JPn Neuroscience

The Journal of Physiology

J Physiol 591.4 (2013) pp 845–858

α2-containing $GABA_A$ receptors expressed in hippocampal region CA3 control fast network oscillations

Tim S. Heistek, Marta Ruiperez-Alonso, A. Jaap Timmerman, Arjen B. Brussaard and Huibert D. Mansvelder

Department of Integrative Neurophysiology, CNCR, Neuroscience Campus Amsterdam, VU University, Amsterdam, The Netherlands

Key points

- Hippocampal oscillations are thought to be important for memory encoding and retrieval and depend on inhibition via GABA synapses.
- $GABA_A$ receptor subunits are differentially expressed throughout the hippocampal circuitry. Here we address which subunit controls cholinergically induced fast network oscillations and where it is expressed.
- By selectively increasing and decreasing the function of α1 and α2 subunits, we find that hippocampal oscillations are controlled by α2 subunits expressed in CA3.
- Synapses from fast spiking interneurons to pyramidal cells in CA3 that provide the perisomatic inhibition necessary for fast network oscillations contain $GABA_A$ receptors with the α2 subunit.
- Our data suggest that α2-containing GABA receptors in CA3 have an important role in rhythmic hippocampal activity and thereby possibly in cognitive processing.

Abstract $GABA_A$ receptors are critically involved in hippocampal oscillations. $GABA_A$ receptor α1 and α2 subunits are differentially expressed throughout the hippocampal circuitry and thereby may have distinct contributions to oscillations. It is unknown which $GABA_A$ receptor α subunit controls hippocampal oscillations and where these receptors are expressed. To address these questions we used transgenic mice expressing $GABA_A$ receptor α1 and/or α2 subunits with point mutations (H101R) that render these receptors insensitive to allosteric modulation at the benzodiazepine binding site, and tested how increased or decreased function of α subunits affects hippocampal oscillations. Positive allosteric modulation by zolpidem prolonged decay kinetics of hippocampal GABAergic synaptic transmission and reduced the frequency of cholinergically induced oscillations. Allosteric modulation of GABAergic receptors in CA3 altered oscillation frequency in CA1, while modulation of GABA receptors in CA1 did not affect oscillations. In mice having a point mutation (H101R) at the $GABA_A$ receptor α2 subunit, zolpidem effects on cholinergically induced oscillations were strongly reduced compared to wild-type animals, while zolpidem modulation was still present in mice with the H101R mutation at the α1 subunit. Furthermore, genetic knockout of α2 subunits strongly reduced oscillations, whereas knockout of α1 subunits had no effect. Allosteric modulation of GABAergic receptors was strongly reduced in unitary connections between fast spiking interneurons and pyramidal neurons in CA3 of α2H101R mice, but not of α1H101R mice, suggesting that fast spiking interneuron to pyramidal neuron synapses in CA3 contain α2 subunits. These findings suggest that α2-containing $GABA_A$ receptors expressed in the CA3 region provide the inhibition that controls hippocampal rhythm during cholinergically induced oscillations.

DOI: 10.1113/jphysiol.2012.243725

(Resubmitted 27 August 2012; accepted after revision 23 October 2012; first published online 29 October 2012)
Corresponding author H.D. Mansvelder: Department of Integrative Neurophysiology, CNCR, Neuroscience Campus Amsterdam, VU University, Amsterdam, The Netherlands. Email: huibert.mansvelder@cncr.vu.nl

Introduction

Gamma-band oscillations (∼30–100 Hz) in the hippocampus are thought to be important for memory encoding and retrieval (Lisman & Idiart, 1995; Montgomery & Buzsaki, 2007) and are usually observed on top of τ oscillations (Bragin *et al.* 1995; Lisman & Idiart, 1995; Csicsvari *et al.* 2003; Cardin *et al.* 2009). Two different gamma generators have been identified for the hippocampus *in vivo*, i.e. from the entorhinal cortex and within the CA3–CA1 region (Bragin *et al.* 1995; Csicsvari *et al.* 2003). *In vitro* studies showed that the local generator of gamma oscillations within the CA3 region can be activated by muscarinic acetylcholine receptor activation (Fisahn *et al.* 1998; Mann *et al.* 2005). These oscillations depend on both AMPA and $GABA_A$ receptors ($GABA_A$-R) in the perisomatic region of CA3 pyramidal neurons (Mann *et al.* 2005) and several pyramidal neuron-targeting interneuron types fire in phase with oscillations (Klausberger *et al.* 2003; Hajos *et al.* 2004). The shape of oscillations in the local field potential (LFP) is mainly determined by large phase-coupled synaptic inhibitory currents on CA3 pyramidal neurons (Oren *et al.* 2006, 2010), whereby cycle-to-cycle variations in inhibition determine the length of a single wave (Atallah & Scanziani, 2009). Inhibitory GABAergic synapse kinetics and subcellular location of $GABA_A$-Rs depends mainly on the α subunits of this receptor (Banks & Pearce, 2000; Hutcheon *et al.* 2000; Nyiri *et al.* 2001; Vicini *et al.* 2001; Klausberger *et al.* 2002; Bosman *et al.* 2005). In the CA1 area, depending on type of presynaptic interneuron, GABAergic synapses on pyramidal neurons contain either mainly α1 or α2 subunits (Nyiri *et al.* 2001; Klausberger *et al.* 2002), while this is not clear for GABAergic synapses on CA3 pyramidal neurons. At present, it is unknown which $GABA_A$-R α subunit is controlling the power and rhythm of cholinergically induced oscillations. Furthermore, it is not known whether these oscillations in CA1 depend on local feedback inhibition or whether $GABA_A$-Rs on CA3 pyramidal neurons determine the shape of the LFP in CA1.

To address this issue, we made use of $GABA_A$-R subunit knockout mice and transgenic mice in which either α1 subunits, α2 subunits or both were insensitive to allosteric modulation at the benzodiazepine binding site (H101R mutation) (McKernan *et al.* 2000; Dias *et al.* 2005), and tested how the kinetics of $GABA_A$-R with either an α1 or an α2 subunit affect hippocampal oscillations. In α1 knockout mice handling-induced tremors are observed (Sur *et al.* 2001), while no behavioural abnormalities have been reported for mutant mouse lines used in this study. We find that inhibitory postsynaptic currents (IPSCs) in pyramidal neurons in CA3 are mediated mainly by $GABA_A$-Rs containing α2 subunits and that kinetics of these receptor types, in contrast to kinetics of α1 containing receptors, control cholinergically induced oscillations in the hippocampus network. The α2 subunit is present at synapses of perisomatic targeting fast spiking interneurons thought to provide the inhibitory input required for fast network oscillations.

Methods

Ethical approval

All experimental methods involving animals were approved by the animal welfare committee of our university (DierExperimentenCommissie), and in accordance with Dutch and European law. The experiments comply with the policies and regulations as required by *The Journal of Physiology* (Drummond, 2009)

Slice preparation

All mutant mice and their wild-type littermates were from an original mixed genetic background of C57BL6 and 129SvEv and were back-crossed to a C57BLl6 background (five to 10 generations). Techniques for creation of mutant lines have been previously described: α1 knockout (Sur *et al.* 2001), α2 knockout (Boehm *et al.* 2004), α1H101R (McKernan *et al.* 2000) and α2H101R (Dias *et al.* 2005). Mice carrying the H101R point mutation in both α1 and α2 were obtained by crossing double heterozygous mice born from homozygous α1H101R and α2H101R intercrosses. Mutant mice and wild-type littermates were identified using PCR analysis. Wild-type littermates were used for comparison with transgenic mice; all other experiments were performed on C57Bl6 mice. After decapitation without anaesthesia, brains were quickly removed and stored in ice-cold artificial cerebrospinal fluid (ACSF) containing (mM): 126 NaCl, 3 KCl, 10 glucose, 26 $NaHCO_3$, 1.2 NaH_2PO_4, 1 $CaCl_2$ and 3 $MgSO_4$ (carboxygenated with 5% CO_2/95% O_2) or a modified slicing solution with low sodium concentrations used for paired recordings and recordings in adult animals containing (mM): 110 choline chloride, 11.6 sodium ascorbate, 7 $MgCl_2$,

3.1 sodium pyruvate, 2.5 KCl, 1.25 NaH_2PO_4, 0.5 mM $CaCl_2$, 26 $NaHCO_3$ and 10 glucose (carboxygenated with 5% CO_2/95% O_2). Horizontal hippocampal slices (400 μm) from young (P13–18) or adult (P55–70) animals were cut in ice-cold ACSF using a microtome (Microm, Waldorf, Germany). After preparation, slices were stored for at least 1 h in ACSF containing 2 mM $CaCl_2$ and 2 mM $MgSO_4$. Next the slices were mounted on planar multi-electrode arrays with 150 μm spacing (MED-P5155, Alpha MED Sciences, Osaka, Japan) or 200 μm (200/30-T-gr and pMEA200/30iR, Multichannel Systems, Reutlingen, Germany) with a polyethylene coating (Sigma-Aldrich, St Louis, MO, USA). Slices were left to attach properly to electrodes for at least 1 h in a chamber with humidified carbogen gas before they were placed in the recording unit. For recording oscillations in adult slices we used perforated arrays with a 200 μm spacing (pMEA200/30iR) and slices were attached on the electrode array by 30 mbar under pressure.

