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Ethosuximide modifies network excitability in the rat entorhinal cortex via an increase in GABA release

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ABSTRACT

Ethosuximide is the drug of choice for treating generalized absence seizures, but its mechanism of action is still a matter of debate. It has long been thought to act by disrupting a thalamic focus via blockade of T-type channels and, thus, generation of spike–wave activity in thalamocortical pathways. However, there is now good evidence that generalized absence seizures may be initiated at a cortical focus and that ethosuximide may target this focus. In the present study we have looked at the effect ethosuximide on glutamate and GABA release at synapses in the rat entorhinal cortex in vitro, using two experimental approaches. Whole-cell patch-clamp studies revealed an increase in spontaneous GABA release by ethosuximide concurrent with no change in glutamate release. This was reflected in studies that estimated global background inhibition and excitation from intracellularly recorded membrane potential fluctuations, where there was a substantial rise in the ratio of network inhibition to excitation, and a concurrent decrease in excitability of neurones embedded in this network. These studies suggest that, in addition to well-characterised effects on ion channels, ethosuximide may directly elevate synaptic inhibition in the cortex and that this could contribute to its anti-absence effects.

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1. Introduction

Generalized, non-convulsive absence seizures are characterised by brief episodes of unconsciousness accompanied by synchronised bilateral spike and wave discharges. Ethosuximide is the drug of choice in the treatment of absence epilepsy (Posner et al., 2005; Glauser et al., 2010). It has a number of potential molecular targets, but its precise mechanism of action has not been elucidated.

One view of the basis of absence epilepsy is that it involves an interaction between a hyperexcitable cortex and a rhythm generator operative via a thalamocortical loop, where rhythmical bursts of activity in the GABAergic neurones of the thalamic reticular nucleus play a central role (see Crunelli and Leresche, 2002a; Manning et al., 2003; Meeren et al., 2005). These bursts of activity are driven by low-threshold (T-type) Ca-currents, and it has been postulated that block of T-type Ca-channels in thalamocortical reticular nucleus neurones may be the basis of the anti-absence effects of ethosuximide (Coulter et al., 1989a,b; Kostyuk et al., 1992; Huguenard, 1999; Gomora et al., 2001). However, there is still controversy over whether this blockade is the whole story (see Crunelli and Leresche, 2002b; Manning et al., 2003). Certainly, ethosuximide is promiscuous in its effects and has been shown to block other ion channels (Leresche et al., 1998; Kobayashi et al., 2009).

Early studies implicated the thalamus as a primary site of epileptogenesis, but increasing attention has centred on a cortical focus in absence seizures (Seidenbecher et al., 1998; Meeren et al., 2002, 2005; Holmes et al., 2004; Sadleir et al., 2006; Polack et al., 2007). It is possible that ethosuximide acts partially, or exclusively, at this level. A weak and delayed reduction of the spike–wave discharges in genetically epileptic rats followed infusion of ethosuximide directly into the thalamus, but systemic injection resulted in immediate cessation of discharges (Richards et al., 2003). Likewise, direct application into somatosensory cortex caused immediate cessation of discharges (Manning et al., 2004;

Abbreviations: aCSF, artificial cerebrospinal fluid; EC, entorhinal cortex; E_{Bg}, global background synaptic excitation; $GABA_Ar$, $GABA_A$-receptor; $GABA_Br$, $GABA_B$-receptor; GAERS, Genetic Absence Epilepsy Rat from Strasbourg; GIRK, G-protein couple inwardly rectifying K-channel; I_{Bg}, global background synaptic inhibition; I:E, inhibition:excitation ratio; IEI, inter-event interval; KS, Kolmogorov–Smirnov test; mIPSC, miniature inhibitory postsynaptic current; sEPSC, spontaneous excitatory postsynaptic current; sIPSC, spontaneous inhibitory postsynaptic current; T-type, low-threshold Ca-channel; VmD, membrane potential distribution method.

