulative duration per min

(Table 3). However, the ILA amplitude was significantly ($p<0.05$) smaller in epileptic mice (-0.33±0.26 mV; n=10) than in non-epileptic mice (-0.97±0.19 mV; n=11).

2.7 Effect of CBX on 4-aminopyridine-induced ILAs in thalamocortical slices

Studies in human and experimental models have pointed out the implication of GJ in epilepsy and the increased expression of connexins in epileptogenic tissue (Nakase and Naus, 2004; Juszczak and Swiergiel, 2009). Interestingly, interneuronal connexins are strongly expressed in the thalamocortical system of mice (Liu and Jones, 2003). We looked to see whether carbenoxolone (CBX), a GJ blocker (Davidson *et al.*, 1986; Juszczak and Swiergiel, 2009), modulates the epileptiform activities differently in the two strains.

Bath application of CBX produced a progressive decrease in the frequency and mean duration of the 4AP-induced ILAs, until they were abolished within 40 mins (Fig. 5A and 5B). The resulting effect is that CBX decreased the cumulative duration of ILAs (Fig. 5C). This effect was significantly ($p<0.02$) faster in BR/Orl (n=7) than in BS/Orl (n=5) since we observed that over a period of 0-20 mins, following application of CBX, the cumulative duration of ILAs was 54.1±9.6 % of control in BR/Orl mice (n=7) and 87.6±8.2 % of control in BS/Orl mice (Fig. 5C). After a prolonged washout of 60-80 mins ILAs could reappear but at a very low frequency.

After CBX, the 4AP-induced GABA receptor-mediated negative potentials remained without a decrease in amplitude, even after disappearance of the ILAs. The frequency decreased progressively until they disappeared during the second hour of application. These potentials did not recover after 2 hours of washout.

3. Discussion

3.1 Connectivity in the thalamocortical slices

The tracing of nervous fibers by carbocyanine in our thalamocortical slices showed that at least part of the connections between the neocortex and thalamus was preserved, in agreement with previous work in mice (Agmon and Connors, 1991) and rats (Biagini *et al.*, 2001; Staiger *et al.*, 1999). Thalamocortical connections were previously shown to be functional both in young (Tancredi *et al.*, 2000; Biagini *et al.*, 2001; Land and Kandler, 2002) and adult animals (>120 days in our study) (Salami *et al.*, 2003). In all slices used in this study we were able to show that the function of the reciprocal thalamocortical connections was maintained, since we recorded both corticothalamic and thalamocortical responses in the same slices.

3.2 Nature of the cortical and thalamic evoked responses

It seems highly likely that the cortical recording sites corresponded to the facial region that encompasses whisker projections (Land and Kandler, 2002). We confirm that the early cortical responses (latency to peak <2.5 ms) are fiber afferent volleys, whereas the late phase (latency to peak >2.5 ms) has postsynaptic origin (Agmon and Connors, 1991; Tancredi *et al.*, 2000; Biagini *et al.*, 2001).

The kinetics of the thalamic field potentials evoked by cortical stimulation was similar to those induced in the VB *in vivo* by cortical stimulation (Mishima, 1992) and mechanical activation of the afferent pathway by displacement of the rat whiskers (Temereanca and Simons, 2003). The early phase N1 is a fiber afferent volley, whereas P2 and N3 have a postsynaptic origin.

3.3 Short-term facilitation and inhibition

The thalamic responses following stimulations of cortical afferences were not facilitated by low frequency paired stimuli. However, facilitation could be observed in different experiments both *in vivo* and *in vitro* with stimuli trains of higher frequencies and longer durations (Lindstrom and Wrobel, 1990; Mishima and Ohta, 1992; von Krosigk *et al.*, 1999). In contrast, we obtained a short-term facilitation of the cortical responses to thalamic stimulation using low frequency stimulations, in keeping with previous data in rats (Mishima and Ohta, 1992; Gigout *et al.*, 2005). Short-term inhibition was not noticed. Luhmann *et al.,* (1995) had previously observed an intracortical inhibition following high intensity stimuli when applied to the underlying white matter/layer VI of rat coronal slices. We verified that intracortical inhibition was present in the thalamocortical slices through subcortical white matter stimulation. However, we did not observe any modulation of corticothalamic responses using paired-pulse protocol.