Electrophysiology

LFPs were measured in four slices simultaneous with four recording units at the same time using the multichannel system or in combination with patch clamp recordings in the MED64 system.

During recording, slices were perfused with ACSF at a flow rate between 4 and 5 ml min^{-1} and were kept at 30°C and in submerged conditions; for adult slices, the flow rate underneath the slice was 1 ml min^{-1}. ACSF containing 25 μM carbamoylcholine chloride (Carbachol; Sigma-Aldrich) was perfused for at least 45 min or added after the mounting of the slices on to the multi-electrode grids. Spontaneous field potentials from all 64 recording electrodes were acquired simultaneously at 20 kHz (Alpha MED Sciences) or with 60 electrodes at 1 kHz (Multichannel Systems, Reutlingen, Germany).

Pyramidal neurons in CA3 and CA1 neurons were recorded in whole cell mode using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA). Borosilicate glass (Harvard Apparatus, Holliston, MA, USA) electrodes with tip resistances of 2–5 MΩ were filled with intracellular solution containing (mM): 140 potassium gluconate, 1 KCl, 10 Hepes, 4 potassium phosphocreatine, 4 ATP-Mg and 0.4 GTP (pH adjusted to 7.3 with KOH). To ensure temporal alignment between the multi-electrode and single cell electrophysiological signals, the current or voltage signals from the patch clamp amplifiers were recorded on one channel of the MED64 system, using a custom-made interface device. IPSCs during oscillations were recorded at +20 mV holding potential.

Local injections of 1 mM tetrodotoxin citrate (TTX; Abcam, Cambridge, UK) and 1 mM zolpidem (Duchefa, Haarlem, The Netherlands) were done with borosilicate glass electrodes with tip resistances of 1–2 MΩ. Injections were done by giving 25–30 mbar overpressure for 1 min in the stratum pyramidale of either CA3 or CA1. Zolpidem was dissolved in DMSO and TTX dissolved in ACSF.

Unitary connections of fast spiking interneurons to pyramidal neurons

Recordings were made from fast spiking interneurons with their soma in stratum oriens/pyramidale and from pyramidal neurons. Electrodes were filled with an intracellular solution containing (mM): 70 potassium gluconate, 70 KCl, 4 Mg-ATP, 4 phosphocreatine, 0.4 GTP, 0.5 EGTA, 10 Hepes and 0.5% biocytin (pH 7.3, KOH).

Glutematergic events were blocked using DNQX (10 μM). Interneuron activity was recorded in current clamp, while pyramidal cells were voltage clamped at −70 mV. In connected cell pairs a spike in the interneuron resulted in an IPSC in the pyramidal neuron. Owing to the high intracellular chloride concentrations, IPSC were observed as inward current. The presynaptic cell was stimulated with suprathreshold current pulses with an interval of 5 s. After recording slices were transferred to a 4% paraformaldehyde solution. Biocytin was revealed with the chromogen 3,3′-diaminobenzidine tetrahydrochloride using the avidin–biotin–peroxidase method (Horikawa & Armstrong, 1988). Slices were mounted on slides and embedded in mowiol (Clariant GmbH, Frankfurt am Main, Germany). Cells were reconstructed using Neurolucida software (Microbrightfield, Williston, VT, USA), using a ×100 oil objective.

Data analysis

Synaptic events were detected using the Mini Analysis Program (Synaptosoft, Decatur, GA, USA). All other analyses were done using custom-made scripts in Igor Pro (Wavemetrics, Lake Oswego, OR, USA) and Matlab (The Mathworks, Natick, MA, USA). Data were down-sampled to 200 Hz–2 kHz for analysis. Fourier transforms were performed using a 2 s Hanning window. Frequency and power of oscillations were calculated by fitting a Gaussian function to the Fourier transformed data, as noise in the Fourier transform might influence the peak frequency of the oscillations. The average power and frequency of the oscillations were calculated separately for the CA3 region and the CA1 region by taking the average of all electrodes in that region. The power of oscillations is transformed by taking the squared root to obtain a normal distribution. Single cell data were only used for cells that showed significant phase coupling between IPSCs and the LFP of the electrode near the soma of the

pyramidal neuron. IPSC τ decay times were calculated by fitting an exponential function to the individual IPSCs, all events with double peaks were rejected from analysis (<10%). All IPSC τ decay times and amplitudes of a single neuron were put in a histogram and fitted with a lognormal function. For statistical analysis the Student's t test or for multiple comparisons an ANOVA with Student–Newman–Keuls *post hoc* test was used. Data are represented as average ± SEM, n is indicated as number of slices for LFP or number of cells for intracellular recordings. Statistical differences of $P < 0.05$ are indicated by asterisks in the figures.

Results

Fast network oscillations are reduced in $\alpha2^{-/-}$ mice

Hippocampal fast network oscillations induced by muscarinic receptor activation depend on rhythmic synaptic inhibition of CA3 pyramidal neurons (Fisahn *et al.* 1998; Mann *et al.* 2005; Oren *et al.* 2010). Which $GABA_A$-R subunits are involved in this rhythmic inhibition of these neurons is not known. $GABA_A$-Rs containing $\alpha1$ or $\alpha2$ subunits are the most abundant synaptic $GABA_A$-Rs in mouse hippocampus (Laurie *et al.* 1992; Fritschy & Brunig, 2003; Prenosil *et al.* 2006). As a first step, to test whether hippocampal fast network oscillations induced by muscarinic receptor stimulation depend on $GABA_A$-Rs containing either $\alpha1$ or $\alpha2$ subunits, we studied oscillations in acute hippocampal slices of mice lacking these subunits. The cholinergic agonist carbachol (25 μM) induced fast network oscillations at a frequency of 20.7 ± 0.3 Hz at 30°C. The frequency of these oscillations depends on temperature and at physiological temperatures the frequency falls into the beta/gamma band range (Dickinson *et al.* 2003; Jansen *et al.* 2011). In $GABA_A$-R $\alpha1$ subunit knockout mice ($\alpha1^{-/-}$) no differences in frequency and power of carbachol-induced oscillations in CA3 were observed compared to wild-type littermates ($\alpha1^{+/+}$ $n = 11$ slices, 6 animals, $\alpha1^{-/-}$ $n = 15$ slices, 6 animals, $P = 0.79$ and $P = 0.43$ respectively, unpaired Student's t test; Fig. 1*A* and *C*). In addition, the spatial distribution of oscillations in the hippocampal slice was not altered in mice lacking $\alpha1$ subunits (Fig. 1*A*, bottom panels). In contrast in mice lacking $GABA_A$-R $\alpha2$ subunits ($\alpha2^{-/-}$), the power of oscillations in CA3 was strongly reduced (26.6 ± 6.3 μV Hz^{-2} in $\alpha2^{+/+}$, $n = 11$ slices, 5 animals, 7.0 ± 1.4 μV Hz^{-2} in $\alpha2^{-/-}$, $n = 10$ slices, 5 animals, $P < 0.05$, unpaired Student's t test; Fig. 1*B* and *D*). The frequency of oscillations and the spatial distribution were not affected (Fig. 1*B* and *D*). These results suggest that $GABA_A$-Rs containing $\alpha2$ subunits are involved in cholinergically induced oscillations.

