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Reduced $GABA_B$ receptor subunit expression and paired-pulse depression in a genetic model of absence seizures

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Neocortical networks play a major role in the genesis of generalized spike-and-wave (SW) discharges associated with absence seizures in humans and in animal models, including genetically predisposed WAG/Rij rats. Here, we tested the hypothesis that alterations in $GABA_B$ receptors contribute to neocortical hyperexcitability in these animals. By using Real-Time PCR we found that mRNA levels for most $GABA_{B(1)}$ subunits are diminished in epileptic WAG/Rij neocortex as compared with age-matched non-epileptic controls (NEC), whereas $GABA_{B(2)}$ mRNA is unchanged. Next, we investigated the cellular distribution of $GABA_{B(1)}$ and $GABA_{B(2)}$ subunits by confocal microscopy and discovered that $GABA_{B(1)}$ subunits fail to localize in the distal dendrites of WAG/Rij neocortical pyramidal cells. Intracellular recordings from neocortical cells in an *in vitro* slice preparation demonstrated reduced paired-pulse depression of pharmacologically isolated excitatory and inhibitory responses in epileptic WAG/Rij rats as compared with NECs; moreover, paired-pulse depression in NEC slices was diminished by a $GABA_B$ receptor antagonist to a greater extent than in WAG/Rij rats further suggesting $GABA_B$ receptor dysfunction. In conclusion, our data identify changes in $GABA_B$ receptor subunit expression and distribution along with decreased paired-pulse depression in epileptic WAG/Rij rat neocortex. We propose that these alterations may contribute to neocortical hyperexcitability and thus to SW generation in absence epilepsy.

Keywords: Absence seizures; $GABA_B$ receptor; Immunofluorescence; Paired-pulse depression; Real-Time PCR; WAG/Rij rats

Introduction

$GABA_B$ receptors regulate neuronal excitability by causing a postsynaptic K^+-dependent hyperpolarization and by modulating transmitter release (Bowery et al., 2002; Enna and Bowery, 2004). Functional $GABA_B$ receptors are heterodimers comprising $GABA_{B(1)}$ and $GABA_{B(2)}$ subunits (Kaupmann et al., 1997). Alternative splicing generates different isoforms of $GABA_{B(1)}$, of which $GABA_{B(1a)}$, $GABA_{B(1b)}$, $GABA_{B(1ac)}$, $GABA_{B(1bc)}$, $GABA_{B(1d)}$, $GABA_{B(1h)}$ and $GABA_{B(1i)}$, are the most highly expressed subunits in the rat CNS (Isomoto et al., 1998; Pfaff et al., 1999; Holter et al., 2005), with $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ representing the vast majority of $GABA_B$ receptors in the brain (Mohler et al., 2001). The $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ complexes differ in their pattern of expression at regional, cellular and sub-cellular levels, thus pointing to distinct functional roles for these two splice variants. A presynaptic localization has been suggested for both receptor subtypes (Kaupmann et al., 1998; Billinton et al., 1999; Brauner-Osborne and Krogsgaard-Larsen, 1999). Moreover, it has been reported that in the $GABA_B$ heteromer the $GABA_{B(1)}$ subunit binds all known $GABA_B$ ligands (Kaupmann et al., 1998) whereas the $GABA_{B(2)}$ subunit is responsible for cell surface trafficking and G protein activation (Margeta-Mitrovic et al., 2000, 2001; Calver et al., 2001; Galvez et al., 2001; Pagano et al., 2001; Robbins et al., 2001).

Studies performed on knockout mice have demonstrated that all classical $GABA_B$ responses are mediated by heteromeric $GABA_{B(1,2)}$ receptors and that the two subunits cross-stabilize each other (Schuler et al., 2001; Prosser et al., 2001; Brown et al., 2003; Gassmann et al., 2004). Indeed, $GABA_{B(1)}{}^{-/-}$ and $GABA_{B(2)}{}^{-/-}$ mice exhibit a loss of all biochemical and electrophysiological $GABA_B$ functions. In addition, these knockout mice present with spontaneous generalized spike-and-wave (SW) discharges (Schuler et al., 2001; Prosser et al., 2001; Brown et al., 2003; Gassmann et al., 2004), which represent the hallmark of seizures in patients with absence epilepsy. These observations suggest that abnormal

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expression or function of $GABA_B$ receptors could contribute to epileptogenesis in primary generalized epileptic disorders.

The neocortex plays a major role in the generation of SW discharges in normal animals treated with convulsants and in GAERS or WAG/Rij rats, which are strains genetically predisposed to produce absence seizures (see for review: Avoli et al., 2001; Crunelli and Leresche, 2002). Indeed, recent evidence points to the perioral area of the somatosensory cortex as the site of initiation of generalized SW activity in both WAG/Rij and GAERS epileptic animals (Meeren et al., 2002; Manning et al., 2004). Several *in vivo* and *in vitro* studies have shown that neocortical hyperexcitability in these genetic models is caused by changes in intrinsic (Strauss et al., 2004; Klein et al., 2004) and synaptic mechanisms (Peeters et al., 1989; Pumain et al., 1992; Luhmann et al., 1995; D'Arcangelo et al., 2002; Pinault, 2003; D'Antuono et al., 2006).

The data obtained from knockout mice suggest that alterations in the expression or function of $GABA_B$ receptors contribute to abnormal neuronal function in these animals. However, in spite of this evidence, little is known regarding the molecular and functional properties of $GABA_B$ receptors in genetic models of absence seizures and, in particular, whether changes in $GABA_B$ receptor function occur in the neocortical networks that initiate SW discharges. In this study, we used Real-Time PCR to analyze the expression levels of mRNAs encoding different $GABA_B$ receptor subunits and confocal microscopy to assess the distribution of $GABA_B$ receptors subunits in the somatosensory neocortex of epileptic (>180-day-old) WAG/Rij rats. In addition, we employed intracellular recordings from neurons in neocortical slices to test the hypothesis that paired-pulse depression, which is presumed to be mainly mediated by $GABA_B$ receptors located presynaptically (Gil et al., 1997; Deisz, 1999; Fukuda et al., 1993; Kang, 1995), is altered in these animals.

Materials and methods

Animals

Epileptic (>180-day-old) WAG/Rij (Harlan, Horst, The Netherlands) and age-matched, NEC (Crl(WI)BR Wistar, Harlan) rats were used according to the procedures established by the Canadian and the European Union Councils of Animal Care. All efforts were made to minimize the number of animals used and their suffering. WAG/Rij rats housed before the experiment had frequent episodes of behavioral arrest (duration=up to 15 s) with mild myoclonic twitches. Previous *in vivo* studies have demonstrated that these clinical events correspond to the occurrence of bilaterally generalized SW discharges at 7–11 Hz (Drinkenburg et al., 1993; Midzianovskaia et al., 2001; Meeren et al., 2002). By contrast, similar behavioral episodes were rarely observed in NEC rats; these animals were not used for any of the experiments reported here.