3.4 Effectiveness of the connections between cortex and thalamus

Surprisingly, the synaptic responses of the thalamocortical connections displayed a lower effectiveness in BS/Orl than those of the BR/Orl. This may be due to decreased thalamocortical and corticothalamic connectivity in the BS/Orl since the fiber response amplitudes were smaller in this strain. However, the decrease in amplitude of the synaptic responses is much larger than that of the fiber responses in BS/Orl mice. A relevant parameter for SWD generation may therefore be the relative strength of the reciprocal connections between the cortex and the thalamus. An imbalance in favor of the thalamocortical influence was revealed in non-epileptic mice since the thalamocortical connection was more efficient than at the corticothalamic connection. In epileptic mice such imbalance was not observed, suggesting that the corticothalamic *versus* the thalamocortical influence is relatively stronger than in non-epileptic

animals. Besides, the increased short-term facilitation of cortical responses to thalamic stimulations in BS/Orl should reinforce the efficacy of the thalamocortical network.

3.5 Comparison between BS/Orl and genetic absence epileptic rats from Strasbourg (GAERS)

In a previous study, we did not observe any significant difference for the effectiveness of the thalamocortical and corticothalamic connections in GAERS, compared to non-epileptic rats (NER) (Gigout *et al*., 2005). However, in the present study we do observe a significant difference for the effectiveness of the thalamocortical and corticothalamic connections between epileptic and non-epileptic mice. This may reflect a genuine difference between the two models. However, due to the larger rat brain sizes and differences of the age at recording, the variability of the rat thalamocortical slices was greater than that of the slices obtained in mice, making it more difficult to reach statistical significance.

In addition, we were not able to record both corticothalamic and thalamocortical responses in all thalamocortical slices from GAERS and NER, which suggests that the function of reciprocal thalamocortical connections could not be maintained in slices of adult rats contrary to results obtained with adult mice in the present study.

3.6 4-aminopyridine-induced ictal-like activities in thalamocortical slices

4AP-induced ILAs were recorded both in the somatosensory cortex and in the VB (undetectable time lags between voltage peaks). These activities were smaller in VB than in the cortex so we considered that, as in rats (Gigout *et al*., 2006b) in which the distance between the cortex and thalamus is larger than in mice, the ILAs were generated in the cortex and recorded in the thalamus by volume conduction. Indeed, 4AP-induced epileptiform activities generated in the

cortex were recorded in the hippocampus (Breustedt *et al.*, 2002) or in adjacent slices (Inaba and Avoli, 2005) by volume conduction.

Unlike the observation that GAERS slices are less responsive to 4AP than slices of NER (Gigout *et al.*, 2006b), both strains of mice had the same sensitivity to 4-AP in the present study. Interestingly, a variable sensitivity to 4AP has been found in the hippocampus of several models of epilepsy (Zahn *et al.*, 2008, 2012). Such differences have been attributed to RNA editing of Kv1.1 (Streit *et al.*, 2011). One can speculate that a similar editing of Kv1.1 may abolish thalamic ILAs generation under 4-AP.

The 4AP-induced ILAs were more robust in deep cortical layers, suggesting that in mice they are generated in infragranular cortical layers, which is in keeping with previous studies in rats (Hoffman and Prince, 1995; Lopantsev and Avoli, 1998; Yang and Benardo, 2002; Gigout *et al.*, 2006b).

The negative GABA-mediated potentials appeared instrumental in triggering ILAs in the neocortex of mice, since they are observed just before their onset. This feature has already been described in the rat hippocampus, entorhinal cortex and somatosensory cortex (Perrault and Avoli, 1992; Avoli *et al.*, 1996; Lopantsev and Avoli, 1998; Gigout *et al.*, 2006b). Interestingly, it was recently been shown *in vivo* that GABAergic interneurones fired during seizures shortly after the discharge of ictogenic neurones (Chipaux *et al.*, 2011).