Zolpidem modulation of fast network oscillations is largely reduced in $\alpha2$H101R mice

The $\alpha1^{-/-}$ and $\alpha2^{-/-}$ mice lack these subunits from early development on, which may result in compensatory developmental adaptations that may compromise interpretation of the results. Therefore, to circumvent these potential problems, we made use of transgenic mice that express $GABA_A$-R $\alpha1$ and/or $\alpha2$ subunits with point mutations that render these receptors insensitive to allosteric modulation via the benzodiazepine binding site (McKernan *et al.* 2000; Dias *et al.* 2005). These mice show normal behaviour and do not suffer

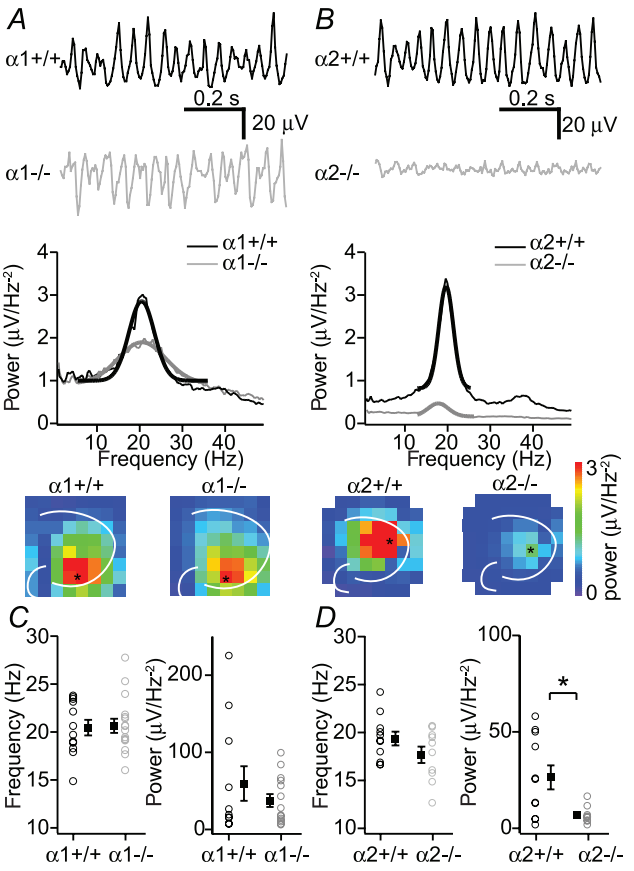

Figure 1. Hippocampal fast network oscillations are strongly reduced in $\alpha2^{-/-}$ mice but not in $\alpha1^{-/-}$ mice
A, example traces of oscillations in CA3 in $\alpha1^{+/+}$ mice and $\alpha1^{-/-}$ mice (top) and corresponding Fourier transforms (middle) and peak power at different location of the hippocampus (bottom). Traces are from electrodes indicated by an asterix. *B*, example traces of oscillations in CA3 in $\alpha2^{+/+}$ mice and $\alpha2^{-/-}$ mice (top) and corresponding Fourier transforms (middle) and peak power at different location of the hippocampus (bottom). *C*, peak frequency and peak power of oscillations in $\alpha1^{+/+}$ mice and $\alpha1^{-/-}$ mice ($n = 11$ for $\alpha1^{+/+}$ and $n = 15$ for $\alpha1^{-/-}$). *D*, peak frequency and peak power of oscillations in $\alpha2^{+/+}$ mice and $\alpha2^{-/-}$ mice ($n = 15$ for $\alpha1^{+/+}$ and $n = 10$ for $\alpha1^{-/-}$). Data show SEM.

from compensatory expression that general $GABA_A$-R knockout mice experience, i.e. function and location of the $GABA_A$-Rs are not affected (Rudolph *et al.* 1999; McKernan *et al.* 2000; Prenosil *et al.* 2006). It is well-established that allosteric modulation of $GABA_A$-Rs, with for instance zolpidem, alters the frequency and power of carbachol-induced fast network oscillations (Fig. 2*A* and *B*) (Palhalmi *et al.* 2004; Cope *et al.* 2005;

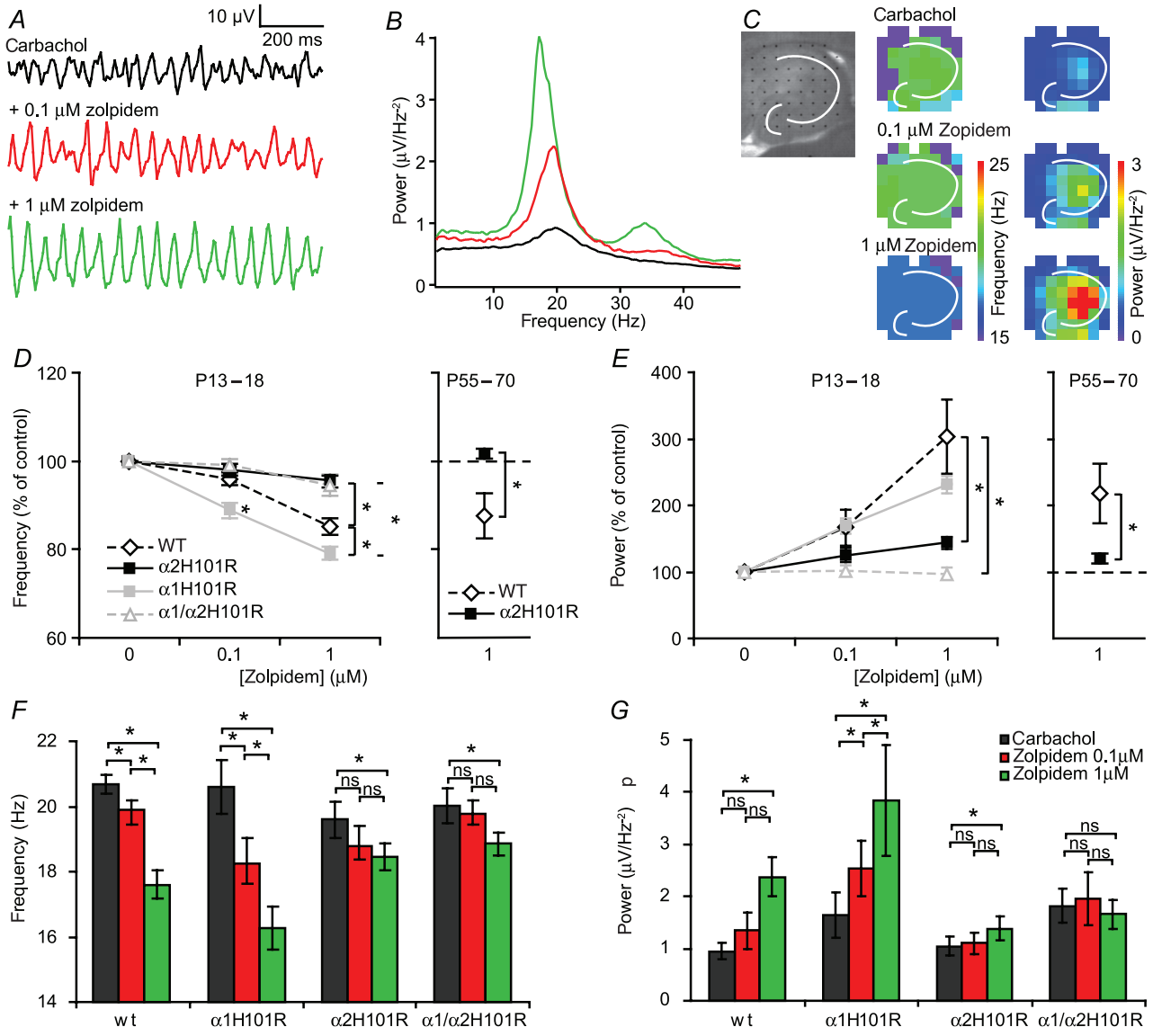

Figure 2. Changes of frequency and power of fast network oscillations by allosteric modulation of $GABA_A$ receptors are absent in α2 H101R mice

A, example traces of oscillations in CA3 in control and different concentrations of zolpidem in hippocampal slices of wild-type mice at 30°C. *B*, Fourier transform corresponding to the traces in A. *C*, left, hippocampal slice placed on a multi-electrode grid. The white lines show the stratum pyramidale and the granular layer of the dentate gyrus. Middle, frequency of fast network oscillations at control and different concentrations of zolpidem in wild-type mice at different electrodes. Right, power of fast network oscillations at control and different concentrations of zolpidem in wild-type mice at different electrodes. *D*, (left) comparison of zolpidem effects on frequency between different transgenic mice in CA3 in young animals ($n = 26$, $n = 17$, $n = 24$ and $n = 18$ for wild-type, α1 H101R, α2 H101R and α1 H101R/α2 H101R respectively), (right) zolpidem effects on frequency in adult animals ($n = 7$ and $n = 8$ for wild-type and α2 H101R respectively). Data are normalized to control. *E*, comparison of zolpidem effects on frequency between different transgenic mice in CA3 in young (left) and adult animals (right). Data are normalized to control. *F*, effects of zolpidem on the frequency of fast network oscillations in different transgenic mice. *G*, effects of zolpidem on the power of fast network oscillations in different transgenic mice. ANOVA test showed differences between genotypes in both frequency and power ($P < 0.05$). Data show SEM. WT, wild-type.