Real-time PCR

The total RNA of the somatosensory cortex of >180-day-old epileptic WAG/Rij and NEC rats was prepared by using Trizol (Invitrogen, Milano, Italy). Briefly, animals were anesthetized with enfluorane and decapitated. Coronal brain slices were cut (450 μm) with a vibratome from a region corresponding to the somatosensory cortex as defined by the plates in Paxinos and Watson (1998) at −0.3 to +0.7 mm from the bregma (also *cf.*, Pinault, 2003; Manning et al., 2004). The total RNA was run on a 2% agarose gel and quantified by densitometric analysis using the Fluor-S, Biorad (Milano, Italy). Total RNA (1 μg) was reverse transcribed using the first-strand synthesis system for RT-PCR (Superscript, Invitrogen), in 20 μl final volume for 1 h at 42°C following the manufacturer's protocol. For specific-primer RT-PCR, 1 μg of total RNA was reverse transcribed using a downstream primer specific for $GABA_{B(1ac)}$ and $GABA_{B(1bc)}$ (5′ GCCAGACACAGATGGGTGAGTC 3′) at an annealing temperature of 55°C.

Relative and absolute quantitative Real-Time PCR was performed in a Real-Time Thermocycler (MX 3000, Stratagene, Milano, Italy) using the Brilliant SYBR Green QPCR Master Mix according to manufacturer's instructions. The dye ROX (Stratagene) was included in each reaction to normalize non-PCR-related fluctuations in fluorescence signal. All PCR reactions were coupled to melting-curve analysis to confirm the amplification specificity. Specific amplification was confirmed by ethidium bromide staining of the PCR products on agarose gel. Nontemplate controls were included for each primer pair to check for any significant levels of contaminants. Specific primers for rat $GABA_{B(1)}$ subunits, $GABA_{B(2)}$ subunit and beta-actin were designed as shown in Table 1 in order to amplify short DNA fragments (110–200 bp in length). $GABA_B$ subunits quantitation data ($GABA_{B(1a)}$, $GABA_{B(1b)}$, $GABA_{B(1d)}$ and $GABA_{B(2)}$) were normalized to expression of housekeeping gene beta-actin. The relative quantitation was calculated with the analysis software that accompanied the thermal cycler (MX 3000, Stratagene). Absolute quantitation was obtained for subunits amplified by primer-specific-RT-PCR ($GABA_{B(1ac)}$ and $GABA_{B(1bc)}$). For each transcript, an absolute standard curve was constructed using 10-fold serial dilutions of previously amplified and gel-purified cDNA fragments. The molecule number was calculated from the linear regression of the standard curve.

Tissue preparation for immunofluorence

Rats were deeply anesthetized with chloral hydrate (500 mg/kg, i.p.) and perfused through the ascending aorta with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3. After perfusion, the brains were removed from the skull and post-fixed in the same fixative overnight at 4°C. After rinsing in phosphate buffer, brains were cryoprotected in 15% sucrose solution followed by a passage in 30% sucrose solution both overnight at 4°C. The brains were then sectioned at 30 μm on a cryostat. Coronal brain sections corresponding to the somatosensory cortex were analysed for immunofluorescence.

Table 1
Nucleotide sequences of the primers used for Real-Time PCR

$GABA_{B(1a)}$ and $GABA_{B(1ac)}$	Forward: 5′ CGACCAGGTGAAGGCCATCAAC 3′ Reverse: 5′ AGCGGCTGGGTGTGTCCATATC 3′
$GABA_{B(1b)}$ and $GABA_{B(1bc)}$	Forward: 5′ CTCTTCTGCTGGTGATGGC 3′ Reverse: 5′ TACTGCACGCCGTTCTGAG 3′
$GABA_{B(1d)}$	Forward: 5′ TCCCGACTGTTGGAGAAGGAA 3′ Reverse: 5′ GGCAACCCACCCTCAGACAC 3′
$GABA_{B(2)}$	Forward: 5′ GGA AAC CCG CAA TGT GAG C 3′ Reverse: 5′ GGC ACA AAC ACC AGG CAG AG 3′
Beta-actin	Forward: 5′ AGGCATCCTGACCCTGAAGTAC 3′ Reverse: 5′ GAGGCATACAGGGACAACACAG 3′

Immunofluorescence

Free-floating sections were incubated in 50 mM ammonium chloride for 15 min to reduce autofluorescence. After rinsing in phosphate buffer, sections were incubated in 10% normal horse serum in phosphate buffer containing 0.2% Triton X-100 for 30 min to reduce non-specific binding. These sections were then incubated with primary antibodies (anti-$GABA_{B(1)}$ and anti-$GABA_{B(2)}$ 1:500, Chemicon, Milan; anti-syntaxin 1 1:500, Synaptic System, Goettingen; Germany; anti-MAP2 1:600, Sigma, Milan) diluted in phosphate buffer containing 2% normal horse serum and 0.2% Tritox X-100, overnight at 4°C. After rinsing, the sections were incubated with labelled secondary antibodies (Alexa Fluor 488 goat anti-guinea pig IgG, Molecular Probes, Rome; Cy3 donkey anti-mouse IgG, Jackson Immunoresearch Laboratories, Milan) for 1 h at RT. After a thorough rinse, the sections were incubated in phosphate buffer containing a Hoechst for 10 min at RT; sections were mounted on slides, and coverslipped with antifade medium. Sections were analysed with a confocal microscope (Nikon Instruments Spa, Eclipse TE 200) equipped with a 405 nm diode.

Electrophysiology

As reported for the Real Time PCR studies, WAG/Rij and NEC rats were anesthetized with enfluorane and decapitated. Neocortical slices (450 μm) were cut coronally with a vibratome from a region corresponding to the somatosensory cortex. Slices were then transferred to a tissue chamber where they lay in an interface between oxygenated artificial cerebral spinal fluid (ACSF) and humidified gas (95%O_2/5%CO_2) at 32°C (pH = 7.4). ACSF composition was (in mM): NaCl 124, KCl 2, KH_2PO_4 1.25, $MgSO_4$ 2 or 4, $CaCl_2$ 2, $NaHCO_3$ 26 and glucose 10. The ACSF contained either the glutamatergic receptor antagonists 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonate (CPP, 20 μM) and 6-cyano-7-nitroquino-xaline-2,3-dione (CNQX, 10 μM) or the $GABA_A$ receptor antagonist picrotoxin (10 μM) and CPP. In some experiments, we also bath applied the $GABA_B$ receptor antagonist 3-*N*[1-(*S*)-(3,4-dichlorophenyl) ethyl]amino-2-(*S*)-hydroxypropyl-*P*-benzyl-phosphinic acid (CGP 55845, 4 μM) was also bath applied. Chemicals were acquired from Sigma (St. Louis, MO, USA) with the exception of CNQX, CPP and CGP 55845 that were obtained from Tocris Cookson (Langford, UK).