3.7 Action of carbenoxolone in thalamocortical slices

CBX applied in cell culture decreases neuronal excitability independently of its effect on GJ (Rouach *et al.*, 2003). However, CBX did not alter neuronal excitability in slices (Köhling *et al.*, 2001; Schmitz *et al.*, 2001; Yang and Michelson, 2001; Gigout *et al.*, 2006a,b). In previous experiments in rats the fiber responses were not decreased in amplitude after CBX application,

suggesting that it does not produce a large unspecific modification of neuronal excitability, thus confirming previous results in the rat cortex (Gigout *et al*., 2006b). However, we cannot completely exclude more subtle non-specific effects.

CBX decreased the frequency and cumulative duration of the 4AP-induced ILAs in thalamocortical slices of BR/Orl and BS/Orl after only 20 mins of application, and made them disappear after 60 mins. These observations demonstrate an antiepileptic action of CBX in this model as observed in various other *in vitro* experimental models (Ross *et al*., 2000; Traub *et al*., 2001; Yang and Michelson, 2001; Dougalis *et al*., 2004; Gigout *et al*., 2006a and b; Juszczak and Swiergiel, 2009). It was shown in addition that *in vivo* CBX reduced the 4AP-induced epileptiform activities, the clonic and tonic phases of audiogenic seizures as well as the SWD in absence epilepsy models (Gajda *et al*., 2003; Gareri *et al*., 2004; Gigout *et al*., 2006a; Proulx *et al*., 2006). Bath application of glycyrrhizic acid (0.1 mM) (a structural analogue of CBX), which is inactive as a gap junction blocker (Davidson *et al*., 1986), did not abolish ILAs in rat thalamocortical slices (Gigout *et al*., 2006b). These results and ours suggest that GJ blockers can be considered as broad-spectrum antiepileptic drugs.

The action of CBX on 4AP-induced ILAs was significantly more rapid in slices from BR/Orl than in slices from BS/Orl, as was observed in GAERS compared to non-epileptic rats (Gigout *et al.,* 2006a). Increased expression of glial and neuronal connexins have been observed in experimental epilepsy models *in vitro* and *in vivo* and in brain tissue from patients with epilepsy (Naus *et al*., 1991; Aronica *et al*., 2001; Condorelli *et al*., 2003; Fonseca *et al*., 2002; Gajda *et al*., 2003). The slower action of CBX in BS/Orl mice may therefore be due to an altered expression of connexins in these animals. Further experiments are needed to clarify these findings.

Surprisingly, CBX did not modify the amplitude, frequency or duration of the GABA-mediated potentials when the ILAs disappeared. Since the cortical GABAergic interneurons are interconnected through GJ (Galarreta and Hestrin, 1999; Gibson *et al.*, 1999) our result suggests that this electrical coupling does not play a crucial role in sustaining the 4AP-induced GABA-mediated synchronized activities. Following longer application of CBX, these potentials decreased in frequency and finally disappear. These findings are in accordance with previous results obtained in hippocampal and cortical slices (Ross *et al.*, 2000; Yang and Michelson, 2001; Gigout *et al.*, 2006b). These observations also show that CBX can (1) disrupt a synchronizing mechanism between the interneurone network and the principal cells (Bikson *et al.*, 2002; Uusisaari *et al.*, 2002), and (2) obliterate the capacity of the pyramidal cell network to generate epileptiform activities.

3.8 Specificity of carbenoxolone

CBX is not a selective GJ blocker, and does not discriminate between neuronal and glial GJ. Therefore, we cannot identify the respective contribution of these cellular elements to the effects reported here. However, 4AP-induced epileptiform discharges are attenuated in slices from connexin 36 (neuronal) knockout mice (Maier *et al.*, 2002). The possible involvement of this connexin is further supported by the observation that an increased expression of connexin 36 mRNA was measured in a 4AP model *in vivo* (Gajda *et al.*, 2003).