Heistek *et al.* 2010). Zolpidem has a higher affinity for $GABA_A$-Rs containing the $\alpha1$ subunit: at 100 nM zolpidem modulates $\alpha1$-containing receptors more than other $GABA_A$-Rs. At 1 μM, zolpidem also modulates $\alpha2/\alpha3$ containing receptors (Pritchett & Seeburg, 1990). In wild-type mice, zolpidem decreased the frequency of oscillations in CA3 in a concentration-dependent manner ($96.1 \pm 1.0\%$ and $87.0 \pm 1.4\%$, $n = 26$ slices, 14 animals with 0.1 and 1 μM zolpidem respectively, Fig. 2*A*–*F*). Furthermore, zolpidem increased the power of oscillations within CA3 to $140 \pm 15\%$ and $247 \pm 32\%$, $n = 26$ slices, 14 animals with 0.1 and 1 μM zolpidem respectively (Fig. 2). These effects of zolpidem were similar in CA3 and CA1 region (Fig. 2*C*, see also Fig. 5). Application of zolpidem did not alter the spatial distribution of oscillations (Fig. 2*C*). To test the involvement of $GABA_A$-R $\alpha1$ subunits, zolpidem was applied to hippocampal slices of mice carrying $GABA_A$-R $\alpha1$ subunits that all had a H101R point mutation ($\alpha1$H101R), which renders this subunit insensitive to zolpidem. In these hippocampal slices, we still observed a decrease in frequency and an increase in power in CA3 ($n = 17$ slices 10 animals, $P < 0.05$, repeated measures ANOVA, Fig. 2*D*–*G*). The decrease in frequency was substantially larger in $\alpha1$H101R mice compared to wild-type mice ($92.3 \pm 1.9\%$ and $81.0 \pm 1.5\%$ of control with 0.1 and 1 μM zolpidem respectively, $n = 17$ slices 10 animals, $P < 0.05$, Student–Newman–Keuls; Fig. 2*D* and *F*). These data suggest that allosteric modulation of $GABA_A$-Rs through the benzodiazepine binding site on $\alpha1$ subunits is not involved in the changes in oscillation frequency and power induced by zolpidem.

In contrast, in mice expressing $\alpha2$ subunits with a H101R point mutation ($\alpha2$H101R), the effects of allosteric modulation of $GABA_A$-R function by zolpidem in CA3 were strongly reduced compared to wild-type animals ($P < 0.05$, Student–Newman–Keuls). At 0.1 μM zolpidem there was no effect on frequency or power of oscillations; at 1 μM, zolpidem only a small but significant decrease in frequency ($96.7 \pm 1.3\%$, $n = 24$ slices, 15 animals, $P < 0.05$) and a moderate increase in power were observed ($126.2 \pm 9.0\%$, $P < 0.05$) (Fig. 2*D*–*G*). These results suggest that allosteric modulation of $\alpha2$ subunit-containing $GABA_A$-Rs mediates the effects of zolpidem on oscillation frequency and power.

In double mutant mice with the H101R point mutations in both the $\alpha1$ and $\alpha2$ subunits ($\alpha1/\alpha2$H101R) results were not significantly different from those in $\alpha2$H101R mice ($P > 0.05$, Student–Newman–Keuls), and in these mice 1 μM zolpidem did not increase the power of oscillations ($n = 18$ slices, 8 animals, $P = 0.51$, repeated measures ANOVA, Fig. 2*D*–*F*). As the expression of α subunits might be developmentally regulated, we also tested the effects of zolpidem in adult mice (P55–70). Similar to the findings in young animals, the effects of 1 μM zolpidem on frequency ($101.5 \pm 1.2\%$) and power ($121 \pm 7\%$) in adult $\alpha2$H101R mice were reduced compared to adult wild-type mice ($87.7 \pm 5.0\%$ and $218 \pm 41\%$, $P < 0.05$, Student–Newman–Keuls).

Together, these data show that allosteric modulation of $GABA_A$-Rs containing $\alpha2$ subunits can alter the frequency and power of carbachol-induced oscillations in hippocampus, while the contribution of allosteric modulation of $\alpha1$ subunit containing receptors to oscillations is marginal.

Location of $GABA_A$ receptors involved in modulating hippocampal oscillations

To determine where the $\alpha2$ subunit-containing $GABA_A$-Rs that affect the frequency and power of oscillations are located, fast network oscillations were manipulated specifically either in CA3 or CA1 region. Local application of 1 mM TTX in the stratum pyramidale of the CA3 region blocked carbachol-induced oscillations within 1 min in the entire hippocampal slice (power decreases to $6 \pm 1\%$, $n = 13$ slices, 4 animals; Fig. 3*A* and *B*; $P < 0.05$, paired Student's t test). In contrast, local injection of TTX in the stratum pyramidale or in stratum radiatum of the CA1 region did not significantly affect oscillations in either the CA1 or the CA3 region ($n = 7$ slices, 2 animals; Fig. 3*A* and *B*; $P = 0.24$, paired Student's t test). To make sure that the TTX application in CA1 was effective, we subsequently applied TTX in CA3, which blocked oscillations in all slices. These data indicate that action potential firing of CA1 neurons within the stratum pyramidale may not be the major determinant of local fast network oscillations during cholinergic receptor activation. Instead, synaptic inputs from CA3 neurons may underlie oscillatory field potentials in CA1, which is in agreement with earlier studies showing that the generator of the oscillations is in CA3 (Fisahn *et al.* 1998; Mann *et al.* 2005).

Next, we tested whether allosteric modulation of $GABA_A$-R function in either the CA3 or CA1 region of the hippocampus affected the frequency of oscillations in these regions. Zolpidem injection (1 mM) in CA3 decreased the frequency (to $83.8 \pm 4.5\%$, $P < 0.05$ in both CA3 and CA1, paired Student's t test) and increased the power (to $129 \pm 51\%$ in CA3 and by $168 \pm 89\%$ in CA1, $P < 0.05$, paired Student's t test) of oscillations in the whole hippocampus ($n = 5$ slices, 3 animals; Fig. 3*C* and *D*), while zolpidem injection in the CA1 region had no effect on the oscillations, neither on the oscillations in CA3 nor in the CA1 region ($P = 0.31$ and $P = 0.28$ respectively, paired Student's t test, $n = 6$ slices, 2 animals, Fig. 3*E* and *F*). The solvent DMSO had no effect on frequency and power of oscillations ($n = 6$ slices, 3 animals, $P = 0.37$ and $P = 0.81$, Fig. 3*D*). These data indicate that allosteric modulation of $GABA_A$-Rs located in the CA3 region affect