Intracellular recordings were made from cells located at >900 μm from the pia with pipettes filled with either 3 M KCl + 75 mM lidocaine, *N*-ethyl bromide (QX314) (tip resistance = 70–120 MΩ) or with 2M K-acetate + 75 mM QX314 (tip resistance = 80–140 MΩ) in a neocortical region corresponding to the perioral somatosensory cortex. Signals were fed to an Axoclamp 2A amplifier (Axon Instruments, Union City, CA, USA) with an internal bridge circuit for passing intracellular current. The bridge balance was routinely checked. Signals were fed to a computer interface (Digidata 1322A, Axon Instruments) for subsequent analysis with the Clampfit 9 software (Axon Instruments). The neuron resting membrane potential (RMP) was measured after withdrawal from the cell, while the membrane apparent input resistance (R_i) was calculated from the peak of voltage responses to hyperpolarizing current pulses (amplitude <0.5 nA). Cells included in this study had $R_i \geq 30$ MΩ and RMP more negative than −65 mV. Overshooting action potentials were generated by all neurons at the beginning of the intracellular recording before QX-314 took its action (*cf.*, D'Antuono et al., 2006).

A bipolar stainless steel electrode was used to deliver paired-pulse stimuli (50–150 μs; <300 μA; interstimulus intervals = 50–1200 ms; intertrial intervals = 15 s). In each experiment we used stimuli of strength capable of giving *quasi* maximal responses. Glutamatergic responses were recorded with KCl + QX314-filled electrodes following electrical stimuli delivered in the white matter during bath application of ACSF containing picrotoxin and 4 mM $MgSO_4$ to depress NMDA receptor-mediated mechanisms along with epileptiform activity. "Monosynaptic" $GABA_A$-receptor mediated IPSPs were analyzed in the presence of glutamatergic antagonists (CPP + CNQX) by using K-acetate + QX314-filled electrodes; focal electrical stimuli in these experiments were delivered intracortically at <500 μM from the recording electrode while neurons were hyperpolarized by steady current injection to obtain depolarizing IPSPs.

Statistical analysis

Values throughout the text are expressed as mean ± S.D. and *n* indicates the number of neurons or brain tissue samples analyzed. Statistical analysis was made with the Student's *t* test. Data were considered significantly different if $p < 0.05$.

Results

$GABA_{B(1)}$ mRNA levels are reduced in WAG/Rij rat neocortex

To establish whether neocortical hyperexcitability in WAG/Rij rats was associated with changes in the expression of $GABA_B$ receptor subunits, we analyzed with Real-Time PCR the mRNA levels of $GABA_B$ subunits that are presumed to be most abundantly expressed in the rat somatosensory cortex. Different $GABA_{B(1)}$ isoforms contain unique sequences that are generated by alternative splicing (Fig. 1A). Therefore, we exploited these differences in order to design specific primers and thus to assess the expression of each isoform. As shown in Fig. 1B, we found a large decrement in mRNA levels for most $GABA_{B(1)}$ splice variants in epileptic WAG/Rij neocortical tissue ($n = 5$ rats) as compared with NEC ($n = 5$ rats). Thus, $GABA_{B(1b)}$, $GABA_{B(1ac)}$ and $GABA_{B(1d)}$ transcripts were decreased by 59.8 ± 3.3%, 61.0 ± 3.1%, 57.5 ± 4.1%, respectively, while $GABA_{B(1bc)}$ was reduced by 90.25 ± 1.4%. In contrast, $GABA_{B(1a)}$ and $GABA_{B(2)}$ were not significantly changed. Therefore, these results indicate that specific splice variants of the $GABA_{B(1)}$ subunit are expressed at lower levels in the epileptic WAG/Rij rat somatosensory neocortex when compared to NEC animals.

$GABA_{B(1)}$ fails to localize in the distal processes of WAG/Rij rat neocortical pyramidal cells

It was previously shown that the perioral region of the somatosensory cortex plays a major role in the initiation of SW discharges in rodent genetic models of absence seizures (Meeren et al., 2002; Manning et al., 2004). Hence, we analyzed the protein levels and the distribution of $GABA_B$ receptors by immunofluorescence in coronal brain sections obtained from NEC and epileptic WAG/Rij animals ($n = 6$ for each group), focusing on the perioral somatosensory cortex. The $GABA_{B(1)}$ distribution was assessed using an antibody directed against the C terminus of $GABA_{B(1)}$ (Fritschy et al., 2004); therefore, this antibody was not able to distinguish the different $GABA_{B(1)}$ isoforms.