CBX is known to have other pharmacological actions, such as inhibition of 11-β-hydroxysteroid dehydrogenase and mineralocorticoid agonist effects (Jellinck *et al.*, 1993). However, the anticonvulsant action of CBX is not mediated by these effects since the mineralocorticoid antagonist spironolactone was unable to block the ability of CBX to depress

spontaneous epileptiform activity in hippocampal brain slices (Ross *et al*., 2000) or to block its action *in vivo* in a model of atypical absence epilepsy (Proulx *et al*., 2006).

In conclusion, we have observed in our mouse model of absence epilepsy (1) that the relative influence of the corticothalamic connections (compared to those of the thalamocortical) is stronger in epileptic mice compared to non-epileptic mice. (2) an increased short-term facilitation of cortical responses to thalamic stimulations in epileptic mice, which should reinforce the efficacy of the thalamocortical network. (3) that gap junction-dependent cortical network synchronization is modified in epileptic mice, since carbenoxolone decreased the frequency and the mean duration of the 4AP-induced ILAs with a slower time course in the epileptic mice. GJ blockers specific for connexin 36 may represent a new promising therapeutic window for the development of broad-spectrum antiepileptic drugs.

4. Experimental Procedure

4.1 Slice preparation and stimulations

Experiments were carried out on BR/Orl and BS/Orl 124 ± 9 days (n=29). Animals were housed under controlled standard conditions (light/dark cycle, 7:00 AM–7:00 PM lights on), with food and water available *ad libitum*. All animal experiments have been carried out in accordance with directive 2010/63/EU and agreed by the Ethics Committee of the "Centre Paul Broca" and the "Bureau de l'Experimentation Animale-INSERM". Adequate measures were taken to minimise pain or discomfort.

Mice were decapitated under deep anesthesia and their brains were quickly removed and placed in cold, oxygenated artificial cerebrospinal fluid (ACSF; see 4.6). Combined thalamocortical slices (400 μm thickness) were obtained using published procedures (Agmon and Connors, 1991; Gigout *et al*., 2006b). The slices were then transferred to an interface-type tissue chamber where they rested at the interface between oxygenated ACSF and humidified gas ($95\%O_2/5\%CO_2$) at 32–35° C (pH = 7.4).

Extracellular field potentials were recorded with ACSF-filled electrodes (resistance between 2 and 8 MΩ) positioned under visual control in neocortex or ventrobasal thalamus (VB). Signals were fed to high-impedance preamplifiers (WPI M-707) and processed through second-stage amplifiers (gain 1000) with filtering capability (analog filters Neurolog; DC-3 kHz). Field potential recordings were digitized (5 kHz) (interface 1401 Plus, C.E.D., Cambridge, UK), stored and analyzed off-line (program Spike 2, C.E.D, Cambridge, UK).

Constant current stimulations (80μs; 0-1 mA) were delivered through a bipolar stainless steel electrode. The time intervals were 10 s between single shock and 30 s between paired or repetitive stimuli. The two shocks intervals of the paired stimuli were set between by 5 to 100 ms. Repetitive stimulations consisted in 20 stimulations applied at frequencies ranging from 1 to 10 Hz. Slices showing reciprocal responses were retained for subsequent experiments using 4AP.

4.2 Protocols of stimulations and isolation of synaptic responses

In preliminary experiments, the responses obtained in control conditions were subtracted from the response obtained from the same slices perfused with a kynurenic acid-containing-solution (Kyn, 1mM), an antagonist of glutamatergic synaptic transmission. This procedure allowed distinguishing the fiber from the synaptic components in the evoked responses, the

remaining component being considered to represent fiber activity, as proved by its disappearance after further application of tetrodotoxin.

4.3 Responses analysis

For each component of the response, the onset latency, latency-to-peak, duration and amplitude were measured (Table 1). The area under the curves was also measured, allowing quantification of the connection "efficacy" (see below).

The threshold intensity inducing a just detectable response was first determined and the stimulus intensity was increased until the amplitude of the synaptic component reached a plateau, usually for I~0.8 mA. The area under the curve of the fiber and synaptic responses were measured and normalized to that of the maximal response for each stimulation intensity. Linear regression in the initial linear range of each curve was used to calculate the slope. This input-output index was named connection efficacy and can be used to evaluate the efficacy of the connections. Finally, the threshold and the intensity of stimulation generating a response equal to 50% of the maximum response (I_{50}) were determined.