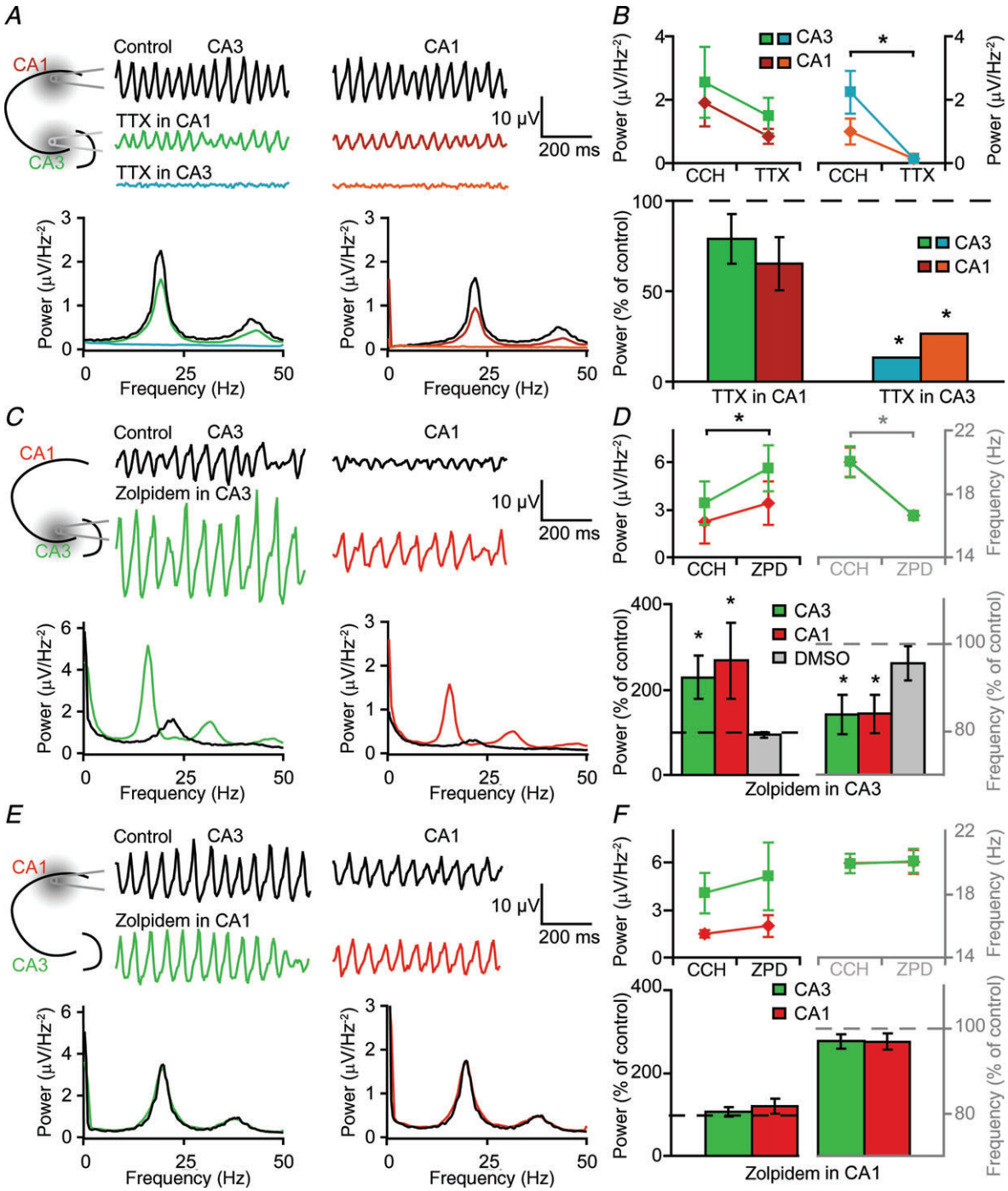

Figure 3. Local injection of TTX and zolpidem
A, schematic view (top left) of experimental setup. TTX (1 mM) was injected with a glass electrode in either CA3 or CA1. Example traces (top right) and corresponding Fourier transforms (bottom) in control and after TTX application in different areas. *B*, effects of TTX injection in CA3 ($n = 14$) and CA1 ($n = 7$) on the power of oscillations in CA3 and CA1 (top), and normalized change in power by TTX (bottom). *C*, experimental setup (top, left), example traces (top,right) and Fourier transform (bottom) in control and after injection of 1 mM ZPD in CA3. *D*, effects of ZPD injection in CA3 ($n = 5$) and on the power and frequency of oscillations in CA3 and CA1 (top), and normalized change in power and frequency by ZPD or DMSO injection in CA3 (bottom). *E*, experimental setup (top, left), example traces (top,right) and Fourier transform (bottom) in control and after injection of 1 mM ZPD in CA1. *F*, effects of ZPD injection in CA1 ($n = 6$) and on the power and frequency of oscillations in CA3 and CA1. Data show SEM. CCH, carbachol; TTX, tetrodotoxin citrate; ZPD, zolpidem.

the frequency of cholinergically induced fast network oscillations in the hippocampus.

Reduced allosteric modulation of inhibitory postsynaptic currents in CA3 pyramidal neurons in α2H101R mice

Earlier work demonstrated that cholinergic fast network oscillations in hippocampus are set by recurrent excitation and perisomatic fast synaptic inhibition of CA3 pyramidal neurons (Mann *et al.* 2005; Oren *et al.* 2006). A logical consequence of the findings above is that $GABA_A$-Rs mediating fast synaptic inhibition received by CA3 pyramidal neurons during oscillations should have a strong α2 subunit contribution and allosteric modulation of α2 subunit-containing $GABA_A$-Rs should affect the kinetics of fast synaptic inhibition during oscillations. To test this, we recorded spontaneous GABAergic IPSCs in CA3 pyramidal neurons during carbachol-induced oscillations of different mutant mice and quantified the effects of zolpidem on IPSC kinetics. Both in wild-type and α1H101R mice, the decay time kinetics of IPSCs increased by application of 1 μM zolpidem (to 140 ± 7% ($n = 7$ cells, 4 animals) and 147 ± 10% ($n = 6$ cells, 3 animals) respectively; Fig. 4*A*, *B*, *D*–*G*). In α2H101R and α1/α2H101R mice, the effect of zolpidem on IPSC kinetics was strongly reduced compared to wild-type animals (117 ± 4%, $n = 8$ cells, 5 animals and 115 ± 6%, $n = 7$ cells, 3 animals, both $P < 0.05$, Student–Newman–Keuls, Fig. 4*C*, *D*, *F* and *G*). These data suggest that α2 subunit-containing $GABA_A$-Rs are strongly involved in fast synaptic inhibition in CA3 pyramidal neurons during oscillations.

Spontaneous IPSCs recorded from CA1 pyramidal neurons during fast network oscillations had faster decay time kinetics on average than CA3 pyramidal neurons (7.2 ± 0.4 ms and 9.2 ± 0.8 ms respectively, $P < 0.05$, Student's t test; Fig. 5*A* and *B*). Although the inhibitory input did not follow every cycle of the oscillations as in CA3 pyramidal cells, the IPSCs were phase locked to the LFP in CA1. Surprisingly, in contrast to IPSCs received by CA3 pyramidal neurons in α2H101R mice, IPSCs recorded in CA1 pyramidal neurons of these mice during fast network oscillations showed substantial allosteric modulation by zolpidem (169 ± 14%, $n = 11$ cells, 5 animals, $P < 0.05$, repeated measures ANOVA; Fig. 5*C*, *F*–*I*) and comparable to zolpidem modulation of IPSC kinetics in wild-type animals (169 ± 7%, $n = 12$ cells, 5 animals, $P = 0.98$). In the same slices, the modulation of frequency and power of field potential oscillations by zolpidem in the CA1 region of α2H101R mice was strongly reduced compared to wild-type animals ($P < 0.05$, Student–Newman–Keuls; Fig. 5*D* and *E*). These results are in line with findings that α2 subunit-containing $GABA_A$-Rs located in the CA3 region control field potential oscillations in CA1. In CA1 pyramidal neurons of α1H101R mice, allosteric modulation of IPSCs by 100 nM zolpidem was reduced ($n = 11$ cells, 5 animals, $P < 0.05$, Student–Newman–Keuls; Fig. 5*F*–*I*). At the same time, the effects of zolpidem on the frequency of oscillations in CA1 were larger than in wild-type mice ($P < 0.05$, Student–Newman–Keuls; Fig. 5*A*), confirming that IPSC kinetics in CA1 pyramidal neurons are most likely not controlling the properties of oscillations in CA1. In α1/α2H101R double mutant mice, zolpidem effects on decay time kinetics of IPSCs received by CA1 pyramidal neurons were absent (112 ± 8%, $n = 5$ cells, 3 animals, $P \geq 0.26$, $P < 0.05$, repeated measures ANOVA; Fig. 5*H* and *I*). As zolpidem did affect IPSC kinetics in α2H101R while the LFP oscillations in CA1 were unaffected, it is unlikely that the α2 subunit-containing GABA receptors contribute substantially to inhibitory currents in CA1 pyramidal neurons during oscillations. In α1H101R mice, modulation of IPSCs by zolpidem was reduced, suggesting that the rhythmic inhibitory inputs on CA1 pyramidal neurons during cholinergic fast network oscillations are predominantly mediated by $GABA_A$-Rs containing α1 receptors.