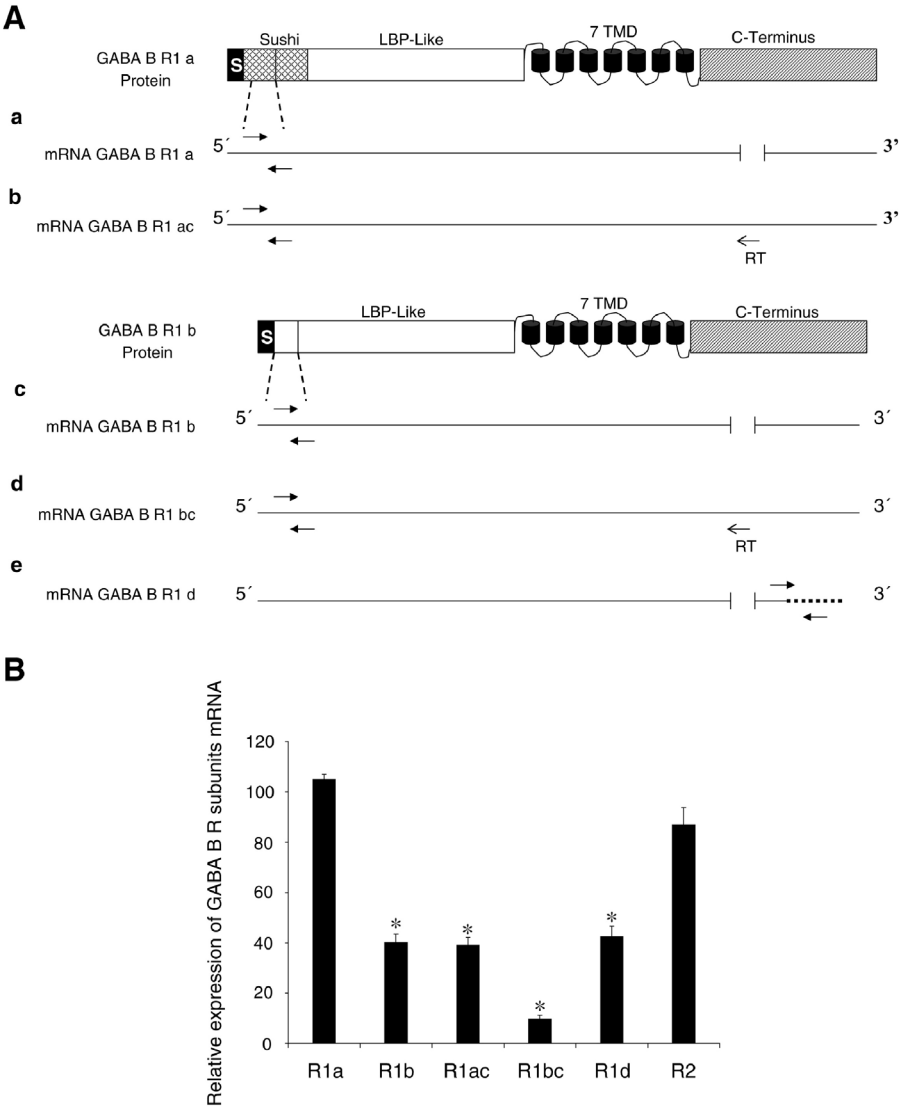

Fig. 1. (A) Strategy used for Real-Time PCR on $GABA_{B(1)}$ alternatively spliced subunits. The schematic diagram illustrates the organization of $GABA_{B(1a)}$ and $GABA_{B(1b)}$. RT-PCR analysis was performed on cDNAs obtained from total RNA with oligo dT (a, c, e) or with a specific primer (b and d). $GABA_{B(1a)}$ and $GABA_{B(1ac)}$ were specifically amplified using primers at the N-terminus of cDNA on oligo dT-RT-PCR and specific-primer-RT-PCR, respectively (a and b). $GABA_{B(1b)}$ and $GABA_{B(1bc)}$ products were generated using a different primer set hybridizing at its N-terminus on oligo dT-RT-PCR and specific-primer-RT-PCR, respectively (c and d). $GABA_{B(1d)}$ PCR fragment was obtained with a specific primer set at the alternative C-terminus as indicated by the dotted line. The position/orientation of PCR primer hybridization sites are indicated by arrows. The position of specific-RT primer hybridization site, indicated by open arrows, is within the 93-bp insertion exclusively present in $GABA_{B(1ac)}$ and $GABA_{B(1bc)}$. Abbreviations: S, signal peptide; LBP-like-leucine-binding protein; Sushi, short consensus repeats or sushi domains; 7TMD, seven-transmembrane domain; RT, reverse transcriptase. (B) Expression of $GABA_B$ receptor subunit mRNAs in the somatosensory cortex of epileptic WAG/Rij rats ($n=5$) relative to age-matched NEC (100%; $n=5$). Values represent mean±S.E.M. Asterisks identify $p<0.05$.

As illustrated in Fig. 2, $GABA_{B(1)}$ immunoreactivity was distributed mainly in pyramidal cells (*cf.*, Lopez-Bendito et al., 2002) and the staining level was comparable in the cell bodies of NEC and WAG/Rij rats. $GABA_{B(1)}$ immunoreactivity was homogeneously distributed along the apical dendrites of deep layer pyramidal neurons in NEC animals; in contrast, the labelling in WAG/Rij neurons outlined the soma and the proximal dendrites but not the distal processes. Since neurons located in the superficial layers are smaller, it was difficult to establish whether similar changes occurred there as well. $GABA_{B(1)}$-positive cells could also be identified in other sectors of the somatosensory cortex such as the forelimb region. These neurons displayed differences in $GABA_{B(1)}$ immunoreactivity between WAG/Rij and NEC that were similar to those observed in the perioral area (not shown).

We also investigated the distribution of immunoreactivity for the dendritic proteins MAP2 (Fig. 2A), for the postsynaptic protein PSD 95 (not shown), and for the presynaptic protein, syntaxin (Fig. 2B) in order to evaluate whether the structure of apical dendrites or the distribution of synaptic terminals on pyramidal cells were altered in the WAG/Rij neocortical tissue. As revealed by immunofluorescence, the distributions of MAP2 and syntaxin (as well as PSD95, data not shown) immunoreactivity were indistinguishable in WAG/Rij and NEC neocortex. Therefore, these data indicate that the changes in $GABA_{B(1)}$ subunit immunoreactivity between WAG/Rij and NEC did not reflect gross changes in cortical