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Gülhan Aker et al., 2010). Polack et al. (2007) suggested that spike–wave discharges were initiated by pyramidal neurones in layer V, and found that hyperexcitability in this region was normalised by systemic ethosuximide (Polack and Charpier, 2009). The molecular mechanism of this action of ethosuximide could involve actions at one or more of the ion channel targets noted above, but it is also feasible that alterations of inhibitory or excitatory synaptic transmission could be involved. For example, Luhmann et al. (1995) showed an impairment of GABA inhibition in the cortex of WAG/Rij rats, a genetic model of absence epilepsy. Also, a number of mutations in $GABA_A$-receptors ($GABA_Ar$) have been linked to childhood absences (see Galanopoulou, 2010; Macdonald et al., 2010). One mutation, in the γ2-subunit, has been shown to elicit ethosuximide-sensitive absence seizures when expressed in mice, associated with a reduced expression of the subunit and a decrease in $GABA_Ar$ mediated events in somatosensory cortex (Tan et al., 2007). Thus, cortical hyperexcitability could be associated with the loss of GABA inhibition and ethosuximide could target cortical GABA transmission in absence epilepsy.

We have recently studied the effects of anticonvulsant drugs on excitatory and inhibitory transmission in the entorhinal cortex (EC). Using patch-clamp recordings of spontaneous postsynaptic currents, we have studied the effects of the drugs on GABA and glutamate release (Cunningham et al., 2000, 2003, 2004; Cunningham and Jones, 2000; Yang et al., 2007). We have complemented these studies using a novel approach (Greenhill and Jones, 2007, 2010) to determine global, network driven, background excitation and inhibition, estimated from the distribution of resting membrane potential fluctuations (VmD) in individual neurones (see Rudolph et al., 2004, 2007). We have studied a number of drugs used to treat tonic–clonic convulsive seizures, some of which are also used in absence epilepsies. Patch-clamp studies revealed diverse effects on spontaneous of glutamate and GABA release, and these were reflected by global changes in excitation and inhibition determined by VmD studies. However, the drugs had the common effect of elevating the ratio between inhibition and excitation in favour of the former (Greenhill and Jones, 2010). Concurrently, they caused a decline in intrinsic neuronal excitability.

We have now determined the effect of the specific anti-absence drug, ethosuximide, on synaptic transmission and excitability in the rat EC. Whilst evidence suggests that the frontal and somatosensory cortices are likely foci in absence seizures (Meeren et al., 2002; Polack et al., 2007), EEG studies indicate that some absence and related seizures could originate at temporal sites (e.g. Lombroso, 1997; Holmes et al., 2010; Caraballo et al., 2008; Tucker et al., 2007). In addition, studies in EC-hippocampal slices have suggested that there is altered excitability and susceptibility to epileptogenesis in GAERS (Genetic Absence Epilepsy Rats from Strasbourg; Armand et al., 1998, 1999), and it is also the case that local cerebral glucose utilisation is increased in limbic regions (including EC) as well frontal and somatosensory areas (Nehlig et al., 1998; Carçak et al., 2009) in GAERS. For these reasons, and because our investigations of other anticonvulsants have focussed on this area we decided to continue with it in the present study, although we do not suggest that the EC may be a primary focus in absence seizures.

2. Materials and methods

2.1. Slice preparation

All experiments were performed in accordance with the U.K. Animals (Scientific Procedures) Act 1986, European Communities Council Directive 1986 (86/609/EEC) and the University of Bath ethical review document. The number of animals used was kept to a minimum and every precaution was taken to minimize any suffering and stress inflicted. EC slices were prepared from male Wistar rats (60–100, P28-40) anaesthetised with ketamine (120 mg/kg) plus xylazine (8 mg/kg). Rats were decapitated and the brain removed and immersed in artificial cerebrospinal fluid (aCSF; see below for composition) at 4 °C. Slices (400 μM) were cut using a Campden Vibroslice and stored in aCSF bubbled with carbogen (95% O_2/5% CO_2) at room temperature. To increase neuronal survival and viability, ketamine (4 μM) was included in the cutting solution, and the antioxidants n-acetyl-L-cysteine (6 μM) and uric acid (100 μM), added to both cutting and storage solutions. For patch-clamp recordings slices, were transferred to a recording chamber perfused (2 ml/min) with oxygenated aCSF at 31–32 °C on an Olympus BX50WI microscope. Neurones were visualized using DIC optics and an infrared video camera. In VmD experiments slices were transferred to a recording chamber where they were held at the interface between a continuous perfusion of oxygenated aCSF (1.5 ml/min) maintained at 32 ± 0.5 °C and warm moist carbogen gas. Intracellular recordings were made "blind" from slices visualized with a binocular microscope (Wild M8). We have found (Woodhall, G.L. and Jones, R.S.G., unpublished observations) that the use of antioxidants produces robust and long-lasting slices, but does not have any apparent effect on the pharmacology of glutamate or GABA transmission. Nevertheless, in both recording situations slices were allowed to equilibrate in the recording chamber for at least 1 h prior to recording to allow for washout of these agents. The perfusion and storage aCSF contained (in mM): NaCl (126), KCl (3.25), NaH_2PO_4 (1.4), $NaHCO_3$ (19), $MgSO_4$ (2), $CaCl_2$ (2), and D-glucose (10). For cutting the slices at 3–4 °C, $NaHCO_3$ was increased to 25 mM to maintain pH at acceptable levels (7.3).