Unitary GABAergic synapses between fast spiking interneurons and pyramidal neurons in CA3 contain α2 subunits

Several studies indicated that parvalbumin expressing fast spiking interneurons play a critical role in the generation of gamma oscillations (Fuchs *et al.* 2007; Cardin *et al.* 2009; Gulyas *et al.* 2010), while synapses from cholecystokinin expressing interneurons are silenced by carbachol (Gulyas *et al.* 2010). Electron microscopy studies in CA1 showed that synapses in pyramidal cells from axo-axonic cells and cholecystokinin basket cells contain mainly GABA-Rs with α2 subunits, while fast spiking basket cells contain α1 subunits (Nyiri *et al.* 2001; Klausberger *et al.* 2002 but see Kasugai *et al.* 2010). If the expression of α subunits is the same in the CA3 region, our data seem to oppose the idea that fast spiking interneurons provide inhibition during oscillations. Therefore, we tested whether the synapses between perisomatic targeting fast spiking interneurons and pyramidal neurons in CA3 contain the α2 subunit by making recordings of connected pairs of these two cell types in wild-type, α1H101R and α2H101R mice.

Fast spiking interneurons had their soma in stratum pyramidale or stratum oriens and were identified based on their spike pattern after depolarizing current injections (Fig. 6*D*). Biocytin reconstruction of these interneurons showed that their axon projected either to stratum oriens/pyramidale or to stratum oriens/radiatum

(Fig. 6*A*). Application of zolpidem increased the decay kinetics of IPSCs induced by a single spike in the fast spiking interneuron to 154 ± 7% ($n = 9$ cells, 7 animals, Fig. 6*B*, *C* and *E*). In contrast, in α2H101R mice the increase in decay time constant by zolpidem (122 ± 10%, $n = 8$ cells, 4 animals) was reduced compared to both wild-type animals and α1H101R mice (α1H101R 168 ± 6% $n = 3$ cells, 2 animals, $P < 0.05$ ANOVA, with Student–Newman–Keuls *post hoc* test; Fig. 6*B*, *C* and *E*). Given the important role of perisomatic targeting fast spiking interneurons in the generation of fast network oscillations (Mann *et al.* 2005; Oren *et al.* 2006; Fuchs *et al.* 2007; Cardin *et al.* 2009; Gulyas *et al.* 2010), our data may suggest that GABA$_A$-Rs containing α2 subunits in fast spiking interneurons to pyramidal neuron synapses in the CA3 region mediate the fast synaptic inhibition that controls the frequency of hippocampal fast network oscillations.

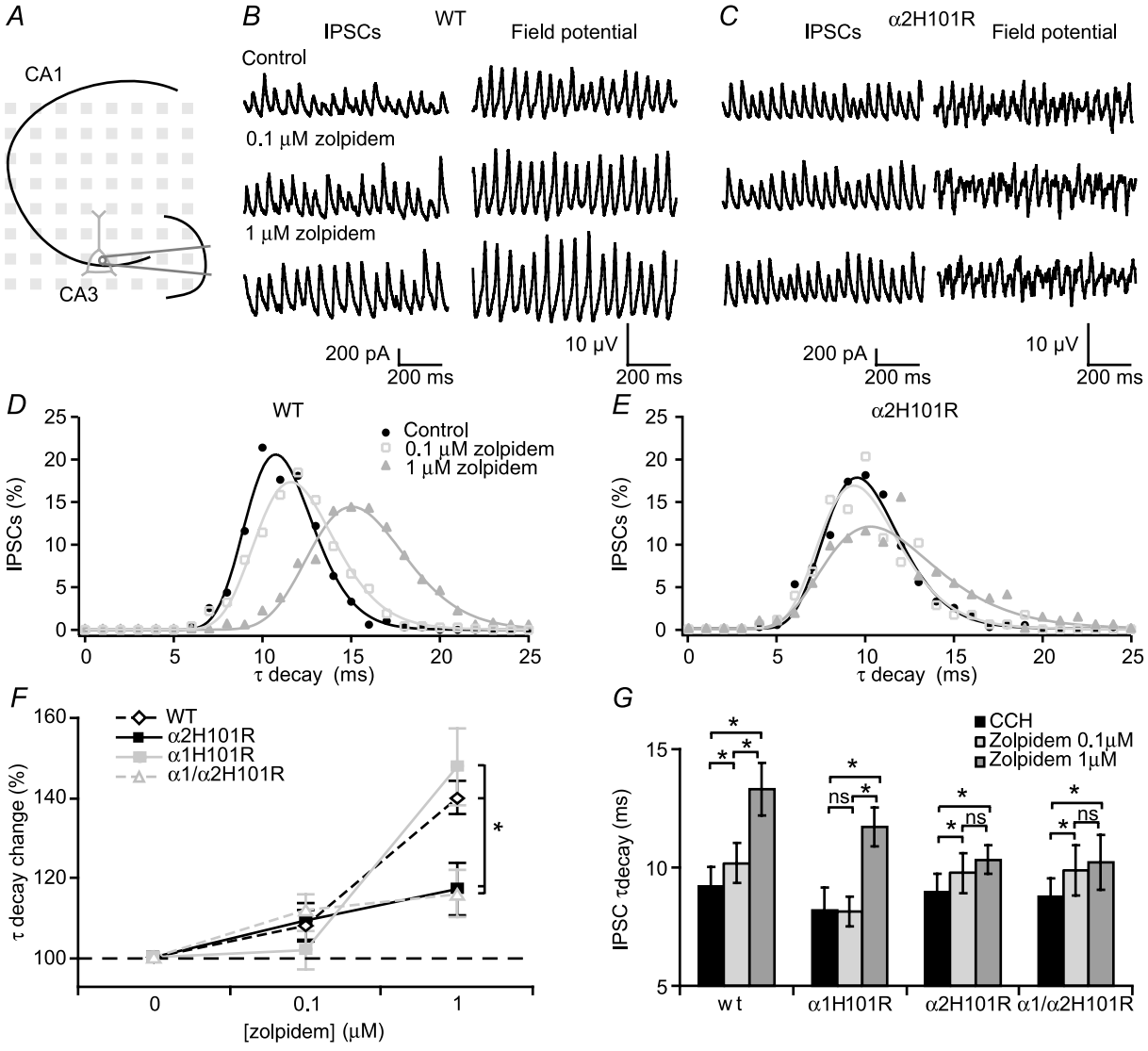

Figure 4. Zolpidem effects on decay time kinetics in CA3 pyramidal cells
A, experimental setup. *B*, left, example traces of IPSCs from a CA3 pyramidal cell in control and increasing concentration of zolpidem from a WT mouse. Right, corresponding local field potential. *C*, same as in *B*, but now in a pyramidal cell from the α2H101R mouse. *D*, histogram of IPSC decay time kinetics fitted with a lognormal function (line) in a WT mouse. *E*, histogram of IPSC decay time kinetics fitted with a lognormal function (line) in a WT mouse. *F*, effects of increasing concentrations of zolpidem on IPSC decay time kinetics in different transgenic mice. ANOVA test showed differences between genotypes ($P < 0.05$, $n = 7$, $n = 6$, $n = 8$ and $n = 7$ for wild-type, α1H101R, α2H101R and α1H101R/α2H101R, respectively). Data are normalized to control. *G*, changes in decay time kinetics by zolpidem. Data show SEM. CCH, carbachol; IPSC, inhibitory postsynaptic current; WT, wild-type.