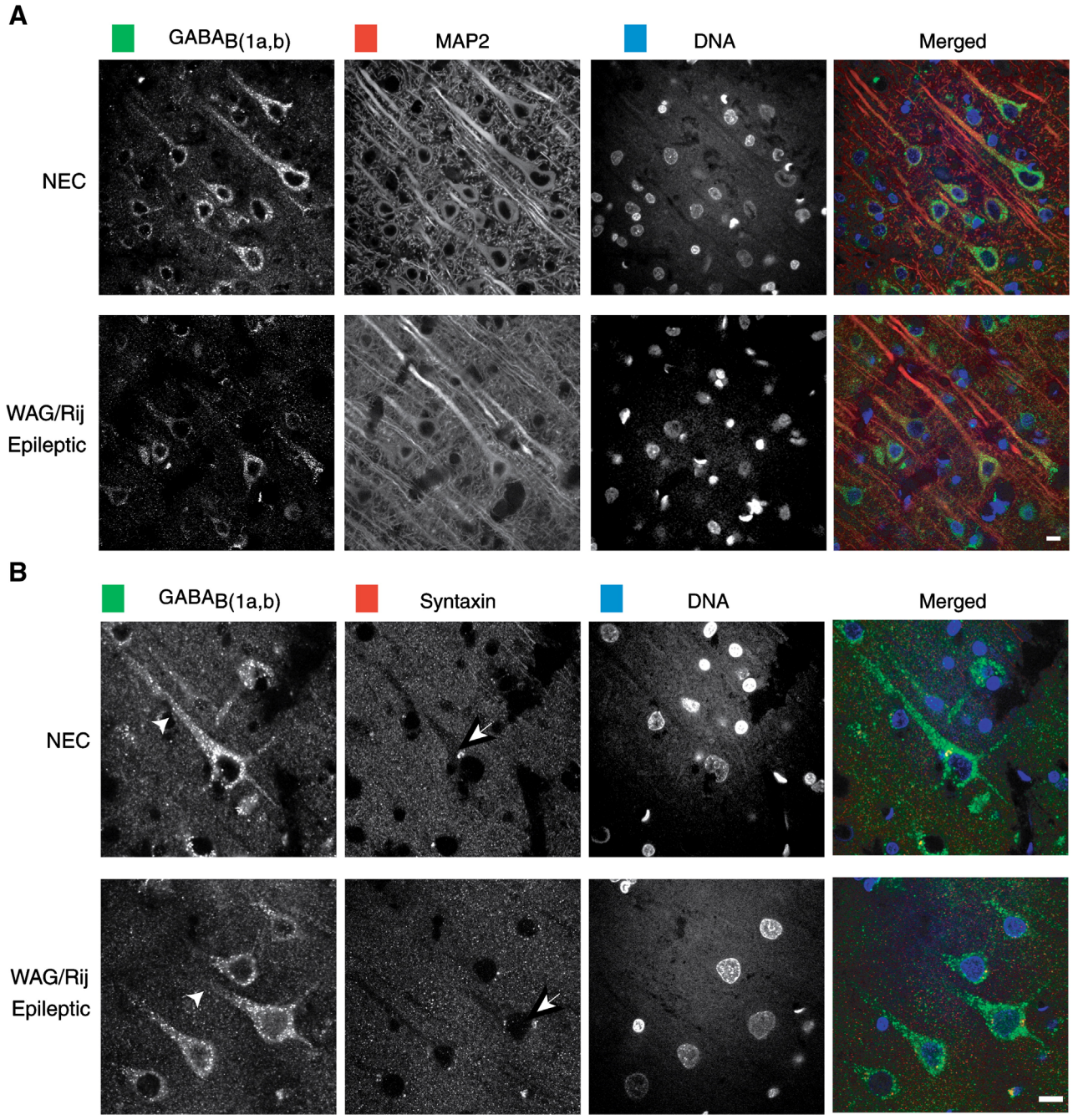

Fig. 2. Triple immunofluorescence for $GABA_{B(1a,b)}$ (green), MAP2 (red) and DNA (blue) (A) or $GABA_{B(1a,b)}$ (green), syntaxin (red) and DNA (blue) (B) in the peri-oral region of the somatosensory cortex of NEC and WAG/Rij coronal brain sections ($n=6$). Note that $GABA_{B(1a,b)}$ immunoreactivity was distributed mainly in pyramidal cells and was intense in cell soma and apical dendrites of NEC pyramidal neurons. In contrast, $GABA_{B(1a,b)}$ labeling of pyramidal cell dendritic processes was decreased in epileptic WAG/Rij (arrowheads in B). Arrows in B indicate coexistence of $GABA_{B(1a,b)}$ subunits with syntaxin in presynaptic terminals contacting neuronal somata. Scale bar: 10 μm.

morphology. Finally, we found that $GABA_{B(2)}$ antibody stained the soma and the neuropil in all regions of the somatosensory cortex of both NEC ($n=3$) and WAG/Rij ($n=4$). As shown in Fig. 3, coronal sections of the neocortex obtained from both types of rodents displayed similar patterns of distribution for $GABA_{B(2)}$ as well as for MAP2 immunoreactivity. Together, these results indicate that WAG/Rij rats exhibit specific alterations in the subcellular distribution of $GABA_{B(1)}$ receptor subunits, as compared with NECs.

Paired-pulse depression is reduced in the epileptic WAG/Rij rat neocortex

Next, we tested the hypothesis that changes in $GABA_B$ receptor subunit expression would result in changes of paired-pulse depression of the glutamatergic and/or GABAergic responses generated by neocortical neurons in NEC and WAG/Rij brain slices. First, we analyzed the paired-pulse glutamatergic

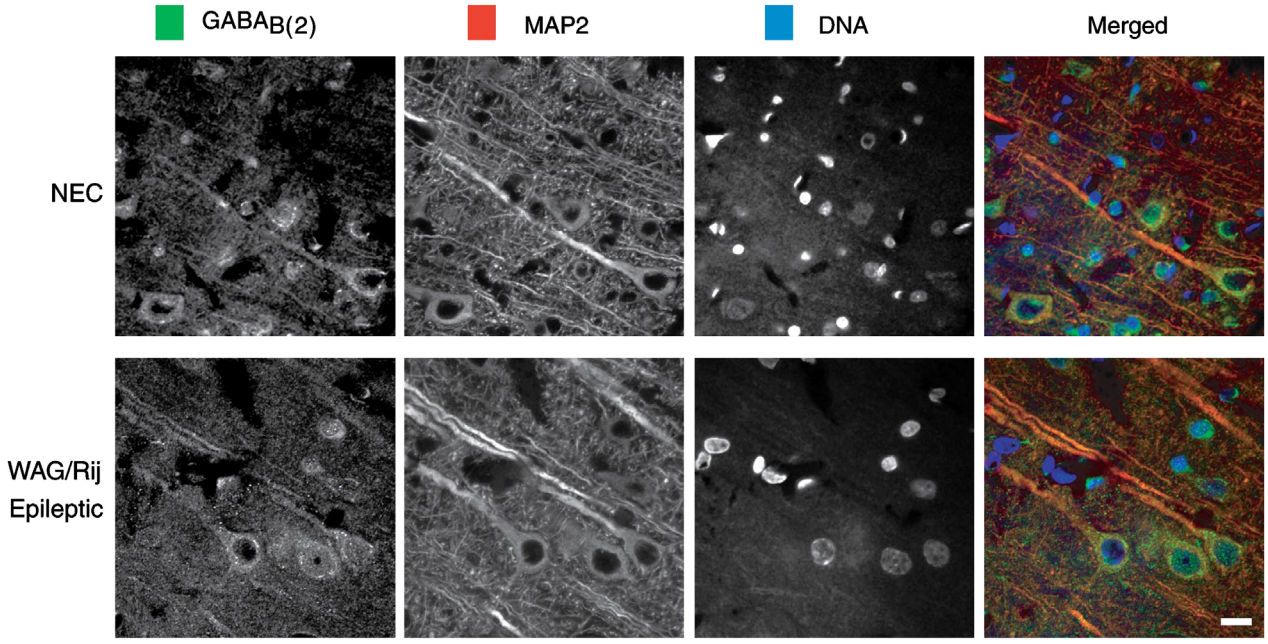

Fig. 3. Triple immunofluorescence for $GABA_{B(2)}$ (green), MAP2 (red) and DNA (blue) in the perioral region of the primary somatosensory cortex of NEC ($n=3$) and WAG/Rij ($n=4$) coronal brain sections. Note that $GABA_{B(2)}$ immunoreactivity was present throughout the length of the apical dendrite in both NEC and epileptic WAG/Rij animals. Scale bar: 10 μm.

depression (*cf.*, Kang, 1995; Gil et al., 1997) by using electrodes containing KCl+QX-314. In addition to reducing current through voltage-gated Na^+ channels (Connors and Prince, 1982), QX-314 blocks postsynaptic $GABA_B$ receptor-mediated conductances (Nathan et al., 1990) thus allowing us to assess the sole presumptive contribution of presynaptic $GABA_B$ receptors located on glutamatergic terminals to paired-pulse depression. The ASCF used in these experiments contained picrotoxin to block $GABA_A$ receptors and CPP+4 mM Mg^{2+} to abolish NMDA receptor-mediated currents, while the stimulating electrode was placed in the white matter.

Consistent with previous studies (Kang, 1995; Gil et al., 1997), paired-pulse stimulation in NEC neocortical slices ($n=10$) reduced the amplitude of the response induced by the second stimulus at intervals of 50–800 ms with maximal decreases occurring at around 250 ms (Figs. 4A and C). In contrast, when analyzed in epileptic WAG/Rij neocortical cells, this protocol did not cause any comparable reduction of the second response ($n=8$; Figs. 4B and C). As indicated by the asterisks in Fig. 4C, paired-pulse indexes obtained from neocortical cells recorded in NEC and WAG/Rij slices were significantly different ($p<0.05$) at interstimulus intervals between 100 and 450 ms.