4.4 Visualisation of reciprocal fibers between cortex and thalamus

The pathway of reciprocal fibers between cortex and thalamus was visualized in some thalamocortical slices by deposit of few crystals of the fluorescent lipophilic neuronal marker carbocyanine (perchlorate of 1,1' -dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine: DiI) in the somatosensory cortex or in the VB. The slices were fixed in paraformaldehyde at 4% during 12 hours, washed in 0.1M phosphate buffer during 3 days and stored at 37° C in phosphate buffer

(0.1 M) added with sodium azide. The migration of DiI was followed by fluorescence microscopy.

4.5 Solutions and substances

The ACSF composition was (in mM): NaCl 124, KCl 3, NaH2PO4 1.25, MgCl2 1.5, CaCl2 1.5, NaHCO3 26 and glucose 10. Chemicals for ACSF were acquired from Sigma (St. Louis, USA).

The following drugs were used: 4-aminopyridine (4AP; 50 µM, Sigma), bicuculline methiodide (BMI, 25 µM, Sigma), 3β-hydroxy-11-oxoolean-12-en-30-oic acid 3-hemisuccinate disodium, (carbenoxolone, CBX, 100 µM; Sigma); 6-cyano-7-nitroquinoxaline- 2,3-dione disodium salt (CNQX, 10 µM Tocris Cookson), 3-((R)-2-carboxypiperazine- 4-yl)-propyl-1-phosphonic acid (CPP, 5 µM, Tocris), kynurenic acid (Kyn, 1 mM, Sigma), tetrodotoxin (1 µM, Latoxan). All drugs were dissolved in water, prepared as stock solutions and added to the ACSF just before bath-applications.

4.6 Analysis

Data are presented as mean ± S.E.M. Since only one functional thalamocortical slice was obtained per mouse, the n values correspond to the number of mice. To compare the sensitivity of slices to 4AP we employed the Chi^2 test with Yates correction to determine whether the ILA-generating or ILA-non-generating slices have the same distribution in BS/Orl and BR/Orl mice. For all the others comparisons we used a bilateral t-test and significance was considered if $p<0.05$.

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

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Figure legends

Figure 1 Visualization of fibers connecting the cortex and thalamus in a thalamocortical slice from a 70 days old BS/Orl mouse.

A,B,C,D: Slice on which crystals of carbocyanine had been placed in the somatosensory cortex. The tracer was followed by fluorescence microscopy and reached the thalamus in approximately 10 days. (A) The cortical deposit site showed an intense red fluorescence with a non-specific halo of diffusion. (B) The fibers run initially along the lower cortical layer in the white matter. (B and C) They skirt the hippocampus, go through the internal capsule. (D) They reached the ventrobasal thalamic nucleus through the nucleus reticularis thalami. In this slice, cortical responses to thalamic stimulations had been obtained.

Legend: Cx: cortex; Hipp: hippocampus; IC: internal capsule; nRT: nucleus reticularis thalami; VB: ventrobasal thalamus.

Figure 2 Responses obtained in thalamocortical slices

A. Photograph of a thalamocortical slice. The stimulating electrode was placed in the VB while the recording electrode was in the cortex. To obtain thalamic responses, the electrodes were inverted. Cx: Cortex; Hipp: Hippocampus; Str: Striatum; VB: Ventrobasal thalamus.

B. Responses obtained in the cortex and thalamus in the same slice for both BR/Orl and BS/Orl mice. VB--> CX: Average cortical response to thalamic stimulation (1mA; 80μs). CX-->VB: Average thalamic response to cortical stimulation (1 mA; 80 μs). Negative is down for all traces. *: indicate stimulus artifacts which have been removed for clarity; N1: peak of the negative fiber component of the cortical (or thalamic) response; N2: peak of the negative synaptic component of

the cortical response; P2: peak of the positive synaptic component of the thalamic response, N3: peak of the negative synaptic component of the thalamic response.

C. Example of paired-pulse inhibition of responses obtained in the supragranular cortical layers when stimulating the underlying white matter in BR/Orl non-epileptic mice (left) and BS/Orl epileptic mice (right). *: indicate stimulus artifacts which have been removed for clarity.