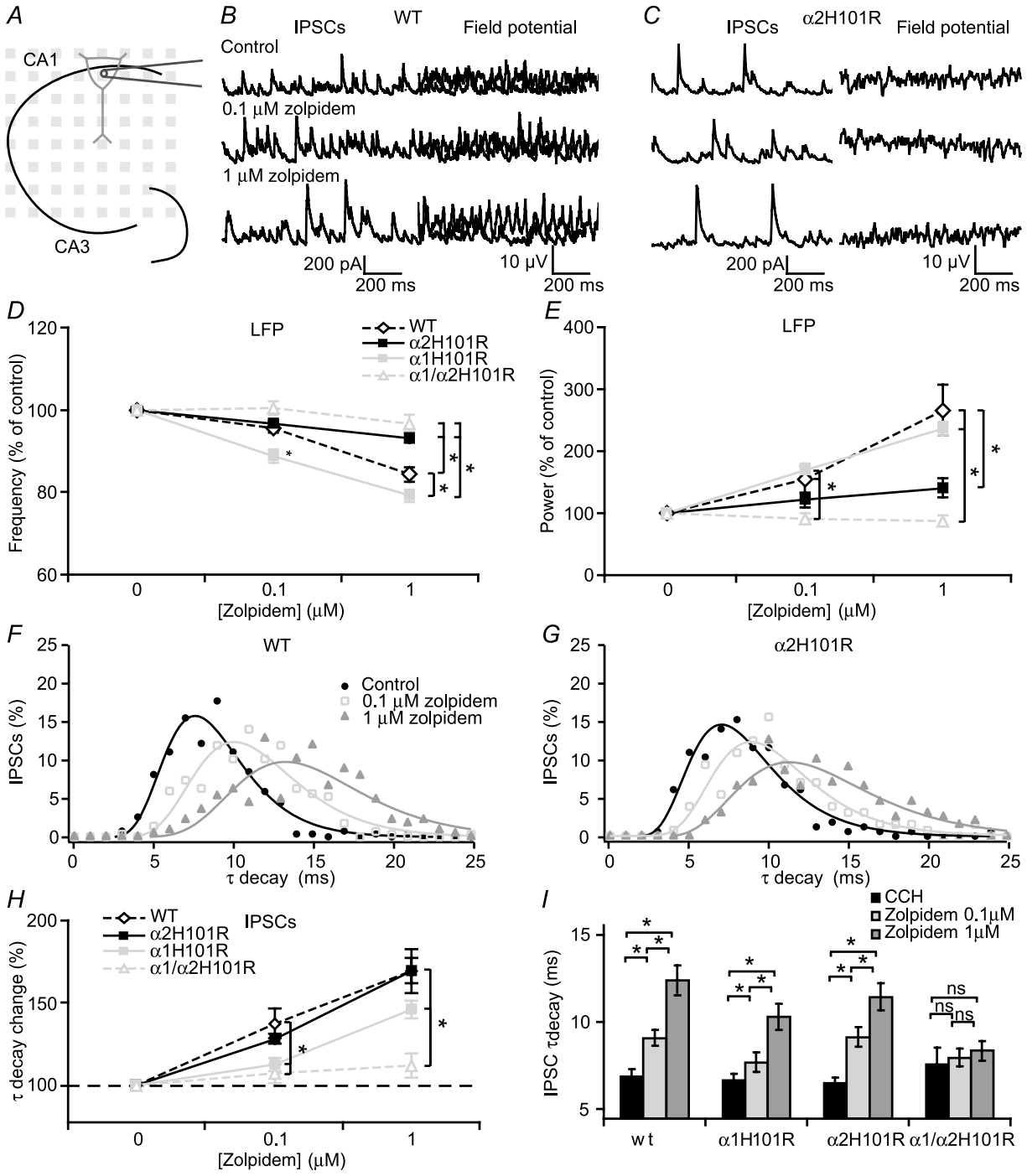

Figure 5. Zolpidem effects on decay time kinetics in CA1 pyramidal cells
A, experimental setup. *B*, left, example traces of IPSCs from a CA1 pyramidal cell in control and increasing concentration of zolpidem from a WT mouse. Right, corresponding LFP. *C*, same as in *B*, but now in a pyramidal cell from a α2H101R mouse. *D*, comparison of zolpidem effects on frequency between different transgenic mice in CA1 ($n = 25$, $n = 15$, $n = 21$ and $n = 18$ for WT, α1H101R, α2H101R and α1H101R/α2H101R, respectively). Data are normalized to control. *E*, comparison of zolpidem effects on frequency between different transgenic mice in CA1. Data are normalized to control. ANOVA test showed differences between genotypes in both frequency and power ($P < 0.05$). *F*, histogram of IPSC decay time kinetics fitted with a lognormal function (line) in a WT mouse. *G*, histogram of IPSC decay time kinetics fitted with a lognormal function (line) in a WT mouse. *H*, effects of increasing concentrations of zolpidem on IPSC decay time kinetics in different transgenic mice ANOVA test showed differences between genotypes ($P < 0.05$, $n = 12$, $n = 11$, $n = 11$ and $n = 5$ for WT, α1H101R, α2H101R and α1H101R/α2H101R, respectively). Data are normalized to control. *I*, changes in decay time kinetics by zolpidem. Data show SEM. CCH, carbachol; IPSC, inhibitory postsynaptic current; LFP, local field potential; WT, wild-type.

Discussion

Many studies have shown that GABAergic inhibition is essential for cholinergically induced oscillations in hippocampus and GABAergic receptor kinetics strongly affects the frequency of these oscillations (Whittington *et al.* 1995; Fisahn *et al.* 1998; Mann *et al.* 2005). Perisomatic synaptic inhibition received by CA3 pyramidal neurons is crucial for hippocampal oscillations *in vitro* (Mann *et al.* 2005; Oren *et al.* 2006). Which $GABA_A$-R subunits mediate this inhibition was not known. It was also not known whether the kinetics of inhibitory synapses on CA3 pyramidal neurons controls the frequency of oscillations in CA1. We find that: (1) hippocampal oscillations are strongly affected in $\alpha2^{-/-}$ mice, but not in $\alpha1^{-/-}$ mice; (2) allosteric modulation of $GABA_A$-Rs containing α2 subunits, but not α1 subunits, affects the kinetics of IPSCs in CA3 pyramidal neurons both during oscillations and by stimulating connected fast spiking interneurons; (3) in CA1 pyramidal neurons, allosteric modulation of $GABA_A$-Rs containing α1 subunits affects the kinetics of IPSCs during oscillations; (4) allosteric modulation of carbachol-induced oscillations in CA3 and CA1 depends mainly on $GABA_A$-Rs with α2 subunits; and (5) only $GABA_A$-Rs located in CA3 affect the frequency of oscillations in hippocampus, and not $GABA_A$-Rs located in CA1. Thus, we conclude that allosteric modulation of $GABA_A$-Rs containing α2 subunits mediates the zolpidem modulation of frequency and power of oscillations. This subunit is expressed at synapses between perisomatic targeting fast spiking interneurons and CA3 pyramidal neurons (Fig. 7).

Current models on mechanisms underlying the generation of fast network oscillations in hippocampus are based on recurrent excitation and inhibition between pyramidal neurons and interneurons. These models predict that the spike timing and spiking probability of pyramidal neurons, and thereby the synchronization of activity in the hippocampal neuronal network, depends on the decay time of inhibitory inputs (Traub *et al.* 2000). In line with the models, positive allosteric modulation that lengthens the decay time of IPSCs in CA3 strongly reduces the frequency of oscillations. By increasing and decreasing α subunit function, we assessed which of these α subunits mediates GABAergic inhibition in the recurrent excitation and inhibition in the CA3 region underlying neuronal synchronization. We find that recurrent inhibition is mediated mainly by α2-containing $GABA_A$-Rs, as both IPSCs in CA3 pyramidal neurons and hippocampal network oscillations in α2H101R mice had lost sensitivity to allosteric modulation.