Next, we delivered paired-pulse stimuli at intervals of 50–1600 ms with a stimulating electrode placed within 500 μm of the recording electrode. Recordings were made with K-acetate+QX-314-filled electrodes during superfusion of ASCF containing the glutamatergic antagonists CPP and CNQX. Since QX-314 abolishes responses caused by activation of postsynaptic $GABA_B$ receptors (Nathan et al., 1990), the stimulus-induced responses recorded under these experimental conditions represented isolated $GABA_A$ receptor-mediated IPSPs (*cf.*, Fukuda et al., 1993; Deisz, 1999). In addition, since QX-314 attenuates I_h (Perkins and Wong, 1995), we could analyze these IPSPs in their reversed form by holding the membrane potential at values more negative than −90 mV. Paired-pulse stimulation protocols in NEC slices ($n=10$) revealed a reduction in the amplitude of the second stimulus-induced IPSP with respect to the first at interstimulus intervals between 50 and 800 ms. Similar to what we observed in pharmacologically isolated glutamatergic responses, this depression was maximal at interstimulus intervals of approx. 250 ms, while the amplitude of the second IPSP recovered to control values at intervals of ≥1200 ms (Figs. 5A and C). In contrast, paired-pulse depression of these presumptive IPSPs was less pronounced in WAG/Rij neocortical neurons ($n=10$) (Fig. 5B). Significant differences ($p<0.05$) between the data obtained from NEC and WAG/Rij slices were observed at interstimulus intervals between 100 and 800 ms (asterisks in Fig. 5C).

Paired-pulse depression in the neocortex has been shown to be reduced by bath application of $GABA_B$ receptor antagonists (*cf.*, Gil et al., 1997; Deisz, 1999; Fukuda et al., 1993; Kang, 1995). Consistent with these early findings, we observed that in NEC slices the $GABA_B$ receptor antagonist CGP 55845 decreased paired-pulse depression of both glutamatergic ($n=4$; Fig. 6A) and inhibitory responses ($n=3$; Fig. 6C). In contrast, the effects induced by CGP 55845 on the paired-pulse depression of glutamatergic (Fig. 6B) and GABAergic events (not shown) were less pronounced in WAG/Rij neocortical tissue ($n=4$ in both cases). Quantification of these data at an interstimulus interval of 250 m is shown in Figs. 6D and E. The percentage changes induced by this $GABA_B$ receptor antagonist under the four different conditions are summarized in Fig. 6F.

Discussion

The prominent role played by neocortical networks in generalized SW discharges has been identified both in humans and in several animal models of absence seizures (Avoli et al., 2001; Crunelli and Leresche, 2002; Blumenfeld, 2005) including WAG/Rij rats (van Luijtelaar and Coenen, 1986). These animals represent

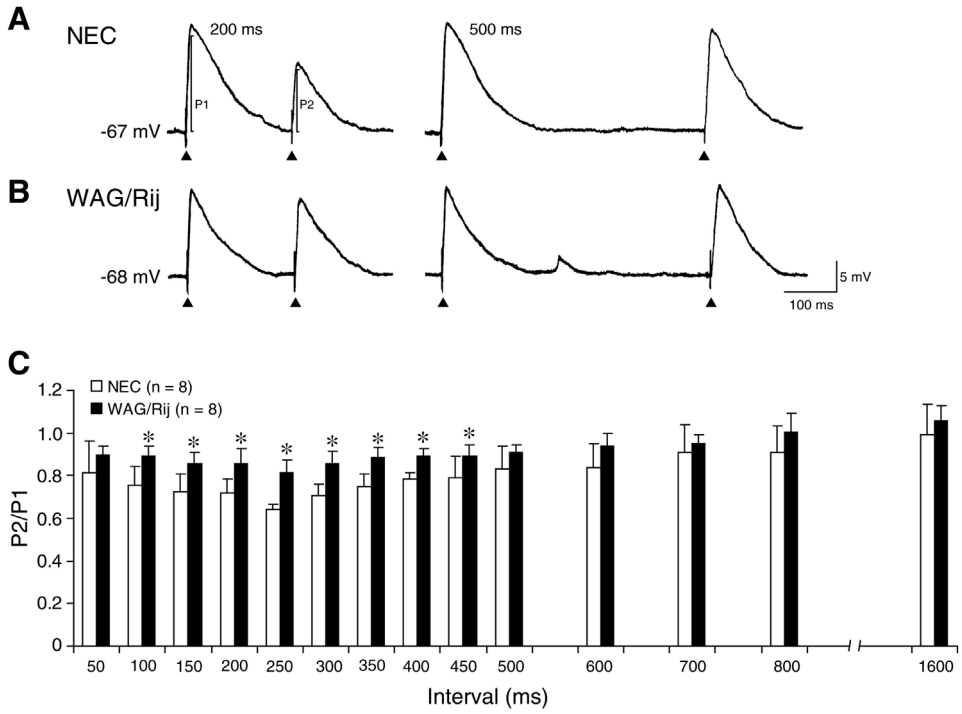

Fig. 4. Paired-pulse depression of excitatory responses recorded with KCl+QX-314-filled electrodes from neocortical neurons in slices obtained from NEC (A) and epileptic WAG/Rij animals (B). In both experiments brain slices were superfused with ASCF containing picrotoxin, CPP and 4 mM Mg^{2+}. Note that reduction in the amplitude of the response induced by the second stimulus is evident in NEC, but not in the epileptic WAG/Rij neocortical neuron. C: Summary graph showing paired-pulse depression of the excitatory responses in NEC ($n=10$) and epileptic WAG/Rij ($n=8$) neocortical neurons. Note that in NEC experiments the response induced by the second stimulus is reduced at intervals of 50–800 ms with maximal decreases occurring at around 250 ms while no comparable reductions are seen in epileptic WAG/Rij neocortical cells In this and following graphs, asterisks indicate interstimulus intervals at which the two groups were significantly different from each other ($p<0.05$).

a genetic model of absence seizures in which SW discharges initiate in the perioral area of the somatosensory cortex (Meeren et al., 2002). Indeed, a major genetic component has been identified in primary generalized epilepsies since the early studies reported by Lennox and Lennox (1960) and Metrakos and Metrakos (1961).

Experimental evidence obtained to date from *in vitro* slice studies indicates that epileptic WAG/Rij neocortical neurons present with: (i) marked reduction in I_h (a cation current that is known to modulate neuron excitability and rhythmicity) (Strauss et al., 2004), (ii) decreased function of $GABA_A$ receptor-mediated post-synaptic inhibition (Luhmann et al., 1995) and (iii) increased NMDA receptor-mediated events leading to prolonged depolarizing responses and to action potential discharge (Luhmann et al., 1995; D'Arcangelo et al., 2002; D'Antuono et al., 2006). The latter mechanism has also been identified *in vivo* both in WAG/Rij (Peeters et al., 1989) and GAERS (Pumain et al., 1992) animals.

The findings reported here demonstrate that the somatosensory cortex of epileptic WAG/Rij rats also exhibits alterations in $GABA_B$ receptor subunit expression and localization. In addition, we have found that paired-pulse depression of pharmacologically isolated excitatory and inhibitory responses is reduced in the WAG/Rij somatosensory cortex. Paired-pulse depression is known to be due to several mechanisms (Zucker and Regehr, 2002) including the activation of presynaptic $GABA_B$ receptors (Fukuda et al., 1993; Kang, 1995; Gil et al., 1997; Deisz, 1999). Therefore, we propose that altered expression and function of $GABA_B$ receptors contributes to the occurrence of absence seizures in this genetic rodent model.