2.2. Whole-cell patch-clamp recordings

Patch pipettes pulled from borosilicate glass were used for recording spontaneous EPSCs (sEPSCs). They were filled with a Cs-gluconate based solution containing (in mM) D-Gluconate (100), HEPES (40), QX-314 (1), EGTA (0.6), $MgCl_2$ (5), TEA-Cl (10), phosphocreatinine (5); ATP-Na (4) and GTP-Na (0.3). To record spontaneous (sIPSCs) or miniature (mIPSCs) inhibitory PSCs, the patch solution contained CsCl (100), HEPES (40), QX-314 (1), EGTA (0.6), TEA-Cl (10), $MgCl_2$ (5), ATP-Na (4) and GTP-Na (0.3). Solutions were adjusted to 275 mOsmol and pH 7.3 with CsOH. Whole-cell voltage clamp recordings (holding potential −60 mV) were made from pyramidal neurones in layer III of the medial division of the EC, using an Axopatch 200B amplifier. Signals were filtered at 2 kHz and digitized at 20 kHz. Series resistance compensation was not employed, but access resistance (10–30 MΩ) was monitored at regular intervals and cells were discarded if it changed by more than ±10%. Liquid junction potentials (EPSC +12.0 mV; IPSCs +10.2 mV) were estimated using pClamp-8 software, and compensated for in the holding potentials. When recording IPSCs, AMPA-receptors and NMDA-receptors were blocked with bath applied NBQX and 2-AP5, respectively.

Data were recorded using Axoscope software, and Minianalysis (Synaptosoft, Decatur) was used for analysis of PSCs off-line. Spontaneous events were detected using a threshold-crossing algorithm. Cumulative probability distributions of inter-event interval (IEI) of spontaneous currents were compared using the Kolmogorov–Smirnoff test (KS). When data were pooled for this analysis, a minimum of 200 events was sampled during a continuous recording period for each neurone under each condition. Mean amplitudes, rise times (10–90%) and total decay times were compared using a paired *t*-test. In some cases, to gain an overall picture of the effects of ethosuximide on the level of spontaneous inhibition or excitation, we estimated total charge transfer associated with sPSCs. This is calculated by measuring the area of sIPSCs or sEPSCs, and is directly proportional to the amplitude multiplied by the decay time (Hollrigel and Soltesz, 1997). We determined charge transfer associated with sPSCs in a set time period of 2 min in control and in the presence of the drug. All error values in the text refer to standard error of the mean.

2.3. VmD estimations

Sharp electrodes, pulled from borosilicate glass and filled with potassium acetate (3 M), were used to make intracellular voltage recordings from pyramidal neurones in layer III of the medial EC using an Axoprobe 1A amplifier (Molecular Devices, Sunnyvale CA, USA) in bridge mode. When membrane potential was stabilised after impalement, estimates of global background excitation (E_{Bg}) and inhibition (I_{Bg}) were derived from membrane potential fluctuations using the VmD method at regular intervals throughout the recordings. This approach was derived by Rudolph et al. (2004) and we have adapted it for recording in EC slices (Greenhill and Jones, 2007). Briefly, neurones were depolarized (for 15–20 s) by injection of two levels of known positive current via the recording electrode. The levels of the currents differed from neurone to neurone, but were maintained the same throughout any individual experiment. One level was chosen to elicit a depolarization to within 1–2 mV of action potential threshold, and the second was adjusted to depolarize the neurone to about half way between this and resting membrane potential. Membrane potential fluctuations at these two levels were fitted to Gaussian distributions (using Prism 4 software, GraphPad, San Diego, USA) and the mean and variance of the membrane potential determined. Leak conductance in each neurone was calculated from the ohmic response produced by a small (0.1 nA, 100 ms) hyperpolarizing current, injected at resting membrane potential. These parameters, together with mean reversal potentials for AMPA-receptors and $GABA_Ar$