Figure 3 Efficacy of thalamocortical (A) and corticothalamic (B) connections

A. Cortical responses to thalamic stimulations. The histogram in black corresponds to the BR/Orl mice and that in red to the BS/Orl mice. The difference between BR/Orl and BS/Orl was significant. (*: $p < 0.01$).

B. Thalamic responses to cortical stimulations. The histogram in black corresponds to BR/Orl mice and that in red to BS/Orl mice. The difference between BR/Orl and BS/Orl was significant. (*: $p < 0.02$)

C. Cortical responses to paired pulse thalamic stimulations (VB→Cx) in BR/Orl and BS/Orl for short interpulse intervals. The paired pulse ratio (amplitude of the second response/amplitude of the first response) is given as a function of the interpulse interval. In black: BR/Orl mice; In red: BS/Orl mice. Bars indicate SEM.

D. Study in of the thalamic responses to paired pulse cortical stimulations (Cx→VB) in BR/Orl and BS/Orl for short interpulse intervals. The paired pulse ratio (amplitude of the second response/amplitude of the first response) is give as a function of the interpulse interval. In black: BR/Orl mice; In red: BS/Orl mice. Bars indicate SEM.

E. Average traces showing paired-pulse facilitation of responses obtained in cortex when stimulating VB both in BR/Orl non-epileptic mice (left) and BS/Orl epileptic mice (right). *: indicate stimulus artifacts which have been removed for clarity.

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Figure 4 Ictal-like activities (ILAs) recorded in thalamocortical slices prepared from adult BR/Orl and BS/Orl mice perfused with 4-aminopyridine (4AP) (50 µM).

A. ILA recorded in somatosensory cortex (Cx) of thalamocortical slices in a BR/Orl. Arrowhead indicates that GABA-dependent negative field potentials are observed at onset of ILA.

B. Simultaneous recordings in somatosensory cortex (Cx) and in ventrobasal thalamus (VB) during occurrence of cortical ILA on thalamocortical slices in a BR/Orl and a BS/Orl.

C. Pharmacological study of the 4AP-induced activities. All traces are from the same slice.

a. Control recording showing spontaneously occurring ILA and GABA-mediated negative potentials.

b. Effect of bath-applied CPP (5µM). ILAs disappeared while GABA-mediated potentials remained.

c. Effect of bath-applied CPP (5µM) +CNQX (10µM) +Bicuculline methiodine (BMI) 25µM. Bicuculline made the GABA-mediated potentials, that remained following CPP+CNQX application, disappear.

Figure 5 Time course of the effect of carbenoxolone (CBX) on Ictal-like activities (ILAs) induced by 4-aminopyridine (4AP; 50 µM) in thalamocortical slices from BR/Orl and BS/Orl mice.

CBX was applied after the slices generated a stable number of s ILAs. This steady state was reached about 1 hour after the beginning of 4AP application.

A. Time course of the CBX effect on the mean duration of ILAs in BR/Orl and BS/Orl.

B. Time course of the CBX effect on ILAs frequency in BR/Orl and BS/Orl.

C. Time course of CBX effect on the cumulative duration of ILAs in BR/Orl and BS/Orl. CBX decreased the cumulative duration of ILAs with a slower time course in slices prepared from BS/Orl than in slices from BR/Orl.

(* :P<0.05).

Table1

Characteristics of the cortical responses to thalamic stimulation recorded on slices obtained from the two strains of mice. The synaptic responses have a longer latency and duration and were of smaller amplitude in the BS/Orl epileptic mice. (*: $p<0.05$).

Table2

Characteristics of the thalamic responses to cortical stimulation recorded on slices obtained from the two strains of mice (*: $p<0.05$). The characteristics of the synaptic responses were not statistically different.

Table3

Percentage of slices generating ictal-like activities (ILAs) and characteristics of the ILAs recorded on slices obtained from the two strains of mice. Statistical difference was not detected for any of these parameters.