Changes in $GABA_B$ receptor subunit composition in WAG/Rij somatosensory cortex

By employing Real-Time PCR analysis we have discovered that mRNA levels for most $GABA_{B(1)}$ subunits were markedly reduced in the somatosensory cortex of epileptic WAG/Rij animals as compared to NECs; in contrast, $GABA_{B(1a)}$ and $GABA_{B(2)}$ mRNA expression levels were unaltered. These changes in subunit mRNAs are likely to lead to specific differences in $GABA_B$ receptor protein expression which in turn could contribute to making the WAG/Rij rat neocortex hyperexcitable and thus prone to generate SW activity. Several investigators have proposed that different $GABA_B$ receptor subtypes possess distinct functional roles (Kaupmann et al., 1998; Billinton et al., 1999; Brauner-Osborne and Krogsgaard-Larsen, 1999); however, this issue has not been clarified to date.

It is also possible that the preserved levels of some $GABA_B$ subunit mRNAs do not correspond to the expression of their respective proteins. Accordingly, Schuler et al. (2001) have reported that KO mice lacking the $GABA_{B(1)}$ subunit are characterized by down-regulation of $GABA_{B(2)}$ protein, even though its mRNA expression level is unchanged. Similarly, a marked reduction of $GABA_{B(1)}$ protein occurs in KO mice that present with no $GABA_{B(2)}$ protein, even though $GABA_{B(1)}$ mRNA was unchanged (Gassmann et al., 2004). In addition, these investigators have found throughout the brain that the remaining $GABA_{B(1)}$ protein was redistributed from the neuropil to the soma (Gassmann

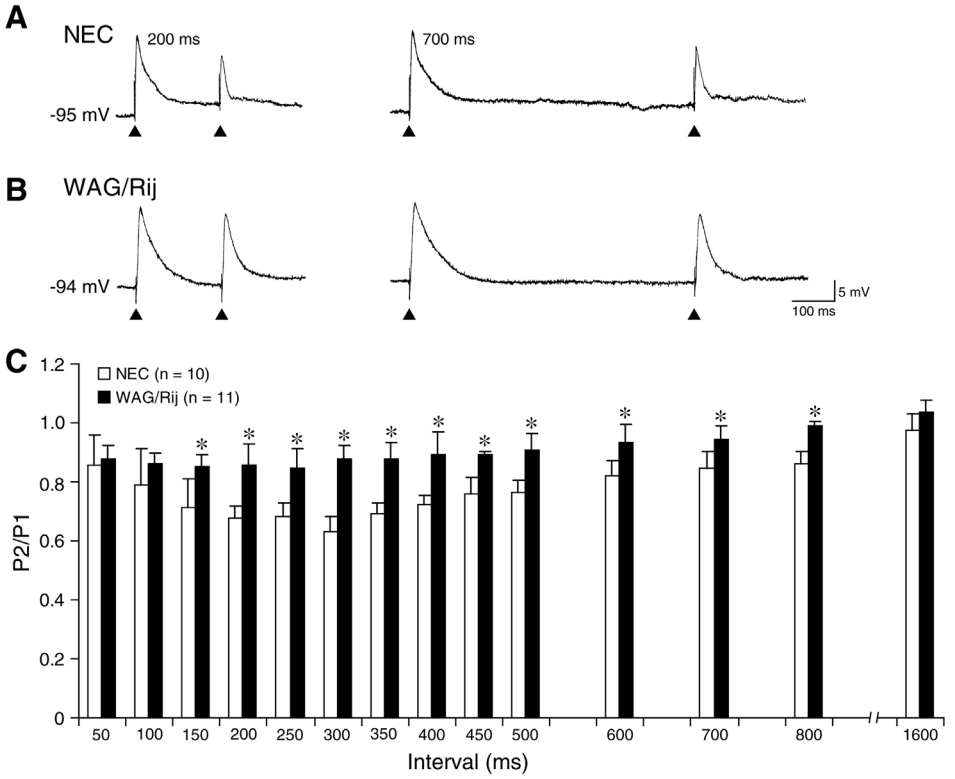

Fig. 5. Paired-pulse depression of neocortical IPSPs recorded with K-acetate+QX-314-filled electrodes from neurons in NEC (A) and epileptic WAG/Rij slices (B). Slices were superfused with ASCF containing CPP+CNQX, while the stimulating electrode was placed within 500 μm of the recording electrode filled with K-acetate+QX-314. Note that since neurons were kept at membrane potentials more negative than −90 mV, IPSP responses are depolarizing. Note also that the amplitude of the responses induced by a second stimulus is reduced in the NEC experiment but not in the epileptic WAG/Rij neuron. (C) Summary graph showing paired-pulse ratio in NEC and epileptic WAG/Rij cells ($n=10$ in both cases). Note that significant differences ($p<0.05$) between the data obtained from NEC and WAG/Rij slices were observed at interstimulus intervals between 100 and 800 ms.

et al., 2004). This evidence would account for the marked decrease in $GABA_{B(1)}$ immunoreactivity detected in our study in the apical dendrites of WAG/Rij neocortical pyramidal cells. Further analysis, with isoform-specific antibodies, will be necessary to confirm this hypothesis. It should also be emphasized that pre- and postsynaptic $GABA_B$ receptor-mediated mechanisms are markedly decreased in the hippocampus (as neocortical cells were not studied) of both types of KO mice. In addition, these animals presented with spontaneous generalized epileptic discharges *in vivo*. This evidence underscores the relation between generalized seizures and $GABA_B$ receptor dysfunction.

Changes in paired-pulse depression in WAG/Rij somatosensory cortex

The alterations in $GABA_B$ receptor subunit expression and localization identified here in WAG/Rij neocortical tissue are likely to contribute to synaptic hyperexcitability (Luhmann et al., 1995; D'Antuono et al., 2006) and thus to the ability of somatosensory networks of these animals to initiate SW dicharges *in vivo* (Meeren et al., 2002). Although the exact functional meaning of changes in subunit composition of $GABA_B$ receptors in the epileptic WAG/Rij rat neocortex remains to be established, our findings indicate that paired-pulse depression of excitatory and inhibitory responses are markedly reduced in the neocortex of epileptic animals as compared with NECs. Activation of presynaptic $GABA_B$ receptors (Fukuda et al., 1993; Kang, 1995; Gil et al., 1997; Deisz, 1999) along with other pre- and postsynaptic mechanisms (Zucker and Regehr, 2002) are known to contribute to paired-pulse depression of stimulus-induced responses in the forebrain.

A weakened presynaptic control would lead to an augmented release of inhibitory and, perhaps to an even greater extent, excitatory transmitters which in turn may facilitate the expression of NMDA receptor-mediated conductances as identified in epileptic WAG/Rij rats (Luhmann et al., 1995; D'Arcangelo et al., 2002; D'Antuono et al., 2006). This view is further supported by preliminary data indicating that paired-pulse depression of excitatory responses is not compromised in young (<90-day-old) non-epileptic WAG/Rij rats (D. Merlo and M. Avoli, unpublished results). Alterations in presynaptic release of neurotransmitters have been reported in animal models of temporal lobe epilepsy (Asprodini et al., 1992; Behr et al., 2002; Benini and Avoli, 2006; Jarvie et al., 1990; Kamphuis et al., 1990) where they are associated with neuronal hyperexcitability.