Although allosteric modulation of the α2 subunit had the strongest impact on the frequency of oscillations,

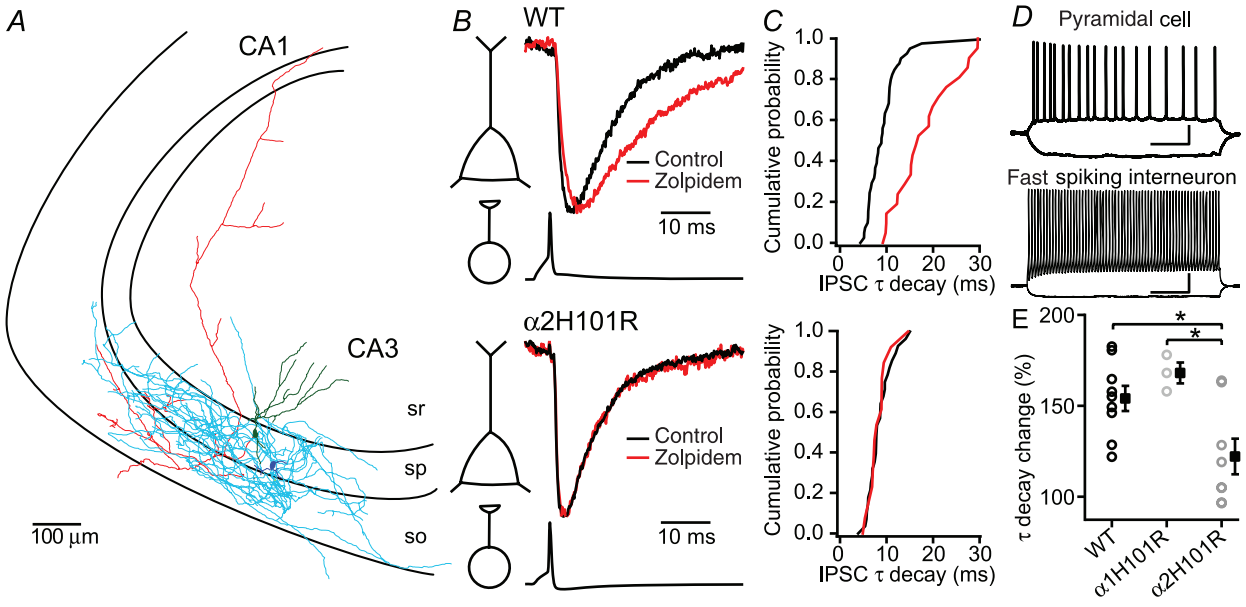

Figure 6. Synapses between perisomatic targeting fast-spiking interneurons and CA3 pyramidal neurons contain α2 subunits
A, reconstruction of a biocytin-filled connected CA3 pyramidal cell and perisomatic targeting fast spiking interneuron (interneuron axon light blue; interneuron soma and dendrites dark blue; pyramidal cell axon red; pyramidal cell dendrites and soma). *B*, peak scaled average IPSC in a pyramidal cell evoked by a single action potential in an interneuron before and after application of zolpidem in WT (top) and α2H101R mice (bottom). *C*, cumulative distribution of τ decay times of the cell pair shown in *B*. *D*, characteristic spike pattern of pyramidal cell and fast spiking interneuron. Scale bar 200 ms, 20 mV. *E*, average change in τ decay time by zolpidem ($n = 9$, $n = 3$ and $n = 8$ for WT, α1H101R and α2H101R, respectively). IPSC, inhibitory postsynaptic current; WT, wild-type.

other α subunits might also contribute to properties of gamma oscillations. As in α1H101R mice the zolpidem modulation of cholinergically induced oscillations is increased, it is possible that the kinetics of α1 subunit containing receptors regulate the oscillations in opposite direction compared to α2 subunit containing receptors. However, the zolpidem modulation in α2H101R and in α1H101R/α2H101R double mutant was similar, showing that in the α2H101R mice α1 modulation does not affect oscillations. Therefore, we suggest that the modulation of receptors with α1 subunits counteracts the modulatory effects of zolpidem on α2 subunits. The α1 and α2 subunit are the most abundant synaptic subunit in the hippocampus, but α3, α4 and α5 are also expressed (Laurie *et al.* 1992; Prenosil *et al.* 2006), although α4 and α5 subunits are not expressed in perisomatic synapses (Nusser *et al.* 1998; Peng *et al.* 2002; Fritschy & Brunig, 2003; Prenosil *et al.* 2006). Furthermore, α4-containing receptors are insensitive to allosteric modulation by zolpidem (Scholze *et al.* 1996) and would under our experimental conditions not be affected. Deletion of either of these subunits does affect properties of fast network oscillations, probably by changing the excitability of neurons due to a decrease in tonic inhibition (Towers *et al.* 2004; Mann & Mody, 2010). The α3 subunit is expressed at low abundance in hippocampal pyramidal neurons (Laurie *et al.* 1992; Prenosil *et al.* 2006). As this subunit is expressed on perisomatic synapses in the hippocampus (Fritschy *et al.* 1998), it is possible that the small remaining decrease in frequency by 1 μM zolpidem in α1H101R/α2H101R mice could be accounted by α3-expressing receptors.

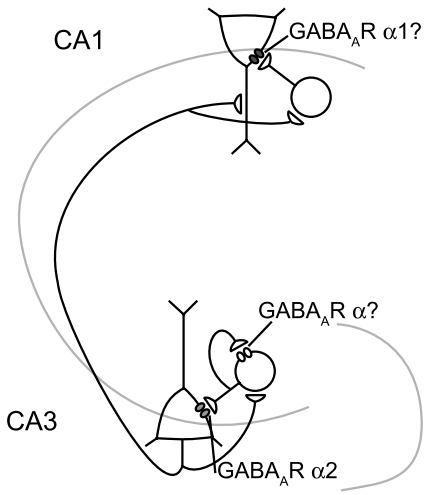

Figure 7. Schematic view of hippocampal networks involved in oscillations
Oscillations are generated in CA3 and depend on feedback inhibition mediated by $GABA_A$ receptor α2 subunits. Oscillations in CA1 reflect excitatory inputs from CA3 and feed forward inhibition mediated by $GABA_A$ receptor α1 subunits.

Several interneuron types have been shown to fire phase-locked with gamma oscillations *in vivo* and *in vitro* (Hajos *et al.* 2004; Gloveli *et al.* 2005; Tukker *et al.* 2007); however, fast spiking basket cells have been shown to be essential for the generation of the cholinergically induced oscillations (Gulyas *et al.* 2010). We found that zolpidem effects on IPSCs from perisomatic targeting fast spiking interneurons in CA3 are mediated through α2 subunits containing receptors. However, in GABAergic synapses on to pyramidal neurons in CA1 contain more α1 subunits in synapses from parvalbumin-positive basket cells (Klausberger *et al.* 2002), while synapses from axo-axonic cells and parvalbumin-negative basket cells contain more α2 subunits (Nyiri *et al.* 2001 but see Kasugai *et al.* 2010). On the other hand, in benzodiazepine insensitive mice it was shown that synaptic input from stimulations near the soma in CA1 are mediated by α2 containing receptors while stimulations at the distal dendrites activate α1 containing receptors (Prenosil *et al.* 2006) and the IPSC decay kinetics of synapses from fast spiking basket cells unto pyramidal cells is equal in CA1 and in CA3 (Bartos *et al.* 2002). Furthermore, it is not known whether the rhythmic GABAergic inhibition in CA1 is mediated via the same interneuron types as in CA3. In CA3 the oscillations depend on local feedback loops (Mann *et al.* 2005), while in CA1 the rhythmic inhibition is likely coming from CA1 interneurons activated via excitatory input from CA3. We showed that allosteric modulation of IPSCs during oscillations in CA1 pyramidal cells depends on the α1 subunit, indicating that the rhythmic inhibitory input might arrive in different subcellular locations than in CA3 pyramidal cells.

Little is known about which $GABA_A$-R subunits are involved in oscillations in other brain areas. Our findings suggest that $GABA_A$-Rs that contain α2 subunits are important for cholinergically induced fast network oscillations in the hippocampus. Whether these subunits play a similar role in neocortical oscillations is not known. Some indications could come from patients with schizophrenia that show decreased gamma band activity in the frontal cortex (Cho *et al.* 2006). Pharmacological treatment with $GABA_A$-R agonists that selectively modulate α2/α3-containing receptors increased gamma power in frontal cortical areas and improved cognitive functioning in these patients (Lewis *et al.* 2008). This may indicate that $GABA_A$-Rs containing α2 subunits may be important for gamma oscillations and cognitive processing in other brain areas as well.

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Author contributions

T.S.H, A.B.B. and H.D.M. designed the experiments. T.S.H., M.R. and A.J.T. performed experiments; T.S.H. analysed the data; T.S.H. and H.D.M. wrote the manuscript. All authors approved the final version.

Acknowledgements

We thank Hans Lodder and Rik Jansen for excellent technical assistance, and Charlotte van Coeverden and Sebastian Falk for help with local application experiments. Funding was received from the Netherlands Organization for Scientific Research (NWO; 917.76.360 and 912.06.148), ERC StG 'Brain-Signals', the Dutch Fund for Economic Structure Reinforcement (FES, 0908 'NeuroBasic PharmaPhenomics project'), EU 7th Framework Programme (HEALTH-F2-2009-242167 'SynSys') and VU University Amsterdam.