Fibers responses (N1)				
	Onset latency (ms)	Latency to peak (ms)	Duration (ms)	Amplitude (µV)
BR/Orl (n=8)	0.9 ± 0.1	1.5 ± 0.1	2.8 ± 0.3	-451 ± 81
BS/Orl (n=7)	0.7 ± 0.1	1.9 ± 0.4	4.4 ± 0.6 (*)	-212 ± 38 (*)
Synaptic responses (N2)				
	Onset latency (ms)	Latency to peak (ms)	Duration (ms)	Amplitude (µV)
BR/Orl (n=8)	3.7 ± 0.3	4.7 ± 0.3	30.5 ± 4.8	-210 ± 24
BS/Orl (n=7)	5.2 ± 0.6 (*)	7.6 ± 2 (*)	49.9 ± 7.9 (*)	-66 ± 9 (*)

Table 1

Fibers responses (N1)				
	Onset latency (ms)	Latency to peak (ms)	Duration (ms)	Amplitude (µV)
BR/Orl (n=7)	0.8 ± 0.1	1.5 ± 0.1	3 ± 0.2	-157 ± 27
BS/Orl (n=7)	1.5 ± 0.2 (*)	1.9 ± 0.2 (*)	2.5 ± 0.3	-111 ± 13 (*)
Synaptic responses (P2)				
	Onset latency (ms)	Latency to peak (ms)	Duration (ms)	Amplitude (µV)
BR/Orl (n=7)	3.8 ± 0.2	5.8 ± 0.4	5.2 ± 0.9	37 ± 9
BS/Orl (n=7)	4 ± 0.2	5.9 ± 0.2	5.9 ± 0.4	44 ± 6
Synaptic responses (N3)				
	Onset latency (ms)	Latency to peak (ms)	Duration (ms)	Amplitude (µV)
BR/Orl (n=7)	8.7 ± 1.1	21.8 ± 2.6	134 ± 21.5	-46 ± 7
BS/Orl (n=7)	9.9 ± 0.2	19.7 ± 1	112.7 ± 11.7	-38 ± 5

Table 2

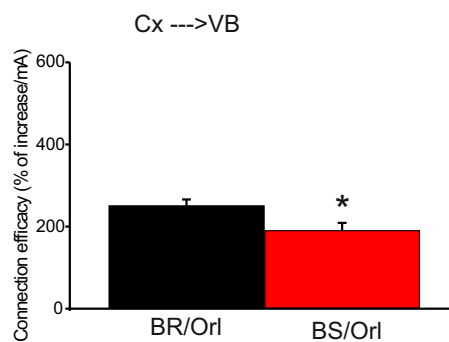|	BR/Orl (n=13)	BS/Orl (n=12)
% of slices generating ILAs	84.6	83.3
Latency of occurrence of ILAs (s)	1085±194	1287±150
Interval between ILAs (s)	156±17	175±31
Mean duration of ILAs (s)	26±4	18±3 (p=0.09)
Cumulative duration of ILAs during a 30 min period (s)	331±59	247±53

Table 3

Figure 1

A

C   BR/Orl   BS/Orl

B   BR/Orl   VB ➤ Cx   BS/Orl

N2
N1

N2
N1

0.2mV
20ms

Cx ➤ VB

P2   BR/Orl   P2   BS/Orl

N3
N1

N3
N1

0.1mV
50ms

1mV
5ms

Figure 2

A VB --->Cx

Connection efficacy (% of increase/mA)

BR/Orl   BS/Orl

B Cx --->VB

Connection efficacy (% of increase/mA)

BR/Orl   BS/Orl

C

■ BR/Orl   ● BS/Orl

PPR

Interpulse interval (ms)

D

■ BR/Orl   ● BS/Orl

PPR

Interpulse interval (ms)

E   BR/Orl   BS/Orl

0.2mV

10ms

Figure 3

A

Cx

0.8 mV

50 s

B

BR/OrI

Cx

VB

2 mV

5 s

BS/OrI

Cx

VB

C a   4AP

0.8 mV   100 s

b   4AP + CPP (5µM)

c   4AP + CPP (5µM) + CNQX (10µM) + BMI (25µM)

Figure 4