Intracellular recordings obtained from epileptic WAG/Rij neocortical pyramidal cells have demonstrated that focal electrical stimuli induce slow hyperpolarizations with reversal potential more negative than −85 mV (Luhmann et al., 1995; D'Antuono et al., 2006) suggesting that they were caused by an increased K^+ conductance. Although the sensitivity of these slow hyperpolarizations to $GABA_B$ receptor antagonists has not been established, previous studies have indicated that they are likely to be caused by

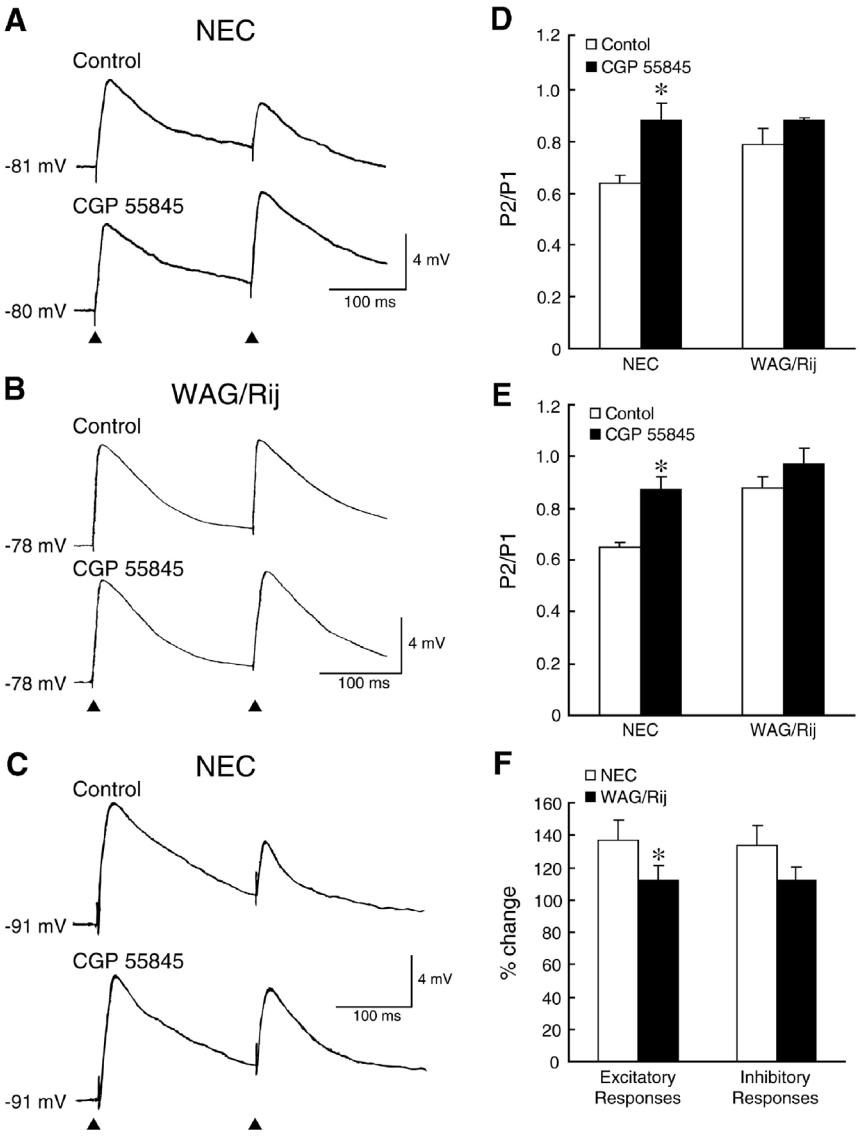

Fig. 6. (A and B) Effects induced by the $GABA_B$ receptor antagonist CGP 55845 on the paired-pulse depression of stimulus-induced glutamatergic responses generated by NEC (A) and WAG/Rij (B) neocortical neurons. Note that CGP 55845 reduces paired-pulse depression in NEC but it does not influence the responses recorded from the WAG/Rij neocortical cell. (C) Effects induced by the $GABA_B$ receptor antagonist CGP 55845 on the paired-pulse depression of stimulus-induced inhibitory responses recorded from a NEC neocortical slice. (D and E) Plots of the absolute P2/P1 values obtained for interstimulus intervals of 250 ms while analyzing the glutamatergic and inhibitory responses in NEC and WAG/Rij neurons under control conditions and during application of CGP 55845. Data were obtained from 4 NEC and 4 WAG/Rij neurons for glutamatergic and from 3 NEC and 4 AG/Rij cells for inhibitory paired-pulse responses. Note the less pronounced effect of this $GABA_B$ receptor antagonism in the WAG/Rij samples. F: Summary plot of the percentage changes induced by CGP 55845 under the four different conditions.

the activation of postsynaptic $GABA_B$ receptors (Andrade et al., 1986; Connors et al., 1988). Hence, this evidence implies that the molecular changes in $GABA_B$ receptor subunits identified in the WAG/Rij somatosensory neocortex may be mirrored by functional downregulation of presynaptic mechanisms, only. Moreover, since the histochemical loss of $GABA_{B(1)}$ was seen in the distal dendrites of pyramidal cells, where $GABA_B$ receptors are presumably located both on presynaptic terminals and on the postsynaptic membrane, the preservation of slow hyperpolarizations in WAG/Rij slices (D'Antuono et al., 2006) suggests the possibility that loss of this subunit may only occur at presynaptic sites. Electromicroscopic analysis will be, however, required for testing this hypothesis.

Interestingly, early studies performed in both GAERS and WAG/Rij rats found that injecting $GABA_B$ receptor antagonists either into the thalamus or systemically, blocked absence seizures (Liu et al., 1992; Snead, 1996; Puigcerver et al., 1996). These data have been interpreted as reflecting the ability of $GABA_B$ receptor antagonists to reduce slow hyperpolarizing IPSPs (and thus their ability to de-inactivate low-threshold Ca^{2+} conductances) in thalamocortical relay cells (Steriade et al., 1993).

Conclusions

Our study identifies some molecular and functional changes in $GABA_B$ receptors in the epileptic WAG/Rij neocortex. These

findings lead us to propose that these alterations may contribute to the generation of generalized SW *in vivo*. Such a conclusion is supported by the evidence obtained by several investigators in knockout animals. $GABA_{B(1)}$−/− and $GABA_{B(2)}$−/− mice exhibit a loss of all biochemical and electrophysiological $GABA_B$ functions and also present with spontaneous generalized SW discharges (Schuler et al., 2001; Prosser et al., 2001; Brown et al., 2003; Gassmann et al., 2004). However, it remains unclear whether the changes in $GABA_{B(1)}$ subunit expression and in paired-pulse depression reported in WAG/Rij rats are genetically determined (and thus developmentally regulated) or whether they are, at least in part, the consequence of SW discharges. Further experiments aimed at establishing whether antiepileptic drug pre-treatment early in life can influence these changes, are needed to answer this question and thus to shed more light on the role of $GABA_B$ receptors in this genetic model of absence seizures.

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